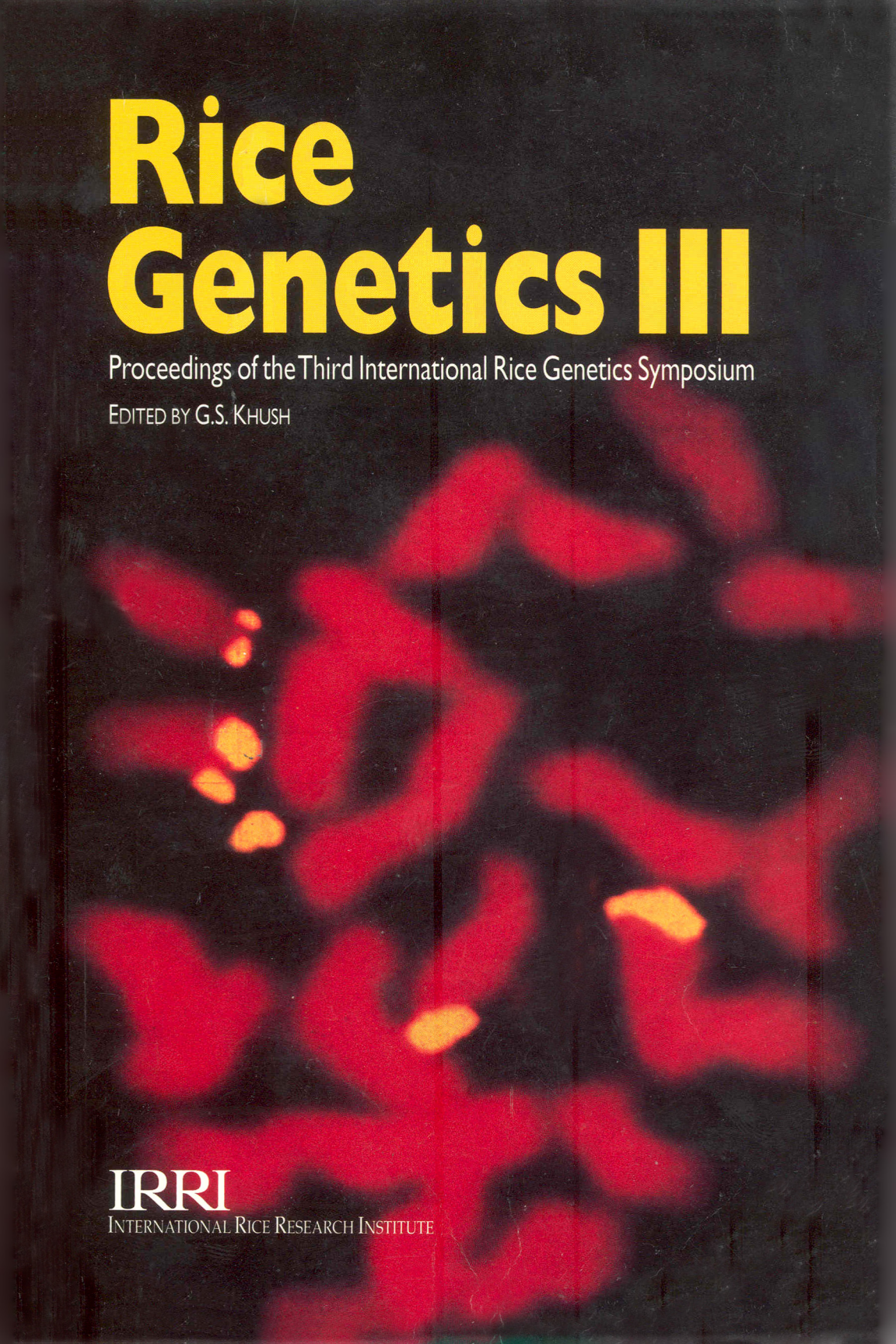
Rice Genetics III

Proceedings of the Third International Rice Genetics Symposium

EDITED BY G.S. KHUSH



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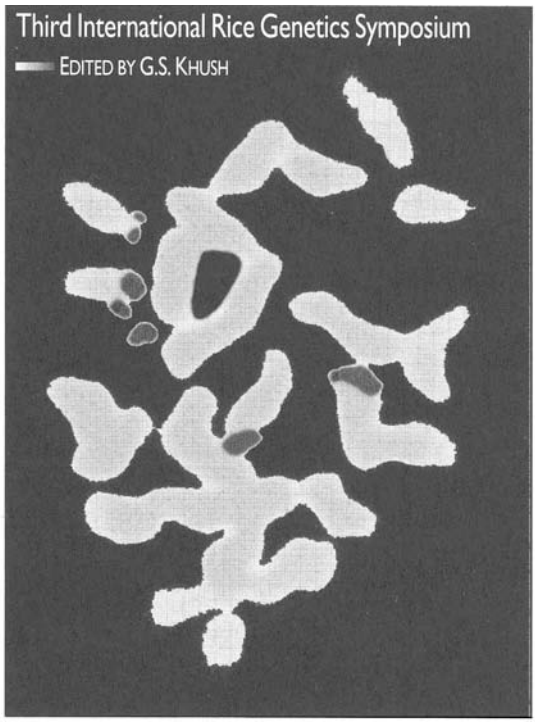

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INTERNATIONAL RICE RESEARCH INSTITUTE

P.O. Box 933, Manila 1099, Philippines

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Foreword

Rice is the principal food of nearly half of mankind and more than 90% of it is grown in developing countries where problems of food supply are more acute. Over the last 30 yr, world rice production has more than doubled—from 257 million t to 534 million t. This phenomenal increase can be attributed to the large-scale introduction of improved rice varieties and technology developed at IRRI and national rice improvement programs through the application of Mendelian genetics and conventional breeding approaches.

These approaches to increasing rice production averted the large-scale famines predicted by some experts. However, the specter of food shortages looms once again. The annual rate of increase of rice production has slowed down to where it is lower than the rate of increase of rice consumers. There are no new areas that can be used for rice farming. Agricultural economists estimate that we will have to produce 70% more rice by 2025 from less land with less labor, less water, and fewer chemicals. New rice varieties that combine higher yield potential with excellent grain quality, resistance to biotic and abiotic stresses, and input use efficiency are desperately needed.

Recent advances in cellular and molecular genetics of rice have come perhaps in the "nick of time" to provide us with new tools to develop the rice varieties of the future. Only 10 yr ago, the status of rice genetics was considered far behind that of other food crops such as maize, wheat, barley, and tomato. However, the last decade has seen a knowledge explosion in this arena and rice is now considered a model plant for such research on cereal crops. Several fortunate developments have led to this happy turn of events.

First, the Rice Genetics Cooperative (RGC) was established in 1985 during the first international rice genetics symposium at IRRI. It has greatly enhanced international collaboration in rice genetics. The RGC has formulated the rules for gene symbolization and nomenclature in rice and monitors the gene symbols and coordinates linkage mapping. It has established two genetic stock centers for preservation and distribution of gene marker stocks and chromosomal variants. It publishes the *Rice genetics newsletter* annually and organizes this prestigious gathering every 5 yr. Perhaps the RGC's most significant achievement to date was the establishment, in 1990, of an

agreed upon system of numbering rice chromosomes and linkage groups—without it, rice genetics would not be where it is today.

Second, the Rockefeller Foundation (RF) established the International Program on Rice Biotechnology, also in 1985, which has contributed greatly in moving the frontiers of knowledge on rice cellular and molecular genetics. Since rice is not an important crop in developed countries, except Japan. there had been very little investment in studying rice genetics in advanced country laboratories. RF has been funding research on rice biotechnology in carefully selected laboratories in developed countries and is also working to build the capacity of developing countries to use rice biotechnology techniques, particularly through the training of scientists. Over the last decade, RF's program has led to many breakthroughs in rice genetics.

Third, the establishment of the Rice Genome Research Program (RGRP) at the Society for Techno-innovation of Agriculture, Forestry, and Fisheries (STAFF) Institute in Japan has moved the frontiers of our knowledge on the rice genome far beyond anyone's expectations. A high-density map of about 2,000 molecular markers has been prepared and numerous genes have been sequenced. The physical map, whose preparation is now under way, will facilitate the cloning of important genes not only from rice but also from other cereals because of the small size of the rice genome and its synteny with those of other cereals.

IRRI's rice biotechnology program fully supports and participates in the activities of the RGC and has strong linkages with the RF and Japanese programs mentioned above. Like the first two symposia in this series, IRRI was pleased to host the third international rice genetics symposium. Around 200 rice scientists attended the first symposium in May 1985, and more than 300 participated in the second symposium in May 1990. More than 500 scientists from 31 countries were attracted to this third symposium.

Along with the dramatic increase in attendance has come a major shift in the complexion of the program. During the first symposium, around 90% of the papers were on classical genetics. About half the papers presented at the second symposium were devoted to classical genetics and half to cellular and molecular genetics. About 80% of the papers in these proceedings and the posters, which are appearing in the three 1996 issues of the *International rice research notes,* address cellular and molecular genetics topics.

I would like to thank my colleagues at IRRI who have devoted a great deal of time in organizing this symposium. Most of the preparation at IRRI was done by Dr. G.S. Khush, who served as organizing secretary. Drs. D.S. Brar, T. Kinoshita, K. Toriyama, and G. Toenniessen contributed advice and support in the preparation and implementation of the symposium plans. Critical review of the plenary papers was done by Drs. R.K. Aggarwal, O. Azzam, J. Bennett, D.S. Brar, M.B. Cohen, S.K. Datta, N. Huang, R.J. Nelson, J.-L. Pham, and S.S. Virmani. The plenary and concurrent papers in the proceedings were edited by G.P. Hettel, T. Rola, S. Siar, and G. Goss.

GEORGE ROTHSCHILD
Director General

Plenary session papers

Economic prosperity in Asia: implications for rice research

M. Hossain

This paper describes the changing economic environment in Asia and the impacts these changes have on input-output markets and the organization of rice production in high- and low-income countries. Emerging problems in Asian rice farming—related to sustaining farmers' incentives in high-income countries and food security in low-income countries—are discussed. The author assesses the priorities for genetic rice research that will be required to develop appropriate technologies needed to address the problems. These priorities are grain quality, resistance to abiotic and biotic stresses, shifting the yield frontier, direct seeding for crop establishment, and incorporating micronutrients.

Rice is the staple food and the principal crop in humid and subhumid Asia. The monsoon climate and the high temperature during periods of heavy rainfall favor rice cultivation in this region. From the Philippines in the east to eastern India in the west and from southern China in the north to Indonesia in the south, rice accounts for 30-50% of agricultural incomes and provides 50-80% of the calories consumed. Because of its importance in providing national food security and generating employment and incomes for the low-income people in society, rice is regarded as a strategic commodity and an important component of culture in many Asian countries. More than 90% of all rice is produced and consumed in Asia. The world rice economy is thus dominated by the economic environment in Asia.

Two contrasting developments in Asia may substantially affect the structure of rice production and marketing. First, the prosperous Asian countries are finding it difficult to sustain producers' interest in rice farming. The move toward free trade in agricultural production, initiated by the recently concluded General Agreement on Tariffs and Trade (GATT), will have important implications on the sustainability of rice farming in these countries. Second, the potential for increased productivity created by the dramatic technological breakthroughs in the late 1960s has almost been

exploited, particularly for the irrigated and favorable rainfed environments. But poverty in low-income countries is still extensive and population continues to grow at a high rate. As rice production is losing the race against population, sustainability of self-sufficiency in rice production is becoming a major issue for the land-scarce, low-income countries.

Rice research: achievements and future challenges

Genetic improvements in the rice plant made a great contribution to achieving food security in Asia over the last 3 decades (Hossain and Fischer 1995, Khush 1995). The large shift in the yield potential of rice and the reduction in the period of crop growth have made up for the limited availability of land in Asia and have empowered farmers to increase rice supplies. In most Asian countries, average rice yields have continuously increased since the mid-1960s as farmers replaced traditional varieties with improved ones. Incorporation of resistance against major diseases and insects in the modern varieties (MVs) has ensured the stability of rice yield and prices and reduced the risks of cultivation and farmers' dependence on harmful agrochemicals.

Since 1966, when IR8 was released, the Asian population has increased by 85% but rice production has doubled leading to some increase in per capita consumption. Technological progress has led to a downward trend in the unit cost of production and prices. The price decline benefits the vast number of urban poor and the rural landless who spend more than half of their incomes on staple foodgrains. Although profits per unit of output have declined in many countries, the rice farmers were able to increase household incomes through an expanded output from two or three crops grown on the same land and a larger size of each crop. The technological progress thus helped maintain a balance in the interests of consumers (lower prices) and producers (higher incomes).

But the race between rice production and population growth is far from over. Asian population is still growing at 1.7% yr^{-1}, and is projected to increase by another 85% before stabilizing by the end of the 21st century. In fact, in the 1990s, Asia will see the largest absolute increase in population (53 million annually) in the history of mankind. Even if per capita rice consumption is kept at the present level, rice supplies must increase at the rate at which population grows; otherwise there will be an upward pressure on prices. Also, as economic growth and better distribution of income increase the capacity of the Asian poor to satisfy their unmet food needs, per capita rice consumption is expected to increase further, particularly in low-income countries. It is projected that during the 1990-2025 period, the demand for rice may increase by another 70%, which will require an increase in production from 480 million t of unmilled rice at present to 810 million t by 2025 (IRRI 1993, Rosegrant et al 1995). Most of the additional demand will come from the low-income countries of Asia, where there is still substantial unmet demand for rice, and the success in population control has remained limited.

It is true that over the last 3 decades the increase in rice production was greater than what is needed for the next 3 decades. So the challenges may appear to many

observers as less daunting. But, easy options for both technological changes and the utilization of the natural resources (i.e., more intensive use of land and water) have already been exploited, and further exploitation of natural resources may turn out to be uneconomic and more harmful for the environment (Rosegrant and Svendsen 1991, Cassman and Pingali 1995). Also, the fast economic growth in different parts of Asia and the difference in economic landscape have been affecting the availability and the costs of agricultural inputs, and the relative prices and profitability of outputs that have important implications on the production and marketing of rice. In view of the changes in economic environment, the rice research community should reconsider the strategy and priority for technology development.

Economic prosperity and organization of rice production

Economic growth in Asia

The remarkable progress of the Green Revolution in rice cultivation went hand in hand with enormous economic progress in many parts of Asia. Economic growth in Asia has been many times faster than that for other regions in the developing world. Since 1970, the average annual rate of growth has been a robust 6.0% annually in Asia, compared with 3.2% in South America and 2.4% in Africa (World Bank 1995). A rate of population growth of more than 2.5% yr^{-1} has eaten up the meager growth in Africa, so most African nations have seen a deterioration or stagnancy in the economic conditions for their people. In contrast, economic growth in many Asian nations has also contributed to notable progress in population control, which has been reflected in faster growth in per capita incomes. With an annual rate of growth of about 5% yr^{-1}, the per capita income of Asian people has doubled every 14 yr since the early 1960s.

The rate of economic progress has, however, been uneven across countries in Asia. The growth has been much faster in East Asia compared with Southeast Asia, which in turn has grown faster than South Asia. In Southeast Asia, economies in the Philippines, Myanmar, Cambodia, and Lao PDR grew much slower than Thailand, Malaysia, and Indonesia. There was also a positive relationship between overall economic growth and the development of the agricultural sector (Fig. 1). But it is the faster growth of the nonagricultural sectors that has contributed to the vast economic prosperity in Asia.

In 1950, Asian countries had almost identical economic standings, with the exception of Japan. But because of uneven economic growth, substantial economic disparity has been created. For example, in the early 1960s, the Philippines had higher levels of income than the Republic of Korea. Now, Koreans have incomes eight times higher than Filipinos. As the economy grew, the importance of agriculture declined, vibrant manufacturing and service sector activities pulled labor force and population from rural to urban sectors, and higher income levels and larger participation of women in economic activities reduced the demand for children and lowered the population growth. The economic disparity among Asian nations, the importance of agriculture, and the structure and the growth of population can be seen in Table 1.

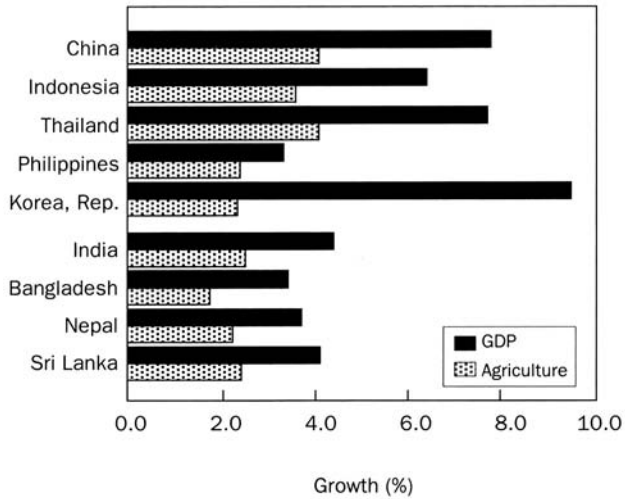

Fig. 1. Growth of economy and agriculture, selected countries of Asia, 1970-93.

Table 1. Economic disparity and the growth and structure of population in major rice-growing countries, 1993-94.

Country	Production of rice unhusked 1994 (million t)	Per capita Income 1993 (US$)	Agriculture's share of the economy, 1993 (%)	Population growth 1990-95 (% yr^{-1})	Urban population 1993 (%)
China	178.3	490	19	1.2	29
India	118.4	300	31	1.9	26
Indonesia	46.2	740	19	1.6	33
Bangladesh	27.5	220	30	2.0	17
Vietnam	22.5	170	29	2.2	20
Myanmar	19.1	59	63	2.2	26
Thailand	18.4	2,110	10	1.4	19
Japan	15.0	31,490	2	0.3	77
Philippines	10.2	850	22	2.4	52
Korea, Rep.	7.1	7,660	7	0.9	78

Source: World Bank (1995).

Effect on input markets

The growing economic prosperity in Asia is a crucial factor that determines the availability of labor, water, and land for rice cultivation. The competing demand for these inputs in various economic activities affected their relative scarcities and prices and changed the relative profitability depending on the intensity of use of these inputs in various economic activities.

Labor and wages. Economic growth brings dramatic changes in the structure of employment, adoption of labor-saving technology, and increase in labor productivity.

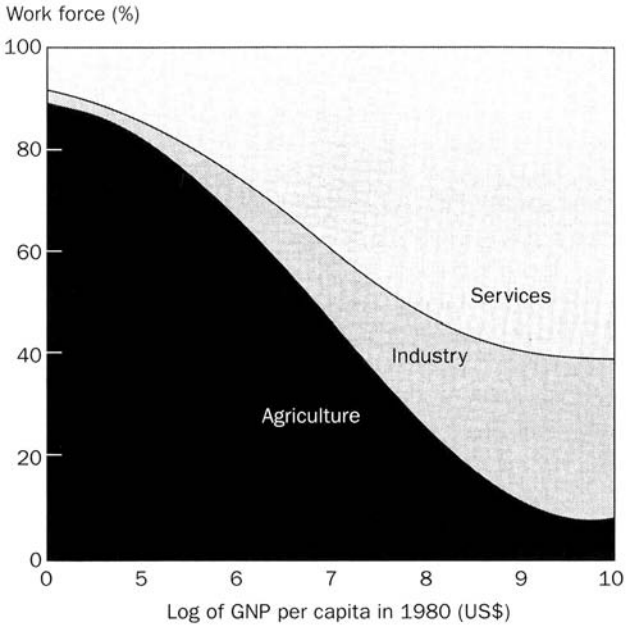

Fig. 2. Relationship between sectoral distribution of labor force and level of economic growth.

The working age population, which is the source of supply of labor, is determined by the rate of population growth and female participation in economic activities. In most developing countries, the rate of growth in the working age population has been around 2-3% annually (World Bank 1995). A faster rate of economic growth than this rate has led to severe competition for labor among economic sectors in Asia, because demand grew at a faster rate than supply. With opportunities for more remunerative employment rising elsewhere, workers move out of low-productivity, low-wage rural economic activities.

The relationship between the sectoral distribution of the labor force and the level of economic growth analyzed by the International Labor Organization is shown in Figure 2. On average, agriculture's share of employment falls from 90% of the total in low-income countries to roughly 5% in high-income countries. The share of industry, which includes manufacturing, construction, and mining, increases from 4 to 35%, and that of services from 6 to 60%. As the economy grows faster, the pull of labor in the manufacturing and services sectors away from agriculture becomes stronger, and after some time, the labor force employed in agriculture declines in absolute terms.

Although the agricultural sector tries to address the problem of labor shortages by adopting labor-saving technologies, it cannot compete with the manufacturing and services sectors in this respect, so the productivity differences continue to grow with economic prosperity. The total agricultural labor force increased from 4.5 to 6.1 million

persons during the 1966-75 period, and then started declining in absolute terms and reached 3.2 million by 1990 (World Bank 1995).

The scarcity of labor is reflected in its price—the wage rate (World Bank 1995). In sub-Saharan Africa, where economies have remained stagnant, the real wages hardly increased during the 1970-90 period. In contrast, in East and Southeast Asia, which experienced more than 5% yr^{-1} growth in per capita incomes, the real wage rate increased by 170% over the 20-yr period. In South Asia, where the economic growth was moderate, the real wage rate increased by only 50%.

Rural-urban migration, increase in labor productivity, and escalating wage rates in the nonfarm sector put an upward pressure on the rural wage rate, which affects the costs of production and profitability in rice cultivation. The growth in the agricultural wage rates over the 1961-91 period for selected Asian countries is shown in Table 2. In the early 1960s, the difference in wage rate across countries was only marginal. In the slow-growing countries, such as Bangladesh, India, and the Philippines, the agricultural wage rates had hardly increased, but there was an escalation in wage rates in Japan and the Republic of Korea.

Availability of water. Water resource development has been the key to increasing rice production in virtually all Asian countries where land is a scarce factor of production. Among all activities involving exploitation of natural resources, irrigation is by far the most important. Asia accounts for 55% of an estimated 253 million ha of irrigated land in the world; China and India alone have more than 100 million ha of irrigated land (Fredericksen et al 1993).

Water has always been regarded as an abundant resource for humid Asia. But with rapidly increasing populations, the substitution of water for scarce land has taken place to meet the food needs. As a result, the perception of abundance of water has been changing in many Asian countries. The per capita availability of water resources declined from 40 to 60% in most Asian countries over the 1955–90 period (Feder and Keck 1994). By common convention, countries are defined as water-stressed when the availability is between 1,000 m^3 and 1,700 m^3 per capita. Projections based on constant availability of water and increasing population suggest that China, India, Sri Lanka, Pakistan, and the Republic of Korea are expected to reach near stress levels by 2025.

An important issue for Asia is the spatial and seasonal dimension of the problem of water availability. Rainfall varies across countries and among regions within a

Table 2. Long-term trend in wage rates, selected Asian countries. (US$ d^{-1}).

Country	1961	1971	1981	1991
Bangladesh	0.46	0.44	0.86	1.39
Philippines	1.39	0.59	1.51	2.16
Korea	0.82	1.86	10.84	32.59
Japan	1.22	8.19	24.16	51.93

Source: IRRI (1995).

country. About half of China receives less than 400 mm of rainfall annually; extensive areas of northwestern and central India and central Myanmar are classified as drought-prone. Most of the Asian region receives rainfall predominantly during a single monsoon lasting from 4 to 6 mo, with almost no rain for the remainder of the year. The marked seasonality of rainfall means that, if reservoir storage is not provided, much of the runoff generated is destined to flow, wasted, into the ocean. The monsoon is often erratic. Floods and seasonal water shortages occur in the same year in many countries. Estimates based on water availability during the dry season show that most countries will be water-stressed within the next 30 yr.

As population increases and economic development intensifies, satisfying the needs for drinking water, sanitation, and industrial activities has to be given higher priority in allocating water resources. As rice is a heavy consumer of water, rice research can contribute to saving this important natural resource, as demands for water in other competing and more productive uses increase with economic growth.

Competing demand for land. Economic prosperity and industrial progress are leading to rapid urbanization and concentration of people in a few large cities. Most of the additional increase in population beyond 2000 will be located in urban areas. By 2025, 53% of the people in Asia will live in urban areas compared with 30% in 1990 (UN 1993). An important implication of growing urbanization is that some of the fertile agricultural land has to be diverted to meet the demand for housing, factories, and roads. Also with urbanization and the associated change in food habits, the markets for vegetables, fruits, and livestock products will grow stronger. The growing market for livestock products will increase the demand for livestock feed (maize) and fodder. The changes in relative profitability will induce farmers to divert riceland to grow more profitable nonrice crops such as vegetables, fruits, and fodder. The area under riceland has already started declining even in low- and middle-income countries such as China, the Philippines, Indonesia (Java), and Bangladesh.

Output market: quantity vs quality

Studies on consumption behavior show that per capita rice consumption largely depends on urbanization, the level of income, and changes in occupational structure. At very low levels of income, people take coarse grains and sweet potatoes as their basic staple, as these are usually the cheapest sources of energy. As income increases, per capita rice consumption grows since people can afford to substitute these low-cost sources of energy for rice. But there is a threshold level of income beyond which rice becomes an inferior good. As incomes rise further, consumers aspire to have a more balanced diet with high-cost quality foods such as vegetables, bread, fish, and livestock products that provide proteins and vitamins, along with calories. So the per capita rice consumption starts declining.

Another notable pattern of rice consumption is that with growing incomes, people express preferences for higher quality rice once their calorie needs have been met. High-income consumers spend more on rice by paying higher prices for the varieties with preferred eating quality.

As rice scientists have had limited success in developing high-yielding cultivars with better eating quality, the price difference between the standard and high-quality varieties has been growing in Asian markets.

Emerging problems in Asian rice farming

Sustaining farmers' incentives in high-income countries

Despite the impressive increase in land productivity, it has been difficult for the fast-growing Asian countries to sustain producers' interest in rice farming. As mentioned earlier, the expansion of the nonfarm sector and the rapidly rising labor productivity have pushed up nonfarm wage rates, which attracted labor from rice farming and increased agricultural wages. Since traditional rice farming is a highly labor-intensive activity, the pressure from the nonfarm sector has pushed up the cost of rice production and reduced profits and farmers' incomes. In Japan, Taiwan, and the Republic of Korea, the constant outflow of the agricultural labor force has caused a continual decline in the farming population (Park 1993). Aging of labor force and depopulation in remote areas have continued, making it difficult to sustain the existing rural communities in some areas.

The competitiveness of rice farming has sought to be maintained through 1) improved farm management practices that increase efficiency in the use of nonland inputs and increase total factor productivity, 2) increased use of capital to replace labor through mechanization of farming operations so that labor productivity could be raised when no further increase in land productivity is possible, and 3) using the price mechanism to transfer income from the relatively well-off rice consumers to the rice producers so that the balance between the rural and urban incomes could be maintained.

The protection of the domestic rice industry encourages high-cost domestic production. The cost of producing rice in Japan is about 17 times higher than in Southeast and South Asian countries and about 10 times higher than in the USA. The recently concluded Uruguay round of trade negotiations has increased pressures to remove rice subsidies and to partially open up rice markets in East Asia. As the economic prosperity in East Asia depends on liberalization of trade of industrial products, these countries may yield to the pressure to move toward free trade in agricultural commodities. In this case, domestic prices of rice will fall and the rice industry will be threatened. These countries will then depend more on imports for meeting their grain needs. They have the economic capacity to pay for such imports, but their riceland would remain fallow and other countries will have to produce an exportable surplus. Under this scenario, the price of rice is expected to increase substantially in the world market, which will redistribute scarce grain supplies in favor of high-income groups, leading to an escalation of poverty in the low-income, rice-growing countries.

Farm household income from rice farming could also be increased through removing legal restrictions on the ceiling of landholding. This will pave the way toward

more enterprising farmers to accumulate land through tenancy arrangements or outright purchase from those who are interested in taking up full-time nonfarm occupations. The accumulation of land will increase farm household incomes even when profits per unit of land remain unchanged or decline. In fact, the main advantage of the American and Australian rice farmers over those in East Asia is the substantially larger farm size.

Sustaining food security in low-income countries

For the low-income countries of Asia, the emerging problem is how to ensure an increase in food supplies to meet the growing demand, and to maintain the capacity of all sections of the population to acquire that food. The challenge will be more acute for those countries that have a large proportion of area under an unfavorable rice-growing environment (Hossain 1995).

The per capita rice consumption is expected to increase faster in the poverty-stricken countries, as people satisfy their unmet food demand with economic growth. The experience of economic development shows that the lower the level of income, the higher the rate of population growth and that success in population control comes with economic prosperity. The low-income countries of South Asia and Southeast Asia (Vietnam, Myanmar, and the Philippines) are projected to have much higher rates of population growth and demand for food than the middle- and high-income countries in East and Southeast Asia. The demand for rice will remain almost the same in the Republic of Korea and will fall by 15% in Japan because of the decline in per capita consumption, but will increase by more than 70% in Bangladesh, Myanmar, and Vietnam and 60–70% in India and Indonesia (Hossain 1995).

The natural resource constraints to increasing rice production are severe for most of the low-income countries in Asia. As the frontier of cultivable land was closed long ago, the per capita availability of arable land has been declining rapidly with growing population. In its rice-growing region, China now supports 17 persons ha^{-1} of arable land; the figure is 13 for Bangladesh, 11 for Vietnam, and 8–10 for India, Indonesia, and the Philippines (Hossain 1995). Only Thailand, Myanmar, and Cambodia have favorable endowments of land, with 2–4 persons ha^{-1}. The population pressure is reflected in the high cropping intensity for foodgrain production. The cropped area under foodgrains per unit of arable land is 148% for China, 132% for Bangladesh, 112% for India, and 108% for Vietnam, compared with about 60% for Thailand and Myanmar.

As per capita rice consumption declines with economic growth, the middle- and high-income countries of Asia should have some surplus rice available for export to the low-income, food-deficit countries. In Japan, peak rice harvest reached 18.8 million t in 1967, but it started declining from that level and fell to about 12 million t in 1992. In Taiwan, China, the peak reached 3.6 million t in 1976; present production is only about 2.0 million t. These countries could have maintained their production levels through export of surplus rice to other countries, but they could not compete in the world market with other exporting countries because of the high production costs

under protected markets. Thus, having an export surplus from middle- and high-income countries in Asia (where per capita rice consumption is expected to decline) to meet the deficits of the low-income countries seems highly improbable.

Thailand, Myanmar, and Cambodia have considerable excess capacity to meet potential shortages in other countries in South and Southeast Asia, and the cost of production will remain competitive for a long time, at least in Myanmar and Cambodia. If rice prices go up, farmers will be encouraged to increase production by investing in irrigation and chemical fertilizers and adopting higher yielding varieties.

But achieving food security through international trade may not be possible due to foreign exchange constraints in the low-income, food deficit countries. Also, since rice production is a major rural economic activity at low levels of income, and land and labor cannot be easily diverted to other economic activities during the monsoon season, low-income households may find it difficult to acquire imported food. If economic conditions of small-scale farmers and landless laborers fail to improve due to stagnant productivity of this most important economic activity, the increase in rice prices will only aggravate the poverty situation in the food-deficit countries.

In short, the ability of the low-income countries to feed its growing population will depend on increasing rice yields in the vast rainfed lowlands than on importing rice from other countries.

Implications for prioritizing research

This section presents the author's assessment of the priorities for genetic research that will be required to develop appropriate technologies needed to address the problems just discussed.

Grain quality

Improving grain quality of MVs is the most pressing need of the time. As Asians become richer, they are not going to eat more rice, but are willing to pay higher prices for rice that tastes better. Farmers are concerned as much with yields as with prices, since both of them determine profits. Rice varieties that combine superior grain quality with high-yielding ability will be in great demand everywhere. With rapid urbanization, the market for superior grains is growing among the high-income consumers of low-income countries. Also, as rice production is becoming uneconomical in high-income countries, the farmers in low-income countries could gain by producing exportable surplus of superior quality grains for the markets in high-income countries.

Research on breeding for quality has to be country-specific. But international genetics research could help identify traits that determine rice quality—the size and the shape of the grain, chalkiness, amylose content, gel consistency, and aroma. Rice breeders in national research systems could then use this knowledge to develop varieties for their respective countries.

Resistance to stresses

Abiotic. Almost half of Asia's riceland is dependent on rainfall and is subjected to both droughts and submergence, sometimes during the same season. Even if sufficient moisture is received over the growing season to support the physiological needs of the crops, the precipitation may not be evenly distributed to satisfy water requirement at various stages of crop growth. The uneven distribution of rainfall may result in temporary flooding and waterlogging from heavy rains particularly in areas with poor drainage, and dry spells in between leading to drought conditions. Even in areas where irrigation facilities are available, the supply of water may be unstable or inadequate to meet the crop's physiological needs, and hence, exposing the crop to the vagaries of the monsoons.

Many traditional varieties have developed traits through centuries of evolution that enable them to withstand these abiotic stresses. Rice scientists have so far had limited success in identifying these traits and incorporating them into high-yielding MVs (Zeigler and Puckridge 1995). The available MVs may do well in normal years, but may perform poorly compared with traditional varieties, if there is a prolonged drought or sudden submergence due to an erratic monsoon. So where the rainfall is unreliable, farmers still grow traditional varieties, which is the main factor behind the low yield and the large yield gap at the national level.

Several national agricultural research systems have recently conducted surveys recording perceptions of experienced farmers and extension workers on losses of rice yields from droughts and temporary submergence (Dey and Upadhaya 1994, Herdt 1996). The losses are estimated at 130 kg ha^{-1} from drought and 110 kg ha^{-1} from temporary submergence. In many countries, yield losses from abiotic stresses are higher than losses due to insects and diseases. Biotechnology has much to offer for the improvement of varieties for abiotic stresses (Bennett 1995). Although locating the minor genes is a difficult task, it is likely that in the long run, this approach will bear fruit, as it relates to many of the traits that plant breeders find difficult to deal with by conventional methods.

If rice research succeeds in incorporating modern traits that help withstand abiotic stresses, MVs will be adopted more extensively in the unfavorable ecosystems. The yield stability of these MVs will reduce risk in rice cultivation, thereby providing incentives to farmers to apply chemical fertilizers in optimum amounts, that will, in turn, lead to further yield increases.

Biotic. Over the last 2 decades, incorporating resistance in MVs against insects and diseases has been a top research priority. Many MVs have already been developed with multiple resistance to insects and diseases (Khush 1995). Host plant resistance is the cornerstone of effective pest management. Combining varietal resistance with biological agents and cultural practices can reduce use of harmful agrochemicals (Heong et a1 1995). Because of the substantial progress made in this area, farmers no longer perceive biotic stresses as major problem areas (Herdt 1996).

But resistant varieties do not remain resistant forever. So research must continue to sustain past developments and to find innovative and more cost-effective ways of controlling biotic stresses. There is a need to continuously identify new genes for

more durable resistance and incorporate them into the improved germplasm. Biotechnology tools can be more effective in characterizing insect and pathogen population structure and in developing more durable host plant resistance through pyramiding of major genes and combining of quantitative traits (Bennett 1995).

Shifting the yield frontier

The yield potential of rice has hardly increased after the introduction of IR8, which kicked off the first generation of MVs. Once the potential yield (the economically optimum yield is much lower than the technical optimum) under the optimum management condition is reached in farmers' fields, the rice yield remains stagnant. In Japan and the Republic of Korea, rice yields have remained stagnant at around 6.5 t ha^{-1} after reaching that level long ago. China will soon approach that limit.

Rice breeders have recently given attention to shifting the yield frontier by changing the plant architecture and incorporating hybrid vigor. Much progress has already been made and it is projected that the potential yield could be increased by another 50% through this method (Virmani 1994, Khush 1995).

Farmers in high-income countries, however, are looking for MVs and farming practices that increase the productivity of labor rather than productivity of the land. Since the population is relatively stagnant and the per capita consumption of rice has started declining, more riceland has to be kept fallow if the yield increases, which will increase the governments' fiscal burdens on account of agricultural subsidies. Also, if liberalization of agricultural trade takes place, even a 50% increase in yield may not be enough to compensate for the drop in prices and thereby to help sustain farmers' incentives in rice cultivation. Hence, the demand for MVs with higher yield potentials may be slack in East Asian countries.

Varieties with higher yield potential will, of course, have a great demand in the low-income countries, as these will enable them to obtain more rice from the irrigated ecosystems where the yield levels of existing varieties are approaching the plateau. But this will further accentuate the regional inequality in agricultural growth contributed by the Green Revolution that has benefited the favorable rice-growing environments. The higher yield may also require larger use of agrochemicals on lands where these are already used in large amounts, and hence will not be environment-friendly. Thus, in view of the equity, food security, and environmental concerns, it is preferable to develop technologies that help reduce the existing yield gaps, than to increase yields for the favorable environments that will help already better-off farmers.

Support direct-seeding method of crop establishment

The direct-seeding method of crop establishment requires less labor and water, and hence is an appropriate technology for economies where labor and water are getting scarce. Puddling of soils for transplanting of seedlings consumes a large proportion of the estimated 5,000 L of water required to produce 1 kg of rice. In rainfed systems, farmers wait for heavy rains that flood rice paddies for puddling the soil. The delay in planting exposes the crop to drought at the reproductive stage of the plant growth and reduces the yield of the nonrice crop that follows rice. Direct seeding introduces

flexibility of timing in crop establishment and contributes to more efficient use of the rain water.

However, to be acceptable to farmers, water science and agronomy research in this area needs to be supported by genetic research for introducing early seedling vigor in rice plants, to save them from sudden submergence during periods of heavy rains. Direct seeding also encourages weed growth, and there is a need to look for genetic traits that make rice more competitive with weeds. Although weeds can be economically controlled by herbicides, new ways of controlling weeds are required because of changes in the weed flora, herbicide resistance, and growing public concerns about the harmful effects of pesticides on human health and the environment (Rola and Pingali 1993, Pingali and Roger 1995).

Incorporating micronutrients in rice

In spite of substantial increases in per capita food intake and impressive reduction in poverty and hunger, the nutritional deficiency-induced diseases are widely prevalent in Asia (WFC 1992). These include child blindness due to Vitamin A deficiency, endemic goiter caused by iodine deficiency, and anemia caused by iron deficiency. The latest estimates based on country surveys suggest that, worldwide, at least 190 million preschool children are at risk of having vitamin A deficiency with about 350,000 victims becoming blind each year. Iodine deficiency usually occurs in mountainous regions and flood-prone areas where iodine easily leaches from the soil. Worldwide, some 1 billion people are at risk with iodine deficiency; 225 million of them have goiter. The problem is most serious in South and East Asia. Bouis (1994) has shown that low micronutrient intakes are a more important constraint to better health and nutrition than low calorie intakes for the Asian population.

As Asian people consume substantial amounts of rice (150 kg annually), small amounts of these micronutrients in rice could effectively address this nutritional problem. Thus, screening rice varieties for availability of the micronutrients and incorporating them into MVs without reducing the yield and quality of rice would definitely be a high-impact area of genetic research, although the probability of research success may be low.

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Establishing the Rockefeller Foundation's priorities for rice biotechnology research in 1995 and beyond

R.W. Herdt

This paper presents the methodology and results of a procedure used to establish the funding priorities of the Rockefeller Foundation's support for rice biotechnology research. Data from a wide range of sources, including new national-level research on rice production losses, are used, together with data on area of land planted to each of the four main rice agroecologies in Asia, Latin America, and Africa, current yields, and estimates of the relative difficulty of achieving various research goals. Priorities are derived by simultaneously considering both the relative importance of constraints and the relative difficulty of overcoming the constraints. Approximately US$443 million could be invested annually in rice biotechnology research to achieve an internal rate of return of 35%, allocated across five categories of challenges and four agroecologies in the developing world.

Since 1983 the Rockefeller Foundation has invested US$65 million developing and applying the tools of biotechnology to rice and creating the capacity for biotechnology research in rice-dependent developing countries. The program continues. The goal is to enable rice-dependent countries to use the most effective means of genetic improvement to produce rice varieties that farmers adopt, with the ultimate objective being better lives for rice-dependent consumers and farmers.

The program helps scientists in rice countries develop collaborative relationships between breeders and biotechnologists in the same country and internationally, to gain facility to routinely use restriction fragment length polymorphism (RFLP) and other gene-mapping techniques, tissue culture, transformation (genetic engineering), and gene cloning. We also fund the application of these tools to help develop desirable traits that will give rice the capacity for superior performance in farmers' fields. Identification of the traits and the genes that confer those traits and marker-assisted breeding are other important elements. After incorporating new traits, materials still

must undergo time-consuming field testing, varietal release evaluations, and seed multiplication before wide-scale adoption is possible. The Foundation's interest is in the results of this process, i.e., we are funding the science to obtain better rices in the hands of farmers and in the mouths of poor consumers. Science capacity is an instrument to help produce more food and produce it more efficiently, not the ultimate objective of the Foundation's support.

This paper describes the priorities guiding the Foundation's current allocation of funds for rice biotechnology, and discusses how those priorities are established. Here, priorities mean an operational statement of how funds ought to be allocated.

The three broad categories of activities are training, tool development, and introduction of traits. We currently use no formal methodology for allocating among the three, but training and tool development were obvious early requirements. In 1987, we developed a quantitative procedure for allocating support among alternative traits and applied it to obtain a rank ordering of traits (Herdt 1991). Priorities for tool development depend on our estimates of the promise of particular tools for genetic improvement in the short to medium run. Training is almost entirely in the laboratories of those seeking genes for traits or developing tools, and so the training priorities follow those for the other two components of activity.

Priorities for the program may now be somewhat different from when we first established them in 1987 for two reasons: 1) a great deal of new biotechnology knowledge has been gained so some research directions seem more promising than they did 7 yr ago, and 2) some rice problems have been alleviated while others have become more pressing.

National priorities for agricultural research have received much attention (e.g., see reviews in Norton and Pardey 1995, Alston and Pardey 1995). Quite a bit of work has been done on national rice biotechnology priorities in the context of the Foundation's program on rice (Evenson et al 1996c). However. none of that work has specifically asked what the Foundation's priorities should be; rather it has taken the perspective of national authorities.

Approach and concepts

The problem

For the Foundation, the problem of priority setting is to determine how much should be invested in each alternative. Early in the program, the tools had to be developed so they would function with rice, and US$20 million, or 40% of Foundation funding was directed to industrialized country laboratories where the tools were being invented during the first 8 yr of the program. Large amounts were invested in wide crossing, transformation, molecular mapping, and tissue culture of rice, but with the techniques developed from that early funding now widespread among participating laboratories, only about 20% went toward those goals during the last 3 yr (1993–95). Some funds are still allocated to enable laboratories to acquire these tools, but to a large extent, the tool development phase has peaked.

Funds for training and development of desirable traits averaged US$2.8 million annually between 1984 and 1992; during the next 3 yr, they averaged US$5 million per year. The close relationship of the Foundation to each of the grantees enables us to encourage laboratories receiving grants for tool or trait development to accept developing country scientists as graduate students and postdoctoral fellows to actually do the tool development. This contributes to trait and tool development and training at the same time.

Quantitative methods have been used to allocate funds among work on various traits. In essence, the approach is to come as close as possible to the economically optimal allocation in which the ratios of marginal value products to marginal factor costs are equated (Herdt 1991, Evenson 1996).

Constraints and research problem areas

Constraints prevent farmers from obtaining higher levels of productivity; they are the conditions that desirable traits are designed to overcome. Constraints of two general types have been identified: losses and opportunities. Losses are constraints that reduce yields—pests, drought, temperatures that restrict growth, inadequate nutrients, and so forth. Opportunities are ideas for how to raise the potential yield, or how to reduce inputs without reducing yields. Both would lead to increased productivity.

The first critical step in applying quantitative methods is the identification of all possible constraints as targets for research. The second is to estimate the difference between maximum yield and actual yield (the yield gap). This latter requires quite specialized knowledge of the kind researchers are often best able to provide. However, most researchers have good knowledge about certain constraints and little about others. Insect specialists may have good knowledge about whether a particular damage is caused by particular insects, but have little knowledge about the effects of soil conditions. Soil scientists may be able to identify the several different soil-related constraints but little about plant diseases, and so forth.

To exploit the knowledge of researchers about constraints to help set priorities, it is necessary to use a comprehensive approach. We used four steps: 1) ask specialists to estimate the maximum possible yield under given conditions, 2) ask them to estimate the relative effects of the various constraints they are able to identify, 3) ask them to estimate the relative effects of all other constraints, and 4) combine the estimates from as many knowledgeable specialists as possible.

Constraints affect rice grown under different production conditions differently. For example, blast is a serious disease when rice is grown as an upland crop where weather conditions deposit heavy dew, but is a minor problem under most irrigated rice conditions where the tempering effects of irrigation water give smaller diurnal temperature-humidity swings.

Rice is grown under a wide range of agroecological conditions, from well-controlled irrigation to completely upland rainfed situation. The opportunity to increase productivity or prevent yield losses from each constraint is, in general, different for various rice ecologies.

In this analysis, the term "research problem area" (RPA) is used to mean a constraint operating in a given rice production ecology. It is necessary to disaggregate information to the RPA level to understand the relative importance of yield constraints, because of the interaction of constraints and ecology. In contrast, one may estimate the difficulty of addressing each constraint more or less independently of the extent of rice grown in different agroecologies.

Obtaining the solution

Priorities are derived by simultaneously considering both the relative importance of RPAs and the relative difficulty of overcoming constraints.

Intuitively, we use the idea that if it is just as "difficult" for research to solve one constraint (A) as to solve another (B), and if A is responsible for twice the loss or could generate twice the gain as B in all rice ecologies. then it seems rational to invest twice the effort or cost to solve A as B. More rigorously, in economic terms, the optimal combination of research investments is that in which the internal rate of return is equal for each RPA (Evenson et al 1996a).

It seems most convenient to think of constraints as causing yield losses, that is, on a per hectare basis. And, some constraints may affect large areas, on average, while others may affect small areas. The total loss attributable to each constraint, on average, is the product of the number of hectares affected, on average, and the loss per hectare, on average.

The difficulty of addressing a problem or exploiting an opportunity is reflected in the time and money expected to be required to address it. Some problems may be more difficult than others to research either because they have been less explored previously or because previous research has opened many possibilities to be investigated. Also, there is greater uncertainty associated with conducting research on some problems than on others.

The methodology used here to obtain a quantitative set of priorities requires the following data:

- The gap between actual and potential yield in each rice agroecology;
- Yield losses per hectare for each RPA, i.e., a disaggregation of the yield gap;
- Hectares of rice in each rice agroecology;
- Number of years of research required to overcome each constraint, given current annual expenditure, or alternatively the number of years needed to get 50 or 75% of the way toward overcoming each constraint.

Yield gaps

In most Asian countries, farmers' average yields are significantly lower than maximum yields on experiment stations—3-5 t ha^{-1} compared with 6–9 t ha^{-1}. This yield gap has two components. Yield gap I is the difference between maximum yields on the experiment station and maximum yields on farmers' fields. This gap is attributable to inherent differences in soil and other difficult-to-change environmental factors between the experiment station and typical farmers' fields. Production research

can do little to exploit it. Yield gap II is the difference between maximum yields and actual yields on farmers' fields. This gap is a reflection of the yield that could be gained through the invention of technology to overcome constraints. Of course, because such technology has not been invented, no one can know with certainty the maximum yield, but informed judgment can provide estimates of how much is lost to constraints or how much might be obtained by raising the yield potential.

Sources of data

Farmers have deep personal knowledge of losses and potential opportunities on their own farms, and can be good sources of information about yield gap II and what may account for it. A well-constructed sample of farmers could generate information on constraints (losses and opportunities) for the RPAs within their experience. However, because of the heterogeneity across the rice ecologies of most countries, the spatial distribution and sample size required for good national estimates from farmers are quite large and costly to obtain, so most such data sets have limitations.

Extension agents, because they deal with a large number of farmers and cover a large geographic area relative to individual farmers, are able to reflect judgments across broader geographic areas. Researchers should have views representative of a larger extent of rice area because of their responsibility for even broader geographic regions than extension agents.

The earlier Rockefeller Foundation priorities work relied largely on the insights of scientists knowledgeable about Asia, with considerable experience at IRRI (Herdt 1991). Since then the Foundation has sponsored work by social scientists in China, several regions of India, Bangladesh, Nepal, Indonesia, Thailand, and the Philippines to compile quantitative judgments by farmers, extension workers, and researchers.

Typically, for pest-caused yield losses, respondents were asked to estimate the proportion of land in each rice ecology damaged by each pest in an average year, and the estimated loss in kilograms per hectare suffered on each damaged hectare of riceland, on average. Comparable information was obtained for each yield constraint/opportunity for which the respondents felt qualified to respond. The hectares of rice in each rice ecology were multiplied by the per hectare yield loss/opportunity associated with each RPA. That gave a measure of the absolute quantity of rice that might be obtained from addressing each RPA. Aggregating across losses/opportunities gave the total and from that the proportion attributable to each loss/opportunity was determined. Aggregate losses divided by the total area of rice gave an estimate of the loss per hectare, averaged across the entire area. Similar values were compiled for each major geographic region.

Difficulty of alternative research activities

Different research goals have different levels of difficulty. For example, the goal of incorporating nitrogen-fixing capacity in a cereal grain is acknowledged by most to be far more difficult than incorporating the semidwarf trait. Most research goals fall between these two extremes. Each researcher-respondent was asked to estimate the number of years that would be required to develop technology that would save 25 and

75% of the yield loss/opportunity associated with each RPA. It was also recognized that for some RPAs, some technology that partially offset the constraint had already been developed, so an adjustment was made for research achievement.

The earlier study relied on insights from IRRI scientists and 10 U.S.-based scientists, who had a combined total of more than 120 scientist-years of experience in the Asian tropics. Evenson et al (1996a) used estimates obtained from 7 IRRI scientists and 10 Asian scientists in national programs. These two sets of data plus the insights gained from interaction with the Foundation's Scientific Advisory Committee and experience with grantees were combined by Foundation staff to generate a new set of estimates of time required to achieve the goals identified for each constraint, especially for this paper.

The analytical procedure used determines the amount of investment in each RPA that would generate a given internal rate of return from each research activity (Evenson 1996), disaggregated for the four rice ecologies (irrigated, rainfed, flood-prone, upland), the five developing country regions (India, China, Southeast Asia, Latin America, Africa), and for 65 RPAs. This gives 4 × 5 × 65 or 1,300 individual RPAs, each with an "optimal" level of research investment. These are condensed for presentation purposes here.

Summary of results

The portfolio of research activities derived here is aggregated into regions to provide the needed guidelines for Rockefeller Foundation purposes. The underlying data come from a loosely coordinated series of country studies (Evenson et al 1996b).

Estimated yield gaps

Yield gap II, the difference between the average yield on farms and the demonstrated average potential yield, ranges from around 3 t ha^{-1} in China and the Philippines to 300 and 400 kg ha^{-1} in the poorest growing conditions of Nepal and Thailand. In India, it reaches 2 t ha^{-1} and in Bangladesh 1.3 t ha^{-1}. It is important for researchers to understand yield gap II—what accounts for it, and how the factors contributing to the gap can be overcome, if that is possible. Yield gap I has a much wider range, but it is of less interest than gap II because gap I is caused largely by soil and other environmental differences that seem impossible to overcome by technology or other practical means.

Contributions to yield gap II

Within a country or region, relatively few individual factors accounted for a high proportion of the specific factors identified as constraints. In China, for example, 18 individual factors were identified, with several of the least important contributing less than 1% of yield gap II. In Bangladesh, the least important of the top 20 constraints occurred, on average, in less than 1% of the rice area over 10 yr and resulted in an average of about 4 kg of lost yield per hectare.

Table 1. Estimated yield losses, by group of constraints, main rice season.

Region	Contributions (kg ha^{-1}) to yield gap II by groups of contraints					
	Insects	Diseases	Drought	Soils	Other abiotic[a] stresses	Others
China[b]	92	88	n.a.	635	557	91
East India[c]	104	93	177	74	81	167 (weeds, lodging birds)
West Bengal[d]	43	213	n.a.	n.a.	772	—
South India[e]	215	137	44	72	7	87 (weeds, lodging, nutrients
Bangladesh[f]	135	111	172	27	106	22 (weeds)
Indonesia[g]	399	24	n.a.	n.a.	n.a.	189 (rats)
Thailand[h]	78	12	n.a.	n.a.	n.a.	5 (rats)
Nepal[i]	810	560	210	150	10	150 (rats) weeds, lodging)
Philippines[j]	—250[k] —		198	n.a.	407	90 (variety, others)

[a]Cold, heat, submergence, "weather". [b]Single season rice. [c]Wet season, rainfed lowland. [d]Rainfed lowland aman; submergence is the other abiotic stress. [e]All ecosystems. [f]Rainfed lowland aman; submergence is the other abiotic stress. [g]Irrigated ecosystem, from survey of 640 households in 32 villages; from percentage damage to an assumed 3.5 t ha^{-1} yield. [h]All ecosystems, assuming 10 million ha of rice area. [i]All ecosystems, extension workers' views. [j]Wet season, rainfed lowland; typhoon damage is the other abiotic stress. [k]Insects and diseases.

Sources: China, Lin and Shen (1996); E. India, Widowsky and O'Toole (1990); W. Bengal, Saha et al (1996); South India, Ramasamy (1996); Bangladesh, Dey et al (1996); Indonesia, Jatileksono (1996); Thailand, Seetboonsarng (1996); Nepal, Upadhyaya et al (1996); Philippines, Hossain et al (1996).

The most important constraints were typically quite different across countries, seasons, and rice ecosystems as reflected in Table 1. In some cases, losses from drought, temperature extremes, and other abiotic factors were large, while in other cases, losses from biotic factors (insects and diseases) were much larger. The number of data points prevent any but the most aggregate representation here, but some observations on Table 1 may still be of interest, keeping in mind that the contributions reported there are for one important rice production season or rice ecology and do not fully represent the respective nations.

Insects and diseases cause yield losses estimated at around 100 kg ha^{-1} in many locations. In China, these are relatively small losses compared with the other categories, but China has an effective system for developing resistant varieties, an elaborate system for monitoring ricefields and well-developed methods for controlling pests (Lin and Shen 1996). In China, most pests are controlled so the remaining losses are small. All three of the studies reporting on India and Bangladesh indicate that insects and diseases are quite important there, in stark contrast to China. Insects and diseases

caused important losses for all the other countries, although the farm survey data from the Philippines study made it impossible to separate the two. Losses reported for Nepal appear to be considerably larger than for other locations.

Drought and other abiotic stresses were quite significant as a whole but differed considerably across countries. In China, nutrient supply, cold temperatures, and other abiotic stresses are estimated to cause losses of more than 1 t ha^{-1}. Submergence is an important problem in rainfed lowland parts of West Bengal, Bangladesh, and other places in eastern India. Drought is an important contribution to losses in India and Bangladesh, even in the wet season lowland conditions reported for eastern India. Although most farmers are using modern varieties that are semidwarf, lodging is reported in a number of places.

Other pests including weeds, birds, and rats are a continuing problem for many rice farmers in some areas. Researchers, accustomed to thinking in terms of modifying genetics to address constraints, suggest little to offset these challenges.

Optimal research portfolios

Table 2 shows a summary of our estimates of the optimal portfolio of research investment using the best current national data available on yield losses and the current probabilities of research success. It shows that a 35% rate of return would be expected from US$52 million spent annually on biotechnology approaches to insect control in the irrigated agroecology and US$75 million on insect control across all agroecologies. For diseases, the total is US$98 million; for improved biological efficiency, it is US$179 million; and for biotechnology approaches to overcoming abiotic stresses, US$71 million.

In total, US$443 million could be allocated as indicated in Table 2 and it would be expected to generate a social internal rate of return of 35% annually, if the expectations of scientists are correct. To put this number in perspective, Asia produces some 350 million t of rice annually which, at a conservative price of US$200 t^{-1}, has a value of more than US$70 billion. Thus, US$443 million amounts to investing about 0.6 of 1% of output value annually in rice biotechnology. Research directed at irrigated ecosystems should receive about 50% of the total, with deep water nearly half as important as rainfed. In contrast to earlier information, upland has a lower priority than deep water.

Table 2. Biotechnology research investment (US$ million) yielding an internal rate of return of 35% with losses as specified by national studies and RF 1995 probabilities of success.

Constraint	Irrigated	Rainfed	Deepwater	Upland	Total
Insects	52	17	2	4	75
Diseases	76	15	2	5	98
Other pests	8	4	2	6	20
Abiotic stresses	36	9	6	20	71
Bioefficiency	164	10	1	4	179
Total	335	55	13	39	443

By far the largest gains from biotechnology research appear possible from improving biological efficiency in irrigated conditions. This result can be traced to the belief that bioefficiency gains are potentially very large even though it is recognized that they will take somewhat longer to achieve than incorporation of resistance to some specific pests. Overcoming diseases, insects, and abiotic stresses should all continue to receive considerable research support.

Table 3 compares the ranking of the 25 most important specific research goals based on expected productivity gains, as determined by the Foundation's original study and as determined by the latest available data and analysis. The big difference between the original analysis (left columns) and the latest (middle columns) is the much greater range of investment per constraint in the earlier analysis. The earlier data indicated that tungro virus should receive twice as much as the next ranked constraint and 10 times as much as the 10th-ranked constraint. (In making its allocations, the Foundation never concentrated its investments so drastically.) The more recent analysis still has tungro as the most important research area, but only 30% more than the second most important and four times as important as the 10th-ranked constraint.

The right columns of Table 3 show the optimal portfolio, excluding China. The most notable observation one can make from comparing the three sets of data is their broad consistency. The seven research problem areas ranked as most important using the earlier data are all among the top 11 RPAs in the second set of results and are identical with the top seven when China is excluded (right columns). Fifteen of the top 25 RPAs appear on all three lists.

Implications and conclusions

The methodology first applied by Evenson (1996) and used here is an advance over earlier methods for setting priorities among alternative rice research possibilities (Herdt 1991). The new methodology generates an optimal portfolio of research investments while earlier methodology generated a rank ordering of problem areas based on losses/gains. However, the quantitative results may, because they give the impression of greater precision, be misleading if not firmly based on empirical information of equal quality for all locations and research problems. That is a demanding requirement.

The quantitative results are useful pointers for research investment policy. They imply that there are quite large social returns to be made by investing in rice research. Those benefits will be shared between rice producers and consumers, and, perhaps, in the case of hybrid rice or strong enforcement of intellectual property rights, seed producers who incorporate attractive traits into seed. For much of the research, there seems little obvious way to induce private investment. Therefore, the Asian public, all of whom are rice consumers and many of whom are rice producers, have an interest in ensuring continuing investments. Hence, the results support the wisdom of continued significant public investment in rice research.

The allocation of public rice research funds across individual RPAs suggests the value of considerable investment on increasing the biological efficiency of the rice plant. Such research might include hybrid rices adapted to more different conditions,

Table 3. Optimal rice biotechnology research investments in the 25 most important research problem areas, ranked by two different estimates of losses, with and without China: annual research investment expected to provide a 35% internal rate of return.

Losses from Herdt (1991), current expected time to success	US$ million	Losses from national studies, current expected time to success	US$ million	Losses from national studies, current expected time to success, without China	US$ million
Tungro virus resistance	132	Tungro virus resistance	48	Tungro virus resistance	32
Cytoplasmic male sterility	76	Plant design	37	Brown planthopper resistance	19
Brown planthopper resistance	71	Cytoplasmic male sterility	36	Bacterial blight resistance	18
Upland drought/blast resistance	34	Reduced growth duration	29	Cytoplasmic male sterility	17
Bacterial blight resistance	29	Brown planthopper resistance	28	Yellow stem borer resistance	14
Yellow stem borer resistance	28	Higher photosynthetic efficiency	23	Upland drought/blast resistance	12
Greater lodging resistance	25	Bacterial blight resistance	22	Greater lodging resistance	9
Gall midge resistance	22	Yellow stem borer resistance	20	Drought resistance at anthesis	8
Weed resistance/tolerance	18	Cold, water-logged soil	18	Weed resistance/tolerance	7
Ragged stunt resistance	12	Upland drought/blast resistance	13	Sheath blight resistance	6
Improved seedling vigor	11	Greater lodging resistance	13	Gall midge resistance	6
Sheath blight resistance	10	Improved tillering ability	12	Blast resistance	6
Drought resistance at anthesis	10	Improved pollination ability	10	Drought resistance of seedlings	5
Submergence tolerance	8	Weed resistance/tolerance	10	Improved seedling vigor	4
Apomixis	7	Improved seedling vigor	9	Sheath rot resistance	4
Storage insect resistance	6	Salinity tolerance/resistance	8	Leaffolder resistance	3
Bird resistance/tolerance	6	Drought resistance at anthesis	8	Ragged stunt resistance	3
Salinity tolerance/resistance	5	Gall midge resistance	8	Submergence resistance/tolerance	3
Leaffolder resistance	4	Sheath blight resistance	7	Acid soils resistance	3
Blast resistance	4	Cold tolerance at anthesis	6	Bird resistance/tolerance	2
Striped stem borer resistance	3	Blast resistance	6	Salinity tolerance/resistance	2
Cold tolerance at seedling stage	3	Improved nutrient use efficiency	6	Storage insects resistance	2
Hispa resistance	3	Drought resistance of seedlings	5	Rice bug resistance	2
Grain discoloration resistance	2	Ragged stunt resistance	5	Armyworm resistance	2

apomixis to fix desirable traits, improving the plant's capacity to generate photosynthate and store it in grains, and perhaps redesigning the plant's architecture from the currently widely grown semidwarf to a plant with about the same height but fewer tillers and more grains per tiller (IRRI's "super rice"). Data are not available to indicate the present allocation of research funds to this set of objectives, but indications are that it is relatively modest, so increased investment to this area could be large.

Evenson (1996) suggests that scientists clearly expect biotechnology—the modern techniques for genetic transfers (wide crossing and transgenic)—to provide large gains, but that conventional plant breeding still has much to offer. Unfortunately, his four-technique classification does not indicate the role of molecular marker techniques that can be used to make "conventional breeding" more efficient. For example, advances in characterizing the blast pathogen using molecular techniques have given plant breeders new techniques for the evaluation of plants that promise more durable resistance to blast. Similarly, molecular markers that can be associated with the length and penetration ability of rice roots could help breeders develop more drought-resistant rices. It is clear that a mix of research approaches should be used in the future.

The results are inevitably a reflection of the data that went into the analysis. To the extent that the country studies provided comprehensive lists of research problem areas, good estimates of the constraints and good estimates of the area over which such constraints prevail, they are good indications of the opportunities for research targets. To the extent that the sample of national and international researchers was able to provide valid estimates of the gains possible from research, the progress already made, and the time needed to make further gains, they are good estimates of the potential of research to make gains.

The Rockefeller Foundation will use the data in Table 3 to guide its allocations of rice biotechnology investments over the next few years. We believe the relative investments indicated there are useful indicators of how our funds should be directed. Of course, the advancements made in addressing rice tungro disease over the past 8 yr mean that problem is close to a solution, and hence the investment in that line of work should be phasing down. Similar comments apply, although more tentatively, to brown planthopper, bacterial blight, and stem borer resistance. Progress is just beginning on drought, which the right columns in Table 3 indicate should have a combined investment of US$25 million, so we will continue to make considerable investments on those topics.

This analysis, unlike the earlier one (Herdt 1991) does not explicitly apply extra weights to increase the attention to RPAs that might be thought to be especially "pro-poor" or "pro-environment." The earlier analysis showed that within the range of options that can be conceived by biotechnologists, such weights have relatively little effect on the outcome. That is because the basic determination of relative poverty is ownership or control over resources. Biotechnology will contribute to making rice cheaper and more available to all and hence the poor, who spend larger fractions of income on rice, will gain relative to the wealthy (Hayami and Herdt 1977, David and Otsuka 1994). The best allocation from an equity view is therefore the one that leads to the most rapid improvements in productivity.

"Pro-environment'' biotechnology might be developments that reduce pesticide use or irrigation use or land degradation. Virtually all the traits directed at pests would replace or reduce the use of pesticides and hence are pro-environment. However, this includes nearly every RPA. The one possible exception would be the incorporation of herbicide tolerance genes. The Foundation has explicitly not backed such efforts in its program.

Finally, the list of RPAs explicitly excluded social science subjects like research on policy and agricultural extension. All acknowledge that policy and extension are important factors contributing to the speed with which biological innovations are adopted by farmers. But the purpose of this research was to determine how allocations should be made among biological research opportunities. So the nonbiological subjects were omitted from the list of possible research areas.

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High-density linkage map of rice with expressed sequence tags

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We have constructed a high-density linkage map of rice using an F_2 population derived from the cross between a japonica variety, Nipponbare, and an indica variety, Kasalath. A total of 1,383 markers, which consist of cDNA clones from callus and root, genomic clones as well as RAPD markers, have been mapped covering a distance of 1,575 cM. All cDNA clones have been sequenced and searched for similarities with known proteins and can be referred to as expressed sequence tags on the map. A majority of the genomic clones and RAPD markers was also sequenced to generate sequence-tagged sites. These extensive linkage analyses gave evidence on duplication of chromosomal segments, particularly in the distal region of chromosomes 11 and 12. Additional markers are being mapped using cDNA clones derived from other cDNA libraries such as green shoot, etiolated shoot, and developing seed. Ultimately, we would like to develop a saturated linkage map that will facilitate a more efficient utilization of molecular markers for rice improvement.

One of the most important advances in the field of biotechnology, which promises to revolutionize several areas of plant genetics and breeding, is the wide utilization of molecular markers. In conjunction with phenotypic and biochemical markers, these markers will have great impact in identifying and ultimately isolating genes for various agronomically important traits. In recent years, construction of RFLP linkage maps has been reported in a number of plants (Bernatzky and Tanksley 1986, Chang et al 1988, Rognli et al 1992, Da Silva et al 1993, Kleinhofs et al 1993). In rice, a molecular linkage map covering the entire genome was developed independently by McCouch et al (1988) and Saito et al (1991) with 135 and 322 markers, respectively. Such molecular maps may provide new opportunities for application in plant genetic manipulation, particularly in tagging genes for agronomically important traits with DNA markers. In addition, these maps could also serve as important tools in understanding the evolutionary relationships among different species as shown by the

synteny studies between such crops as wheat and rye (Rognli et al 1992), potato and tomato (Tanksley et al 1992), rice and maize (Ahn and Tanksley 1993), rice and wheat (Kurata et al 1994a), etc.

In the Rice Genome Research Program (RGP), we are constructing a high-density linkage map of rice with markers spaced at very close intervals throughout the genome. Most markers in this map have been sequenced to generate expressed sequence tags and sequence-tagged sites (STSs), and as such will be a model system for overall analysis of genome structure and function in plants. So far, a map with 1,383 DNA markers at an average interval of 300 kb and distributed along 1,575 cM on the 12 linkage groups has been reported by Kurata et al (1994b). Mapping of more DNA markers is currently in progress to generate a saturated map. This paper summarizes such results as well as some of the most recent findings in restriction fragment length polymorphism (RFLP) mapping at RGP.

Materials and methods

Plant materials

The parent strains consisted of a japonica variety, Nipponbare, and an indica variety, Kasalath. A single cross was made to obtain an F_2 population and 186 individuals were used for analysis of segregation of DNA polymorphism.

DNA manipulation

Total DNA was extracted from the green leaves of parental lines as well as the F_2 progenies by the CTAB method (Murray and Thompson 1980). Then 2 µg total DNAs were each digested with one of eight restriction enzymes, *Bam*HI, *Bgl*II, *Eco*RV, *Hind*III, *Apa*I, *Dra*I, *Eco*RI, and *Kpn*I, overnight at 37 °C. The digested samples were applied in 0.6% agarose gel, electrophoresed for 12 h and transferred in a positively charged nylon membrane by capillary blotting. These were used for hybridization with probes labeled with horseradish peroxidase according to the protocol of ECL direct nucleic acid labeling and detection system (Amersham).

DNA probes

The probes used for hybridization consisted mainly of cDNA clones, genomic clones, and RAPD markers all derived from japonica cultivar, Nipponbare. The cDNA clones consisted of randomly selected clones from callus and root cDNA libraries. The nucleotide sequence from the 5' end for 300–400 bp was determined and translated into an amino acid sequence. Then a similarity search at the protein level was performed in the NBRF-PIR data base using the FASTA algorithm. Clones showing an optimized matching score of more than 150 with amino acid sequences in other organisms were considered as functionally identical clones. All sequenced clones are registered and deposited at the DNA Data Bank of Japan (DDBJ).

The genomic clones used for mapping consisted of random genomic clones, YAC-end clones, *Not*I linking clones, and telomere-associated sequences (TELs). The random genomic clones were prepared by ligating *Hind*III or *Pst*I DNA fragments in

pBluescriptII SK+ or pUC vector. The YAC-end clones were derived from both ends of a large size DNA fragment cloned in YAC, amplified by PCR as 200-1000 bp long DNA, and ligated into TA cloning vector PCR™1000. The *Not*I linking clones consisted of *Sau*3AI partially digested 500–4000 bp fragments with *Not*I sites and cloned in pT7T318U vector at the *Bam*HI site. The TELs were obtained using cassette ligation-mediated PCR of *Sau*3A1 DNA digests and cloned in pCRII vector (Ashikawa et al 1994). For mapping of RAPD markers, 60 arbitrarily designed 10-nucleotide primers were initially subjected to RAPD analysis. Then, these primers were paired randomly and were used for detection of RAPD markers. Detection and mapping of RAPD markers and conversion of RAPD to STS markers were described by Monna et al (1994).

Linkage analysis

The segregation patterns and linkage relationships of RFLP in the F_2 population were analyzed using the MAPMAKER/EXP 3.0 software (Lander et al 1987). Multipoint analysis was performed to calculate the linkage of a large number of markers and produce a map of their order along the chromosomes. Recombination values between the markers were transformed into centimorgan (cM) distance by the Kosambi function (Kosambi 1944).

Results and discussion

RFLP map with 883 expressed sequences

To construct an RFLP linkage map of rice, we analyzed 2,950 cDNA clones from callus and root cDNA library. These clones showed various banding patterns such as single bands, double bands, as well as multiple bands with a smeared background in some cases, suggesting either single-copy sequences or repeated sequences in the genome. A total of 883 cDNA clones, which consisted of 465 clones from callus cDNA and 418 clones from root cDNA, showed distinct RFLP and were used for segregation analyses of the F_2 population derived from the cross Nipponbare/Kasalath. The positions of these clones represented by C-number and R-number for callus and root cDNA clones, respectively, are shown in Figure 1. A more detailed version of this map appeared in Kurata et al (1994b) and included such information as the accession number of the sequence data deposited in the DDBJ. In addition to cDNA clones, 265 genomic DNAs, 147 RAPD markers, and 88 other DNAs were also mapped for a total of 1,383 markers distributed along 1,575 cM on 12 linkage groups at an average interval of 1.14 cM.

A similarity search for proteins of other organisms showed that the cDNA clones have a high similarity to genes of a wide range of organisms including dicots, monocots, mammals, and yeast (Table 1). Most of these genes code for isozymes such as alcohol dehydrogenase (*adh*), aspartate aminotransferase (*got*), fructose bisphosphate aldolase (ald), glucose-6-phosphate isomerase (*pgi*), peroxidase (*pox*), etc. In the conventional linkage map, several isozymes have been mapped and assigned to specific chromosomes (Wu et al 1988). In our RFLP linkage map, we determined the loci of

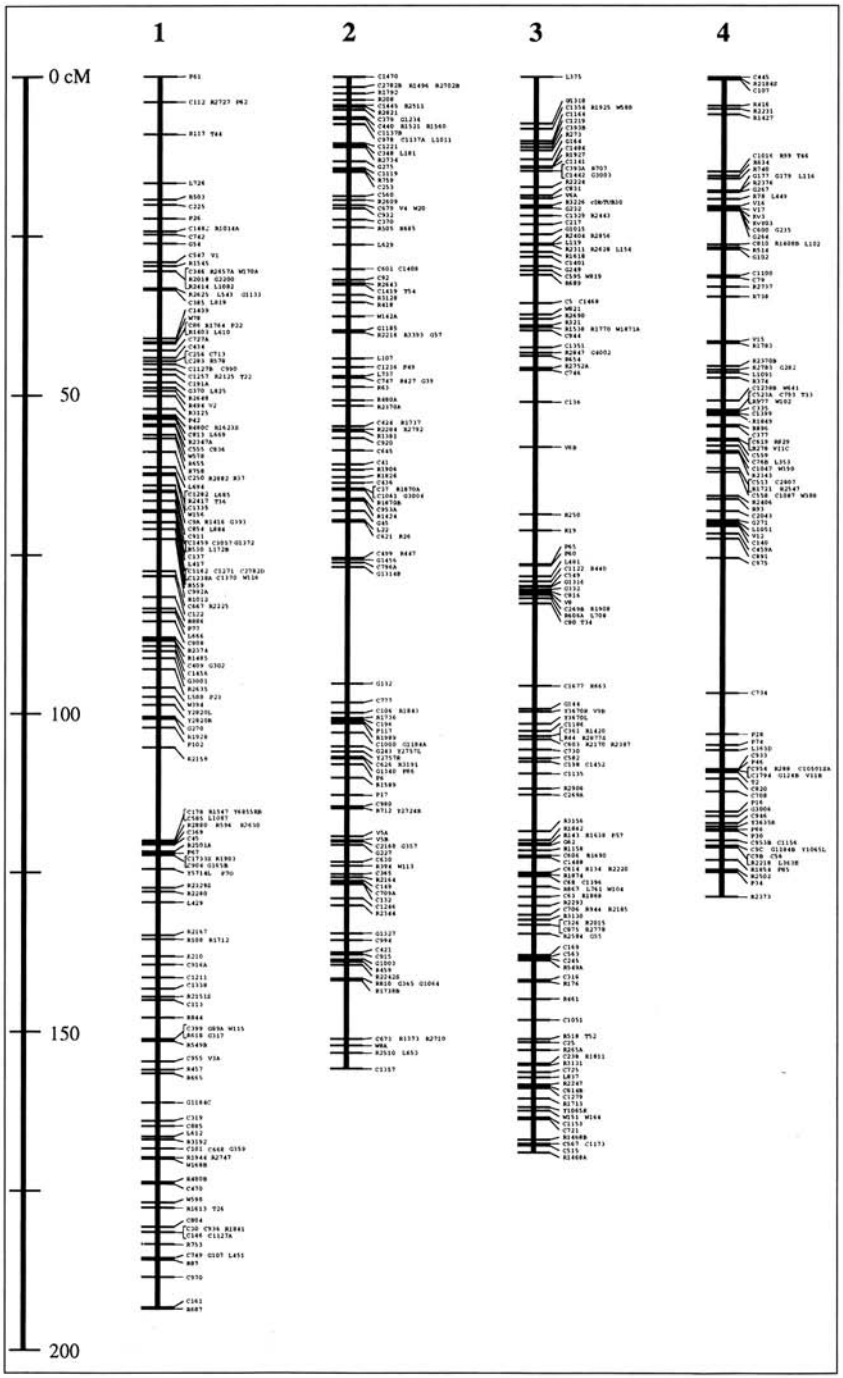
1
2
3
4
0 cM
50
100
150
200

Fig. 1. An RFLP linkage map of rice constructed with 1,383 DNA markers. The markers are designated as follows: C, clones from callus cDNA library; R, clones from root cDNA library; G, random genomic clones; L, *Not*I linking clones; Y, YAC-end clones (L and R after the Y-number indicate left and right end clones); P, RAPD markers; T, RAPD markers converted to STS; TEL, telomere-associated sequences; W, wheat clones; and V, clones from sources other than RGP.

Figure 1 continued

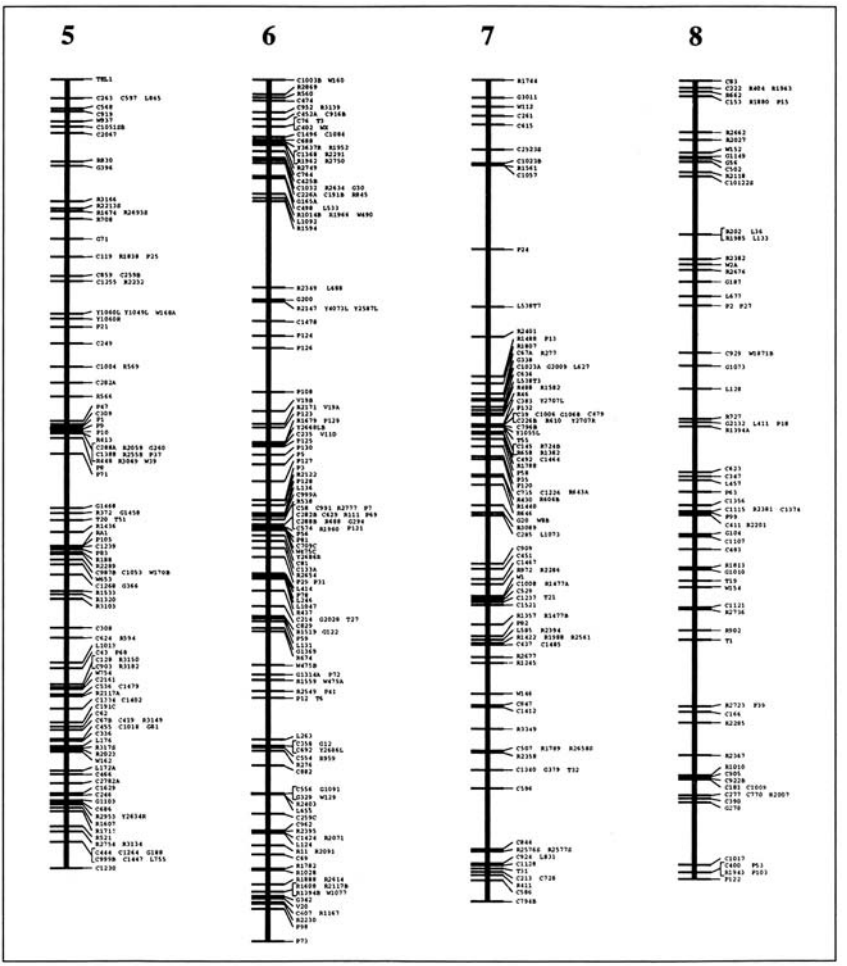

Figure 1 continued

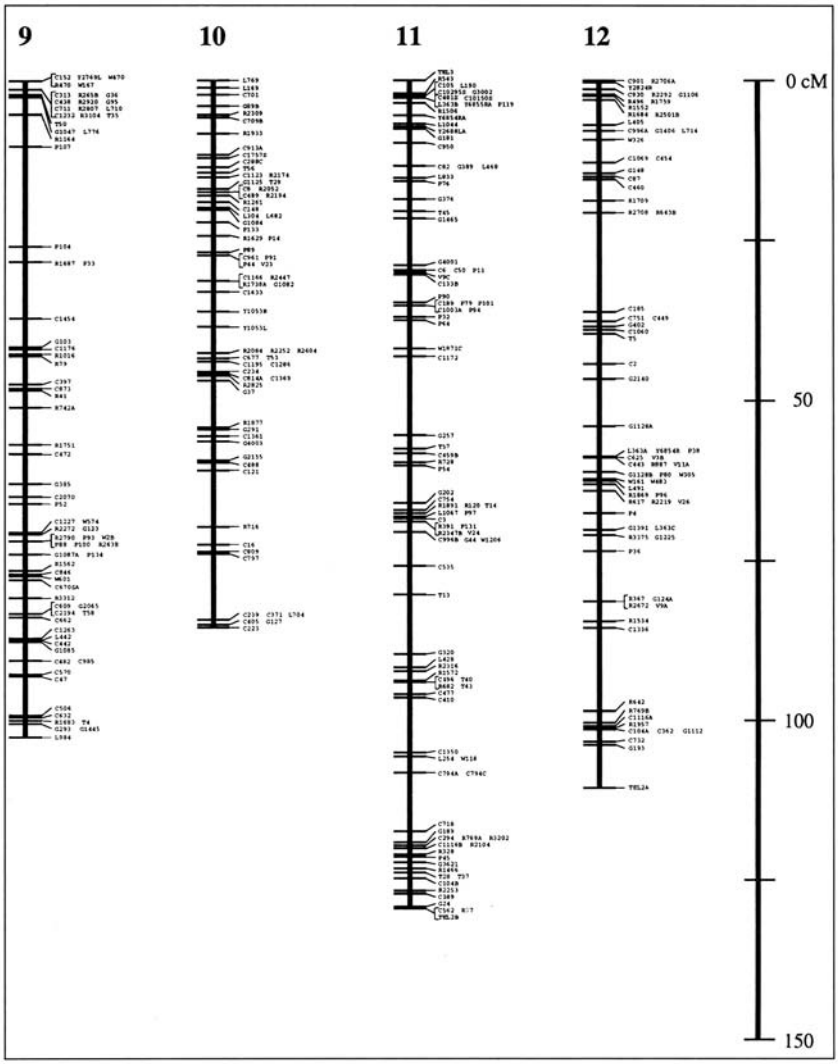

Table 1. Callus and root cDNA clones mapped in rice with similarity to known proteins.

Chr.	Position	Marker	Gene	Protein name	Organism	DDBJ ID No.
1	30.4	R2657A	*ald2*	Fructose bisphosphate aldolase	Rice	D28322
1	41.8	R1764	*got2*	Aspartate aminotransferase	*Bacillus sp.*	D24345
1	42.9	C727A	*gco1*	Glucan endo-1,3-beta-glucosidase	Common tobacco	D15500
1	44.5	C256	*rcs*	Reductase	Soybean	D15203
1	44.5	R578	*hbo*	(S)-tetrahydroberberine oxidase	*Coptis japonica*	D23922
1	49.3	R494	*nia*	Nitrate reductase (NADH)	Tomato	D23879
1	53.1	R1623S	*tub3*	Tubulin beta-2 chain	*Arabidopsis thaliana*	D24277
1	58.9	C250	*got1*	Aspartate aminotransferase	Proso millet	D23735
1	58.9	R37	*glt1*	Glutathione transferase 1	Maize	D32736
1	63.6	C9A	*elf3*	Elongation factor 2	*Caenorhabditis elegance*	D15078
1	64.5	C911	*gtl*	Glutamin:tRNA ligase	Human	D15594
1	69.2	R559	*ppp*	Phosphoprotein phosphatase	Human	D23910
1	70.3	C922A	*gbp*	GTP-binding regulatory protein beta chain	*Chlamydo-monas reinhardtii*	D22667
1	71.9	R1012	*lcl*	Long-chain-acid:CoA ligase	Human	D24049
1	81	R886	*mdh*	Malate dehydrogenase, mitochondrial	Water melon	D24025
1	84.7	C808	*eif2*	Initiation factor eIF-4A	Curled-leaved tobacco	D22665
1	87.7	C409	*sip*	Stress inducible protein STI1	Yeast	D15287
1	90.4	R2635	*soi*	Spil hypothetical protein	Yeast	D24836
1	99.9	R1928	*vcp*	Vasoline-containing protein	Pig	D28306
1	119.1	C585	*secl*	SEC 7 protein	Yeast	D15403
1	119.4	R2630	*hud*	Elav/Sex-lethal related protein	Human	D24832
1	119.4	R596	*glt2*	Glutathione transferase 1	Maize	D28287
1	119.4	R2880	*osb*	Oxysterol-binding protein	Rabbit	D24980
1	119.5	C369	*gdh*	Glutamate dehydrogenase (NAD(P)+)	*Halobacterium salinarium*	D15259
1	121.3	C904	*sall*	SalT protein precursor	Rice	D28208
1	126.4	R476	*ams1*	S-adenosylmethionine synthetase 2	*Arabidopsis thaliana*	D28266
1	126.9	R2280	*ams4*	S-adenosylmethionine synthetase 2	*Arabidopsis thaliana*	D24629
1	133.7	R2167	*ams3*	S-adenosylmethionine synthetase 2	*Arabidopsis thaliana*	D28314
1	137	R210	*cad1*	Cathepsin D	Human	D23806
1	142.1	C1338	*ang*	58K antigen	*Ricketssia tsutsuga-mushi*	D22792
1	149.6	C399	*idh*	Isocitrate dehydrogenase (NADP+)	Alfalfa	D15280
1	155.3	R665	*rac1*	Rac1 protein	Human	D23963
1	165.7	R3192	*spk*	Serine/threonine-specific protein kinase	*Arabidopsis thaliana*	D25110
1	172.4	R480B	*ypt*	Transforming protein, ypt 1, homolog	Maize	D23874
1	180.3	C936	*mtn*	Metallothionein-like protein	*Arabidopsis thaliana*	D15602
1	180.3	C30	*tpi*	Triose phosphate isomerase	Maize	D15092
1	181.6	R753	*sds*	C-5 sterol desaturase	Yeast	D23996
1	184.1	R87	*tin*	Trypsin inhibitor	Rice	D23762

Table 1 continued.

Chr.	Position	Marker	Gene	Protein name	Organism	DDBJ ID No.
2	1.6	R2702B	*hsp5*	Heat shock protein 70	Common tobacco	D23418
2	4.4	C1445	*aux*	Auxin-induced protein	*Arabidopsis thaliana*	D15870
2	6.3	C440	*dfr1*	Dihydrofolate-4-reductase	Garden petunia	D15312
2	6.6	C1137B	*dfr2*	Dihydrofolate-4-reductase	Garden snapdragon	D15715
2	7.4	C1137A	*dfr2*	Dihydrofolate-4-reductase	Garden snapdragon	D15715
2	32.3	C92	*ant*	Adenine nucleotide translocator	Rice	D22519
2	33.1	C1419	*thr*	Thioredoxin reductase (NADPH)	*Escherichia coli*	D13855
2	34.6	R3128	*eno2*	Enolase	Tomato	D25085
2	40.5	R3393	*clc*	Clathrincoat assembly protein	Rat	D24586
2	51.2	R480A	*ypt*	Transforming protein, ypt1, homolog	Maize	D23874
2	55.3	R1737	*prs*	Proteasome XC3 chain	African clawed frog	D24326
2	55.8	R2284	*ams5*	S-adenosylmethionine synthetase 2	*Arabidopsis thaliana*	D24632
2	63.3	R1826	*nab*	X16 protein	Mouse	D24389
2	65	C37	*gpdl*	Glyceraldehyde-3-phosphate dehydrogenase	White mustard	D15096
2	67	R1424	*ste1*	Regulatory protein STE7	Yeast	D24144
2	70.3	C621	*reg1*	14-3-3 protein	Barley	D15430
2	75.9	R447	*sac*	SAC1 protein	Yeast	D23860
2	103.4	C1000	*hsp3*	Heat shock protein 70	Maize	D15636
2	107.2	C626	*cyc*	cyc07 protein, S-phase specific periwinkle	Madagascar	D15433
2	120.6	C2168	*got3*	Aspartate aminotransferase	Proso millet	D16037
2	138.2	C915	*stk*	Kinase-related transforming protein	Mouse	D15597
2	139.3	R459	*gdc2*	Glycine-cleavage system protein H	Garden pea	D23865
2	139.8	R2242S	*tub4*	Tubulin beta-2 chain	Garden pea	D24606
2	142	R810	*ubq4*	Ubiquitin	Garden snapdragon	D25349 D25350
2	151.4	R2710	*urt2*	UTP:glucose-1-phosphate uridyltransferase	Potato	D24887
3	14.7	R707	*qpc*	Ubiquinone binding protein QP-C	Bovine	D23977
3	18.5	C831	*rad6*	RAD6 DNA-repair homolog *Dhr6*	Fruit fly	D22670
3	20.1	R3226	*cof*	Cofilin	Yeast	D25113
3	21.7	R2443	*myb*	Transforming protein, myb, homolog	Maize	D24724
3	21.7	C1329	*pgi*	Glucose-6-phosphate isomerase	*Clarkia lewesii*	D15815
3	26.1	R2856	*cak*	Casein kinase II alpha chain	Maize	D24965
3	26.1	R2404	*eif4*	Initiation factor eIF-5A	Common tobacco	D24702
3	26.3	R2628	*tpa*	Transplantation antigen P198	Mouse	D24830
3	35.4	C1468	*tub2*	Tubulin alpha-2 chain	Maize	D15886
3	37.9	R2690	*act*	Actin 1	Rice	D24576
3	39.2	R1538	*reg4*	14-3-3 protein	Barley	D24218
3	43.2	R2847	*gco2*	Beta-glucosidase	White clover	D24959
3	45.9	C746	*gri*	Glycine rich protein 2	*Arabidopsis thaliana*	D15512
3	79.7	C549	*hsp1*	Heat shock protein 70	Spinach	D22613

Table 1 continued.

Chr.	Position	Marker	Gene	Protein name	Organism	DDBJ ID No.
3	81.2	R1908	*acb*	Endozepine	Yeast	D28303
3	103.7	R2170	*uqn*	NADH dehydrogenase (ubiquinone) chain 2	*Paramecium tetraurelia*	D28315
3	107.2	C1452	*sod*	Superoxide dismutase	Rice	D15675
3	119.6	R1862	*prp*	Prp 16-1 protein	Yeast	D24417
3	121.2	R1158	*snr*	Small nuclear RNA-associated protein	Human	D24080
3	122	R1690	*eif3*	Initiation factor 2 alpha chain	Yeast	D24301
3	128.5	C63	*ubq1*	Ubiquitin fusion protein	Fruit fly	D15108
3	134.2	R2584	*cdh*	Cinnamyl-alcohol dehydrogenase	Kidney bean	D14802
3	150.7	R518	*elf1*	Elongation factor 1 alpha	Tomato	
3	160	R1713	*glt3*	Glutathione transferase III	Maize	D24311
3	166.5	R1468A	*cdc*	CDC2a protein	Rice	D24174
4	4.7	R416	*aox*	Amine oxidase	Rat	D23854
4	15.5	R634	*ocp*	Oryzain alpha chain	Rice	D23944
4	16	R740	*gyk*	Glycerol kinase	*Bacillus subtilis*	D23993
4	19.3	R78	*kin*	ncdD protein	Fruit fly	D23757
4	53.3	R1849	*art*	Arabinose transport protein	*Escherichia coli*	D24407
4	54.6	R896	*gpd2*	Glyceraldehyde-3-phosphate dehydrogenase	Maize	D28294
4	57.4	C559	*ppa*	Inorganic pyrophosphatase	Yeast	D15382
4	59	C1047	*reg3*	14-3-3 protein	Barley	D15663
4	109.2	R288	*ccp*	Cytochrome C peroxidase	Yeast	D23832
4	109.2	C954	*dds*	Dihydrodipicolinatesynthase	Wheat	D15614
4	109.2	C1794	*his1*	Histone H1	Wheat	D22924
4	121.3	C9B	*elf3*	Elongation factor 2	*Caenorhabditis elegance*	D15078
5	27.9	R1838	*dnj*	dnaJ protein homolog	Human	D24399
5	30.9	C259B	*ubq2*	Ubiquitin	Tomato, potato oat	D22550
5	45	R569	*omc*	2-oxoglutarate/malate carrier protein	Bovine	D23915
5	55.5	R2059	*rbp*	Ribophorin	Human	D24495
5	55.5	C1388	*rab11*	GTP-binding protein rab11	Dog	D15842
5	55.5	R2558	*acc*	Acetyl-coA carboxylase	Yeast	D24786
5	95.2	R3182	*hsp6*	Heat shock protein cognate 70	Tomato	D25105
5	95.2	C128	*ubc*	Ubiquitin conjugating protein	Wheat	D15130
5	96.8	C536	*pdc*	Pyruvate decarboxylase	Maize	D15369
5	102.2	C67B	*rif*	ADP-ribosylation factor 4	Human	D22513
5	102.2	C419	*cam*	Calmodulin	Wheat	D15295
5	109	C466	*mpp*	Processing peptidase catalytic chain, mitochondrial	Yeast	D15329
5	113.4	C686	*atp1*	H+-transporting ATP synthase beta chain	Rice	D15470
5	113.7	R2953	*dyl*	Dynamin-like protein	Fruit fly	D25026
5	118	R2754	*cad2*	Cathepsin D	Human	D24912
5	119.6	C1264	*kri*	Ketol-acid reductoisomerase chloroplast	Spinach	D27768
6	2.2	R2869	*pgd*	Phosphogluconate dehydrogenase	*Synechococcus* sp.	D24970
6	9.2	C688	*prt*	Transcription factor for E3	Human	D15472
6	9.8	R2291	*ste2*	Regulatory protein STE7	Yeast	D24636
6	10.1	R2749	*cys*	Cysteine synthase B	Pepper	D24907

Table 1 continued.

Chr.	Position	Marker	Gene	Protein name	Organism	DDBJ ID No.
6	11.2	C764	*hca*	Classll histocompatibility antigen	Human	D15525
6	12.6	C1032	*ag12*	Floral homeotic protein AGL2	*Arabidopsis thaliana*	D15657
6	13.1	R845	*ctl*	Cystathionine gamma-lyase	Yeast	D28293
6	17.9	R1966	*sus*	Sucrose synthase	Barley	D24462
6	34.8	R2147	*sal2*	Salt protein	Rice	D24547
6	57	C235	*hmg2*	High mobility group-like protein NHP2	Yeast	D15191
6	69.8	R111	*fdh*	Formate dehydrogenase	*Pseudomonas* sp.	D23770
6	69.8	C58	*srp*	Signal recognition particle 19K	Human	D15105
6	112	C556	*gdc1*	Glycine-cleavage system protein H	Garden pea	D15379
6	112.1	R2403	*pgk*	Phosphoglycerate kinase, cytosolic	Wheat	D26320
6	115.2	C259C	*ubq2*	Ubiquitin	Tomato, potato, oat	D22550
6	121.5	C69	*eifl*	Initiation factor eIF-4A	Curled-leaved tobacco	D15109
6	126.2	R1888	*ams2*	S-adenosylmethionine synthetase 2	*Arabidopsis thaliana*	D24436
6	127.3	R1394B	*nod*	Nodulation protein	*Rhizobium legumino-sarum*	D24124
6	128.9	R1167	*cat*	Catalase chain I	Maize	D24082
6	128.9	C607	*hmg1*	High mobility group protein	Wheat	D28196
7	40.3	R2401	*thx*	Thioredoxin	*Arabidopsis thaliana*	D24700
7	46.5	R1488	*hxk*	Hexokinase P1	Yeast	D24182
7	49.2	C67A	*rif*	ADP-ribosylation factor 4	Human	D28199
7	54.2	R610	*mak*	MAK16 protein	Yeast	D23935
7	54.2	C479	*sps*	Spermidine synthetase	Human	D22594
7	55.4	C492	*gcw3*	Glysine-rich cell wall structural protein	Garden petunia	D22596
7	88	R2394	*cpk*	Protein kinase, calcium dependent	Soybean	D24697
7	98.5	C1412	*elf2*	Elongation factor1 beta chain	Rice	D15852
7	101.9	R3349	*cvt*	Cystathionine gamma-lyase	Potato	D25146
7	105.3	C507	*cpn*	Probable chaperonin	*Synecho-coccus* sp.	D26192
7	108.4	C1340	*par*	Par gene protein	Common tobacco	D22794
7	124.1	C213	*odh*	Oxoglutarate dehydrogenase	*Escherichia coli*	D15178
7	124.6	R411	*tab*	Tat-binding protein	Human	D23852
7	125.4	C586	*gcw1*	Glycine-rich cell wall structural protein	Garden petunia	D22623
8	1.1	R1963	*map*	Membrane alanyl aminopeptidase	*Escherichia coli*	D28310
8	1.8	R662	*hyp2*	Hypothetical protein 1 (sul 3' region)	*Bacillus subtilis*	D23961
8	2.6	R1880	*acl*	Acyl carrier protein 3	Barley	
8	23.5	R1985	*pkc2*	Protein kinase C homolog	Rice	D24464
8	27.9	R2382	*pat*	Patatin T5	Potato	D24690
8	42.5	C929	*reg2*	14-3-3 protein	Barley	D22692
8	53.9	R1394A	*nod*	Nodulation protein	*Rhizobium legumino-sarum*	D24124

Table 1 continued.

Chr.	Position	Marker	Gene	Protein name	Organism	DDBJ ID No.
8	100.5	R2285	*gdh*	Glucose dehydrogenase (pyrroloquinoline-quinone)	*Acinetobacter calcoaceticus*	D24633
8	109.1	C922B	*gbp*	GTP-binding regulatory protein beta chain	*Chlamydomonas reinhardtii*	D22667
8	111.7	C277	*rpa*	Acidic ribosomal protein 4	Fruit fly	D15212
9	0.8	C711	*pab*	Polyadenylate-binding protein	Human	D15488
9	46.7	C397	*sco1*	SCO1 protein	Yeast	D22575
9	74.6	R1562	*hsp4*	Heat shock protein 82	Rice	D24234
9	75.1	C846	*pkc1*	Protein kinase C homolog	Rice	D15569
9	78.7	R3312	*gco3*	Beta-glucosldase B	*Bacillus polymyxa*	D28326
9	88.4	C985	*hsp2*	Heat shock protein 82	Rice	D22707
9	97	C506	*hmg3*	High mobility group protein	Maize	D22603
9	97.3	C632	*urt1*	UTP:glucose-1-phosphate uridyltransferase	Potato	D15437
10	2.3	C701	*adh2*	Alcohol dehydrogenase	Human	D15481
10	11.7	C913A	*eno1*	Enolase	Tomato	D28210
10	17.6	C489	*atp2*	H+-transporting ATP synthase gamma chain	*Rhodospirilum rubrum*	D15343
10	42.7	R2604	*gcw4*	Glycine-rich cell wall structural protein	Rice	D24186
10	42.7	R2252	*hyp4*	Hypothetical protein YCL59C	Yeast	D24612
10	43.5	C677	*gcw2*	Glycine-rich cell wall structural protein	Rice	D13464
11	9.2	C950	*tum*	Tumor protein	*Arabidopsis thaliana*	D22697
11	65	R120	*ahc*	Adenosyl homocysteinase	Rat	D23773
11	65.8	C3	*sec2*	Sec23 protein	Yeast	D22492
11	91	R1572	*adh2*	Alcohol dehydrogenase	Rice	D24243
11	91.3	C496	*adh1*	Alcohol dehydrogenase	Maize	D15347
11	91.3	R682	*adh2*	Alcohol dehydrogenase	Maize	D23967
11	114	R3202	*cbp*	Calcium binding protein	Mouse	D25111
12	1.4	R2292	*rab5*	GTP-binding protein rab5	Dog	D28317
12	14.5	C1069	*hyp1*	Hypothetical protein	Maize	D15675
12	72.6	R3375	*cla*	Clathrin-associated protein 17	Rat	D25151
12	83	R2672	*elf4*	Elongation factor selB	*Escherichia coli*	D24864
12	87.1	C1336	*ald1*	Fructose-biphosphate aldolase	Rice	D28223
		15 mapped	*pox*	Peroxidase	Horseradish and turnip	
		3 mapped	*his2a*	Histone H2A	Mainly wheat and maize	
		4 mapped	*his2b*	Histone H2B	Mainly wheat and maize	
		4 mapped	*his3*	Histone H3	Mainly wheat and maize	
		5 mapped	*his4*	Histone H4	Mainly wheat and maize	
		24 mapped	*rpl*	Ribosomal protein large subunit	Mainly rat	
		15 mapped	*rps*	Ribosomal protein small subunit	Mainly rat	

these isozymes by mapping cDNA clones derived from callus and root cDNA libraries. Thus, such genes as *got, adh,* and *pox,* which have been assigned in the conventional linkage map by segregation analysis of gene products, could be accurately mapped with their exact locations in the chromosome. In addition, a number of genes, which code for structural proteins such as actin, tubulin and ubiquitin, genes associated with the glycolytic pathway, genes related to the cell cycle, as well as heat shock proteins,

were also mapped. Some of these genes, however, did not necessarily correspond to a specific gene sequence but rather to one of the highly conserved multiple copies in the genome and were mapped in several loci in one or more chromosomes.

Several multigene families such as ribosomal proteins and histones, which have been identified from the large-scale cDNA analysis, have also been mapped. Twenty-four genes of the large subunit ribosomal protein and 15 genes of the small subunit ribosomal protein were found to be widely distributed in the rice genome. We have also identified and mapped the genes for histone proteins, namely, H1, H2A, H2B, H3, and H4 proteins. In human and other animals, these five types of genes formed clusters or repeated tandem units. In rice, however, they were found to be widely distributed in several chromosomes.

Thus, construction of a detailed genetic map using expressed gene sequences may provide a vast amount of information on the structural and functional organization of the rice genome. This could be very useful in identifying a gene of interest as well as in the subsequent stage of manipulation and isolation.

Genomic DNA markers as sequence-tagged sites

The chromosomal distribution of genomic clones classified as random genomic clones (G-number), *Not*I linking clones (L-number), YAC-end clones (Y-number), and TELs were also determined (Fig. 1). One hundred and thirty-seven randomly selected genomic clones were evenly distributed on the map. Most of these genomic clones have been sequenced and registered at DDBJ. Thus, these clones can be referred to as STSs on the map. The YAC-end clones and *Not*I linking clones were used for mapping to determine the nature of these sequences, which was necessary for physical map construction. However, mapping of 33 YAC-end clones (Y-number) and 90 *Not*I linking clones (L-number) did not show any specific features in terms of distribution and chromosomal localization of these clones. Among the mapped YAC-end clones were those containing both ends of the DNA fragment in YAC. These clones were mapped at close proximity to each other so that the physical distance corresponding to the genetic distance in cM can be calculated.

The map positions of TELs isolated using cassette ligation-mediated PCR were also determined (Ashikawa et al 1994). Two of these clones have been located on opposite ends of chromosome 11 so that this chromosome could be completely saturated with DNA markers. Subtelomeric clones have also been mapped on one end of chromosome 12 as well as chromosome 5.

RAPD markers were used to fill such regions on the map with very few markers. More than 150 RAPD were detected between Nipponbare and Kasalath using 1,400 combinations of arbitrarily designed 10-nucleotide primers (Monna et al 1994). One hundred and forty-seven RAPD markers represented by P-number and T-number on the map were mapped on the 12 chromosomes of rice. The T-number markers correspond to RAPD markers, which were converted to STS. More importantly, regions in some chromosomes that cannot be linked by DNA markers had been successfully connected by RAPD markers. The distal regions of chromosomes 1, 6, and 8 were extended by RAPD markers P61, P73, and P122, respectively. These suggest that

RAPD markers can be very useful to fill gaps or to extend the linkage map of each chromosome.

Synteny with the wheat genome

To clarify the relationships of the rice genome with other crops, 60 wheat genomic DNA fragments (W-number) have been mapped on our high-density linkage map in collaboration with the Cambridge Laboratory, John Innes Centre, UK. The results showed that most of these markers have the same linkage order in wheat and rice (Kurata et al 1994a). Furthermore, it has been clarified that rice chromosome 1 corresponds to wheat group 3, rice chromosome 2 to wheat 6, rice 3 to wheat 4, rice 4 and 7 to wheat 2, rice 5 to wheat 1, rice 6 to wheat 7, and rice 9 to wheat 5. This suggests conservation of genome structure between rice and wheat, which are from different Gramineae tribes and differ in both chromosome number and genome size. We are also pursuing reciprocal mapping of DNA probes with other crops such as barley and maize. Eventually, we hope to clarify the extent of synteny and linkage conservation among cereal crops.

Conserved linkage order in chromosomes 11 and 12

Although most of the clones used as probes showed a single-copy band on genomic Southern hybridization, some DNA probes had two or more bands and were located in duplicate or triplicate loci. Seventy-nine probes (6.1% of the total mapped DNA probes) were mapped on more than one locus. Duplicate segments were particularly observed between chromosomes 11 and 12 (Nagamura et al 1995, Fig. 2). Thirteen of the 33 mapped DNA markers at the distal regions of these chromosomes, including a TEL (TEL2), were mapped as duplicate loci. These duplicated segments occupy 10 and 1 1.8 cM in chromosomes 11 and 12, respectively. The other 20 markers in these regions also showed two or more main bands, but only one band was polymorphic, which was mapped in either chromosome 11 or 12. This suggests that RFLP mapping can also be an effective method to clarify chromosomal rearrangements as well as conservation of gene order accompanied by the evolution of a species.

Toward a saturated linkage map and more

At present, we are mapping additional markers in our RFLP linkage map to create a tighter linkage. In addition to callus and root, we are also using cDNA clones from green shoot, etiolated shoot, and developing seed cDNA libraries. As of Mar 1995, we have mapped an additional 521 DNA markers so that our map now has 1,904 DNA markers and a length of 1,556 cM. The average interval between markers is about 0.8 cM. However, there are still several regions in some chromosomes with very few markers as well as long stretches without any markers. Thus, it is necessary to screen for more markers to fill these gaps or to analyze the exact nature of such regions in the chromosomes.

Ultimately, we would like to establish a map with about 2,000 DNA markers at very close intervals necessary for physical map construction and gene tagging. Selection and ordering of YAC clones covering the entire genome to construct a detailed physical

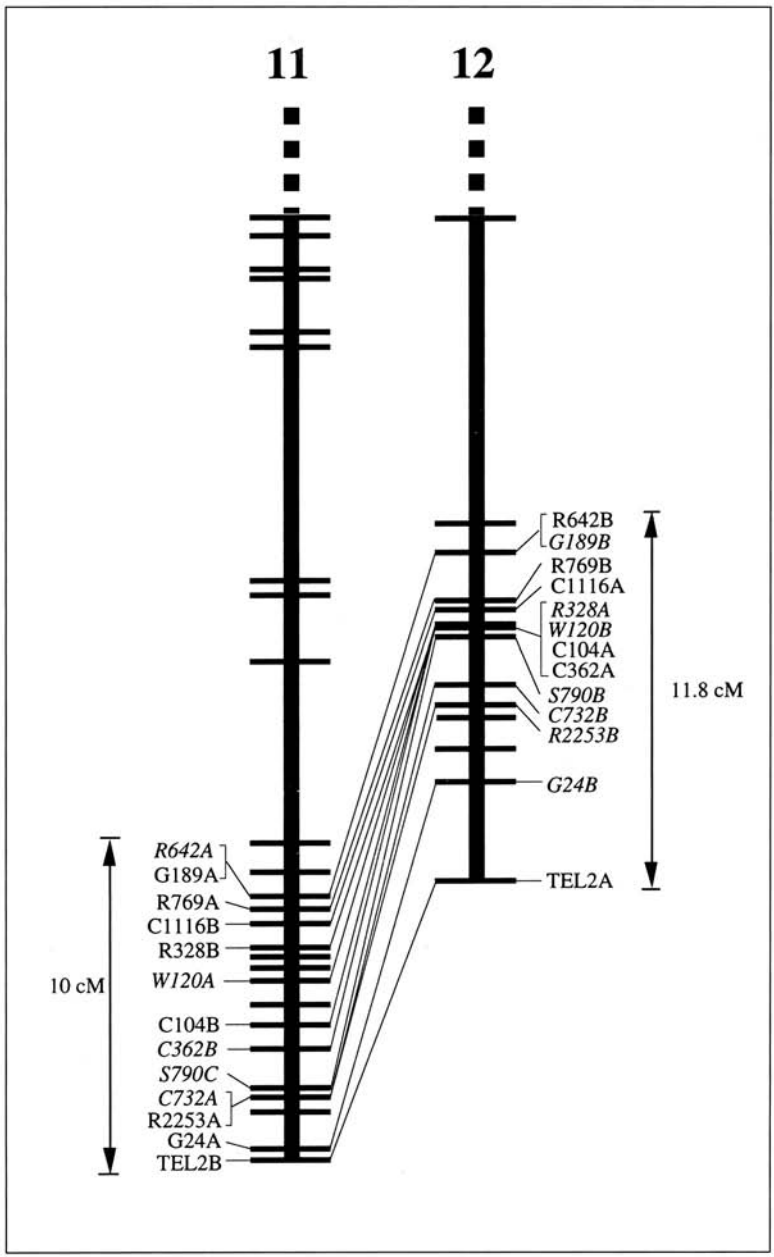

Fig. 2. The distal region of chromosomes 11 and 12 with highly conserved linkage of 13 DNA markers. Marker designations are described in Figure 1. Markers in italics were mapped after the publication of the linkage map in Kurata et al (1994b).

map of rice is in progress. Tagging of genes controlling phenotypical traits, which are important agronomically and for scientific studies, is also under way. We have already identified the chromosomal locations of such genes as *Xa1* (bacterial blight resistance gene) and *Se1* (photoperiod sensitivity gene). Isolation of these genes is expected to progress efficiently through positional map-based cloning with tagged DNA markers by using physically arrayed YAC or cosmid clones.

Thus, a high-density linkage map of rice will have far-reaching applications in understanding genome organization, function, and evolution. More importantly, it is expected to have enormous impact on the more practical aspect of plant genetic manipulation, that is, for marker-aided selection in breeding programs as well as for map-based cloning of agronomically important genes.

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An integrated linkage map of rice

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To furnish significant information for genome analysis of rice, we have tried to integrate the morphological and restriction fragment length polymorphism (RFLP) linkage maps of all chromosomes. Thirty-nine morphological markers and 82 RFLP markers were mapped together based on segregation analysis of 19 F_2 populations derived from the crosses between indica variety IR24 and japonica lines with different morphological markers. Both linkage maps of rice were completely orientated. The gene *d2,* which had been thought to belong to the linkage group of chromosome 4, was linked to some RFLP markers on chromosome 1. Therefore, chromosomal location of *d2* was shifted to chromosome 1 from chromosome 4. Unmapped genes *gl1* on chromosome 5 and *I-Bf* on chromosome 9 were mapped on their respective linkage groups with some RFLP markers. The segregation distortion was detected on chromosomes 1, 2, 3, 6, 11, and 12. In these regions, the japonica/japonica allele frequencies were significantly less than the normal F_2 frequency of 25%. The RFLP linkage maps on chromosomes 2, 3, 6, 7, and 8 were considered not to be saturated. On these chromosomes, some marker genes were estimated to be located beyond the respective terminal ends of RFLP linkage maps.

Rice linkage studies have been conducted for the last 50 yr. Nagao and Takahashi (1963) proposed 12 possible linkage groups corresponding to the haploid chromosome complement of rice (n=12). Cytogenetic stocks such as trisomics and reciprocal translocations were used to establish relationship between these linkage groups and the 12 chromosomes of rice (Iwata 1986).

Recently, restriction fragment length polymorphism (RFLP) linkage maps of rice have been constructed (McCouch et al 1988, Saito et al 1991, Causse et al 1994, Kurata et al 1994). It is important to integrate the morphological linkage map with the RFLP linkage map for various genetic studies involving molecular tagging of genes

and marker-aided selection. Although some efforts to integrate the morphological linkage map and the RFLP linkage map have been reported (Kishimoto et al 1992, Causse et al 1994, Yu et al 1995), the integrated linkage map of all 12 chromosomes of rice had not been accomplished until now. In this study, we describe an integrated linkage map of the 12 chromosomes of rice constructed from the morphological linkage map (Iwata et al 1984, 1989a,b) and the RFLP linkage map (Saito et al 1991).

Materials and methods

Plant materials

Nineteen F_2 populations derived from the crosses between indica variety IR24 and japonica lines with from one to four genetic marker genes were used. Thirty-nine segregating marker genes of these F_2 populations are listed in Table 1.

RFLP analysis

Eighty-two RFLP markers mapped on the molecular map (Saito et al 1991) were used. DNA of F_2 populations were extracted from leaves and digested with six different restriction enzymes (*Bam*HI, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, and *Hin*dIII). Electrophoresis, Southern blotting, and hybridization were performed as described by Saito et al (1991).

Linkage analysis

Linkage analysis was performed on F_2 segregation data. Recombination values were estimated by the maximum likelihood equation (Allard 1956), and the recombination values were converted into genetic map distances (cM) using the Kosambi function (Kosambi 1944).

Table 1. Marker genes used in this study.

Chromosome	Marker genes[a]
1	*fs2, d18, d2, rl2, spl6, eg*
2	*tri, bl1, spl2*
3	*chl1, fcl, dl, sp13*
4	*d11, Ph, lg*
5	*gl1, d1, spl7, nl1*
6	*dp1, spl4, Cl*
7	*g1, d6, spl5, v11*
8	*v8, sug*
9	*lBf, dp2, drp2, Dn1*
10	*spl10, pgl*
11	*z2, v9*
12	*spl1, rl1*

[a] See Kinoshita (1993).

Results and discussion

Among the 172 RFLP markers randomly selected from the molecular map (Saito et al 1991), a total of 147 RFLP markers gave polymorphism in the genomic Southern hybridization patterns of indica variety IR24 and japonica variety Nipponbare. Among the 147 RFLP markers, 82 RFLP markers randomly selected were used for linkage analysis.

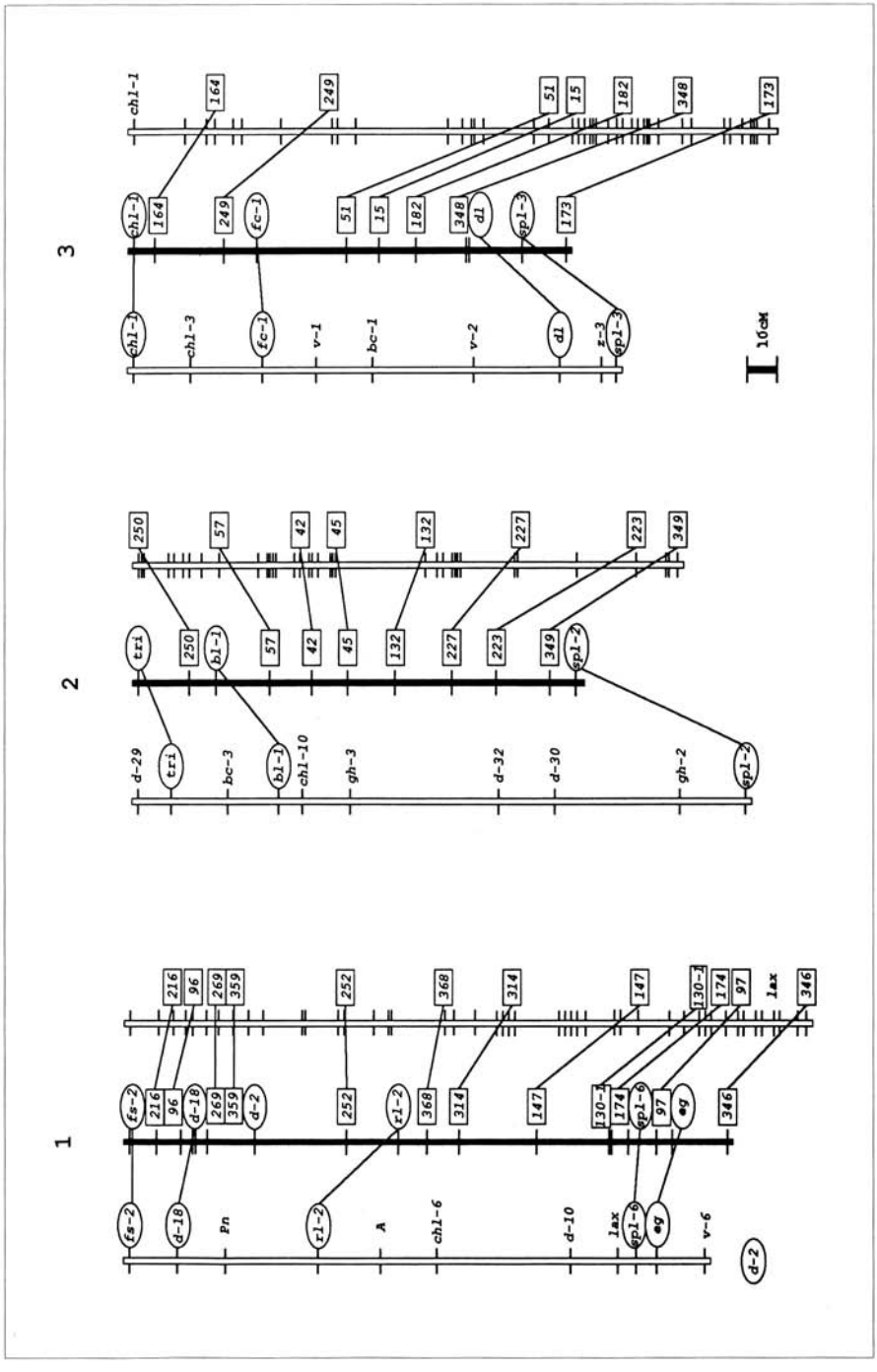
Japonica lines with different morphological markers were crossed to indica variety IR24. The segregation of morphological and RFLP markers was evaluated in 70-140 F_2 individuals. Based on the segregation analysis of these F_2 populations, an integrated linkage map of rice containing 39 morphological markers and 82 RFLP markers was constructed (Fig. 1). When the integrated linkage map was compared with both the morphological and RFLP marker maps, the following was observed.

Gene *d2,* which was previously assigned to chromosome 4, was found to be located on chromosome 1 through detection of linkages between *d2* and several RFLP markers mapped on chromosome 1. Previously, *d2* was estimated to be located on the terminal end of the morphological map of chromosome 4. In this case, gene *lg* was located on the middle of the morphological linkage map of chromosome 4. But Saito et al (1991) located *lg* on the terminal end of the molecular map of chromosome 4. Therefore, a long gap was estimated between *lg* and *d2* on chromosome 4 where there were no RFLP markers. In this study, this gap was closed by shifting the *d2* locus from chromosome 4 to chromosome 1.

Gene *gl1* was located on chromosome 5 and its relationship with other morphological marker genes located on linkage group 5 was established. For chromosome 6, a linear order of *dpl—spl4—XNpb209—XNpb165-1* was determined. As gene *wx* (glutinous endosperm) was located on the terminal end of the morphological linkage map, the arrangement of *wx—dp1—spl4—XNpb209—XNpb165-1* was suggested. On the other hand, Saito et al (1991) located a waxy gene from maize (*cmWX*) on the linear order of *XNpb209—cmWX—XNpb165-1*. Allelic tests between wx and *cmWX* need to be made.

For chromosome 7, *XNpb50* assigned to chromosome 7 by trisomic gene dosage analysis (Ideta et al 1990) was located at the terminal end of the integrated linkage map. The linear order of *XNpb50—gl—d6—spl5* on the integrated linkage map was different from that of *d6—g1—spl5* found on the morphological linkage map. More investigation is needed to determine the order of *d6* and *g1*. For chromosome 9, the locus of the previously unlocated gene *IBf* was determined and the linear order of classical marker genes *IBf—dp2—drp2—Dn1* was established.

The segregation distortion was detected in six regions. In the regions of *fs2—XNpb216—XNpb96—d18—XNpb269—XNpb359—d2* on chromosome 1, *bl1—XNpb57—XNpb42* on chromosome 2, *XNpb164—XNpb249—fc1—XNpb51—XNpb15—XNpb182* on chromosome 3, *XNpb165-1* on chromosome 6, *XNpb389—XNpb78—z2—gmZ410* on chromosome 11, and *rl1—XNpb148—XNpb198* on chromosome 12, the japonica/japonica allele frequencies of both the morphological and RFLP markers showed significantly less than the normal frequency of 25%.


1
2
3
fs-2
d-18
Pn
rl-2
A
chl-6
d-10
lax
gpl-6
•g
v-6
d-2
tri
bl-1
d-29
bc-3
chl-10
gh-3
d-32
d-30
gh-2
gpl-2
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chl-3
fc-1
v-1
bc-1
v-2
dl
z-3
gpl-3
10cM

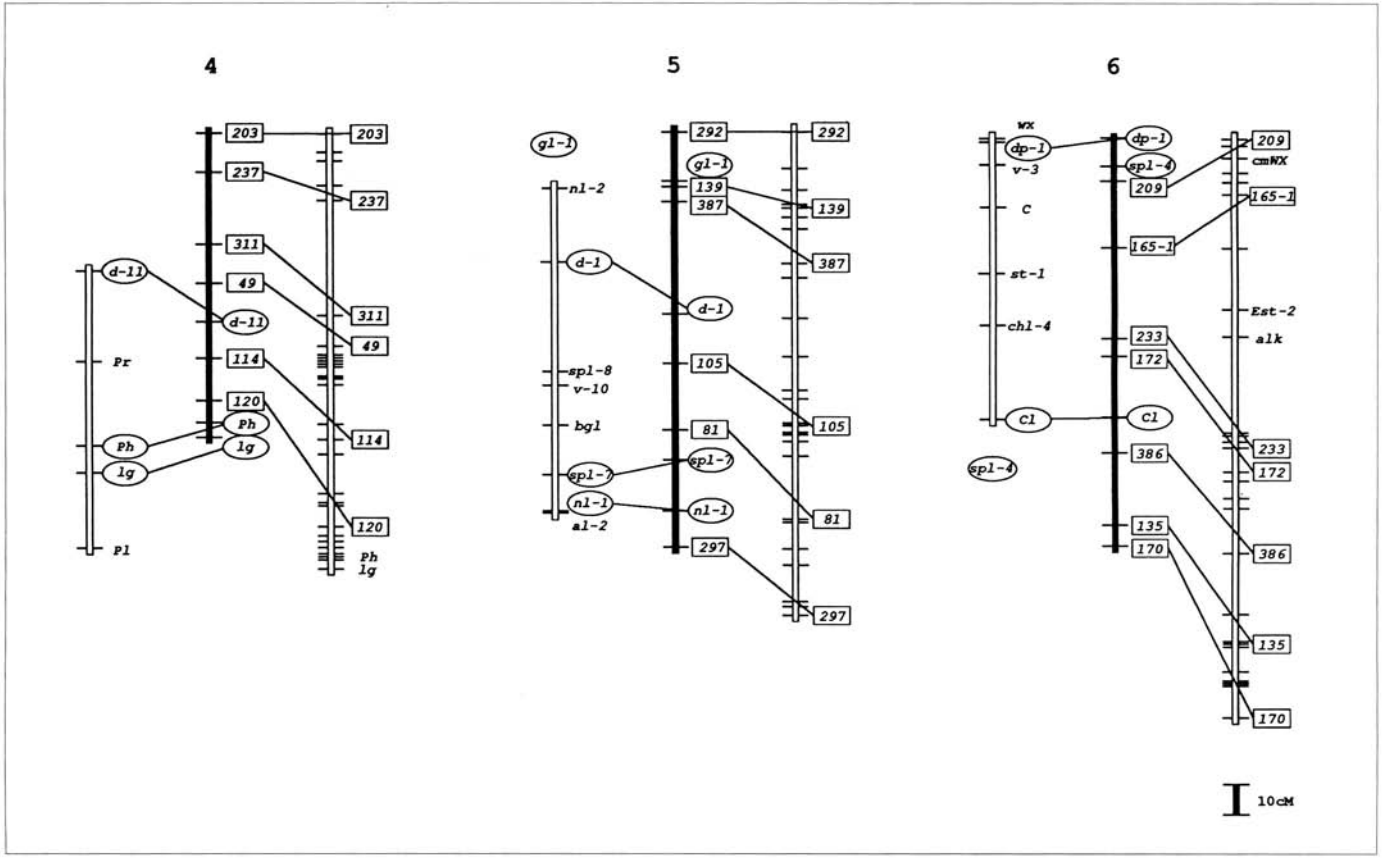
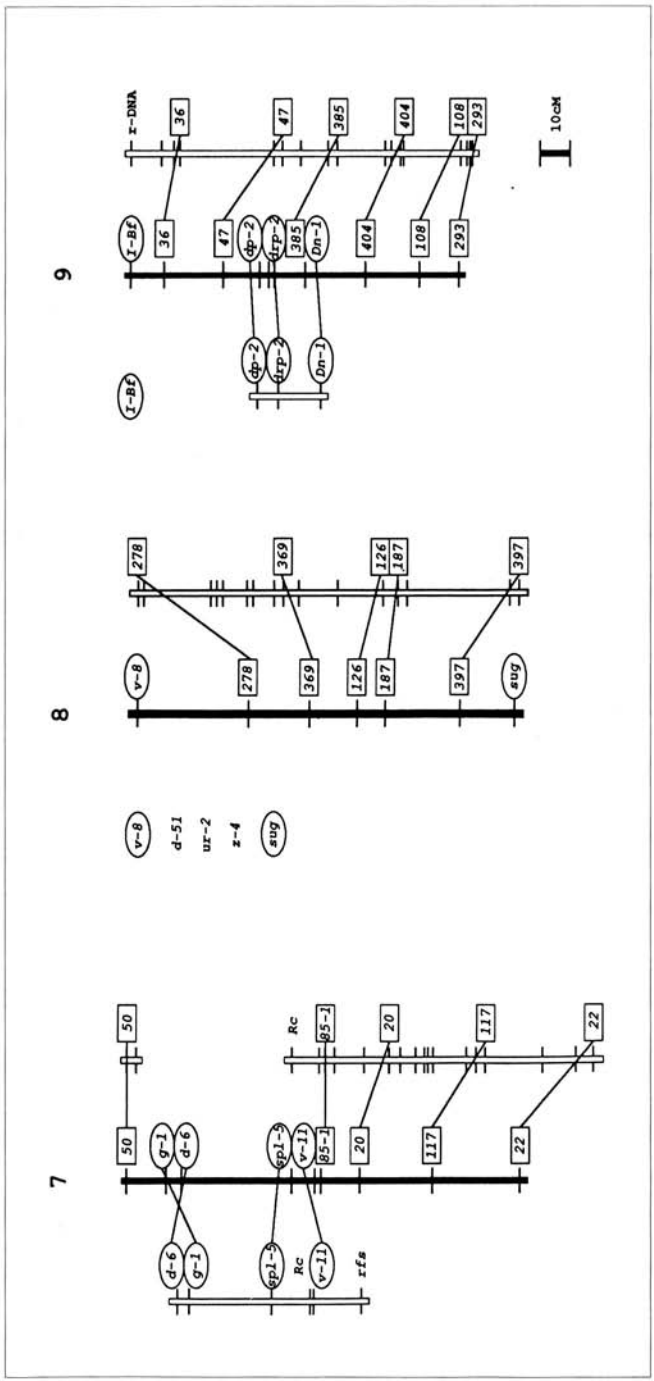


Fig. 1. An integrated linkage map of rice. The classical linkage map is shown on the left and the RFLP linkage map (Saito et al 1991) on the right. Numbers at the top indicate chromosomes. The prefixes of RFLP markers, *XNpb,* are omitted. The loci in ellipses are morphological markers used in this study; those in boxes are RFLP markers.


7
8
9
50
g-1
d-6
gpl-9
v-11
85-1
20
117
22
Rc
rfs
v-8
d-51
ur-2
z-4
sug
278
369
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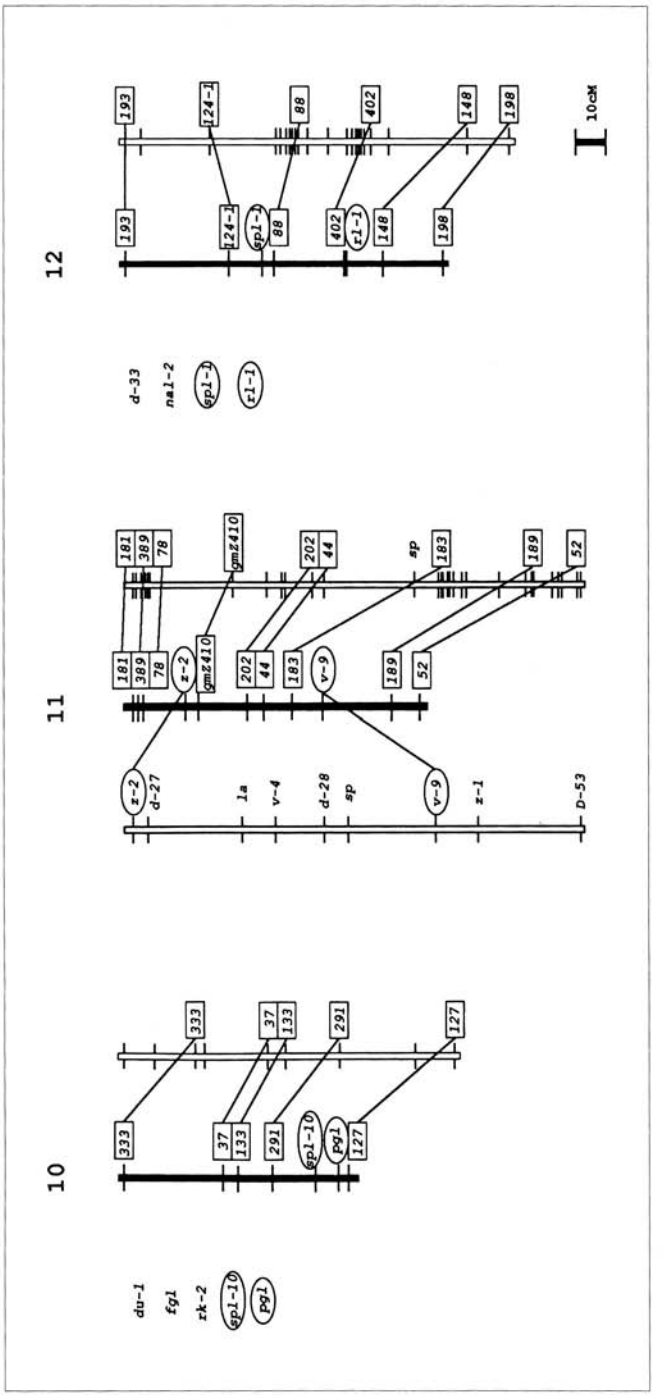
dp-2
drp-2
Dn-1
36
47
385
404
108
293
z-DNA
10cM


10
11
12
du-1
fgl
rk-2
gpl-10
pgl
d-33
na1-2
gpl-2
r1-1
z-2
d-27
la
v-4
d-28
sp
v-9
z-1
D-53
gmz410
10cM

The origin of segregation distortion detected in this study was not examined. However, a gametophyte gene (*ga9*) tightly linked to *d18* on chromosome 1 (Maekawa and Kita 1985), *ga3* (Nakagahra 1972) tightly linked to *XNpb51* on chromosome 3 (Sobrizal 1995), *ga1* (Iwata et al 1964) and *ga4* (Mori et al 1973, Nakagahra et al 1974) located around the *wx—C* region on chromosome 6, and the gametic-lethal gene loosely linked to *la* on chromosome 11 (Tomita et al 1989) can cause segregation distortion. In addition, Nakagahra (1978) had reported segregation distortion of *rl1* on chromosome 12 without revealing its cause. The new segregation distortion found on chromosome 2 needs to be further investigated.

Genes *tri, spl2* (chromosome 2), *chl1* (chromosome 3), *dp1, spl4* (chromosome 6), *d6, g1, spl5* (chromosome 7), *sug* and *v8* (chromosome 8) were estimated to be located beyond the terminal ends of the RFLP linkage map by the three-point linkage test. The RFLP linkage map (Saito et al 1991) was not saturated. Probably, the molecular map constructed by Kurata et al (1994) can cover these regions since it is saturated with more than 1,300 DNA markers.

The morphological linkage map and the RFLP linkage map were integrated for all 12 chromosomes of rice. The integrated linkage map would enhance tagging of genes governing agronomic traits with both the morphological and RFLP markers. Recently, Kurata et al (1994) constructed a high-resolution rice genetic map containing 1,383 DNA markers. The relationship between the RFLP linkage map of Saito et al (1991) and that of Kurata et al (1994) was determined using recombinant inbred lines (Yoshimura et al 1995). A well-saturated linkage map integrated with morphological, isozyme, and RFLP markers would enhance genetic and breeding research aimed at rice improvement.

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Notes

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Centromere mapping and orientation of the cytological, classical, and molecular linkage maps of rice

G.S. Khush, K. Singh, T. Ishii, A. Parco, N. Huang, D.S. Brar, and D.S. Multani

IRRI developed a series of secondary trisomics and telotrisomics and used these for assigning genes and restriction fragment length polymorphism markers to specific chromosome arms and mapped position of centromeres on both classical and molecular linkage maps. Corrected orientations of both the classical and molecular linkage maps were determined and a linear correspondence among the cytological, classical, and molecular maps was established.

Rice is a model cereal plant for molecular and genetic research because

- individual members of its chromosome complement can be easily identified at the pachytene stage of meiosis (Shastry et al 1960, Kurata et al 1981, Khush et al 1984);
- it has a well-developed classical linkage map of more than 170 morphological markers (Khush and Kinoshita 1991);
- the linkage groups have been associated with the cytologically identifiable chromosomes through primary trisomic analysis (Khush et al 1984, Khush 1990);
- comprehensive molecular linkage maps, consisting of more than 2,000 markers, have been produced (Causse et al 1994, Kurata et al 1994b);
- there is a high level of synteny and collinearity of its genome and genomes of other cereals (Ahn et al 1993, Kurata et al 1994a, Van Deynze et al 1995);
- it has a small genome size (4×10^8 bp), which is only three times the size of that of *Arabidopsis thaliana* and 1/37th the size of that of wheat (Arumuganathan and Earle 1991); and
- a transformation and regeneration system, which allows introduction of alien genes in rice genotypes, has been established (Shimamoto 1992).

However, more information is needed about 1) distribution of morphological and molecular markers on the chromosome arms, 2) positions of centromeres on both the

classical and molecular linkage groups, and 3) correspondence between the cytological map and the classical and molecular linkage maps.

Isolation and characterization of secondary trisomics and telotrisomics

The primary trisomic series developed in the background of indica rice cultivar IR36 (Khush et al 1984) served as source material for isolating secondary trisomics and telotrisomics. In primary trisomics, the extra chromosome is present as a univalent in a majority of the sporocytes and it has higher chances of misdivision (Khush 1973). The misdivision of the univalent can give rise to an isochromosome or a telocentric chromosome. A gamete carrying such chromosomes, when fertilized by a normal gamete, results in either a secondary trisomic or a telotrisomic. Large populations of primary trisomics for all 12 rice chromosomes were grown and, based on cytological and genetic analysis, secondary trisomics and telotrisomics were isolated and characterized (Singh et al 1996a). A secondary trisomic forms a ring trivalent at the diakinesis stage of meiosis, which is their unique characteristic. A telotrisomic, on the other hand, can be identified on the basis of the smaller size of the univalent at diakinesis. However, final confirmation of both secondary trisomics and telotrisomics was done at the pachytene stage of meiosis (Singh et al 1996a).

Secondary trisomics for both arms of chromosomes 1, 2, 6, 7, and 11 and for one arm of chromosomes 4, 5, 8, 9, and 12 were identified. However, secondary trisomics for long arms of chromosomes 2 and 7 did not survive. Telotrisomics for the short arms of chromosomes 1, 8, 9, and 10 and for the long arms of chromosomes 2, 3, and 5 were isolated. Thus, secondary trisomics or telotrisomics, representing all 12 chromosomes, were identified. The secondary trisomics, in general, were weaker than the diploid counterparts and the parental primary trisomics and had reduced fertility. Telotrisomics, on the other hand, were vigorous and more fertile than the parental primary trisomics and the secondary trisomics.

Position of centromeres on pachytene chromosomes

The centromere positions on the pachytene chromosomes of rice are difficult to delimit with certainty because the centromeres and the lightly stained chromomeres look alike. However, the pachytene trivalent configurations of secondary trisomics can pinpoint the position of centromeres with certainty. From the observations of pachytene trivalents of secondary trisomics, the centromere positions of 10 chromosomes, as shown in the pachytene ideogram of Khush and Kinoshita (1991), were confirmed but the centromere positions in chromosomes 6 and 12 were in error. Contrary to the locations of centromeres as shown in the ideogram, chromosome 6 is more metacentric and chromosome 12 is submetacentric. Thus, the positions of the centromeres of these two chromosomes were revised (Fig. 1).

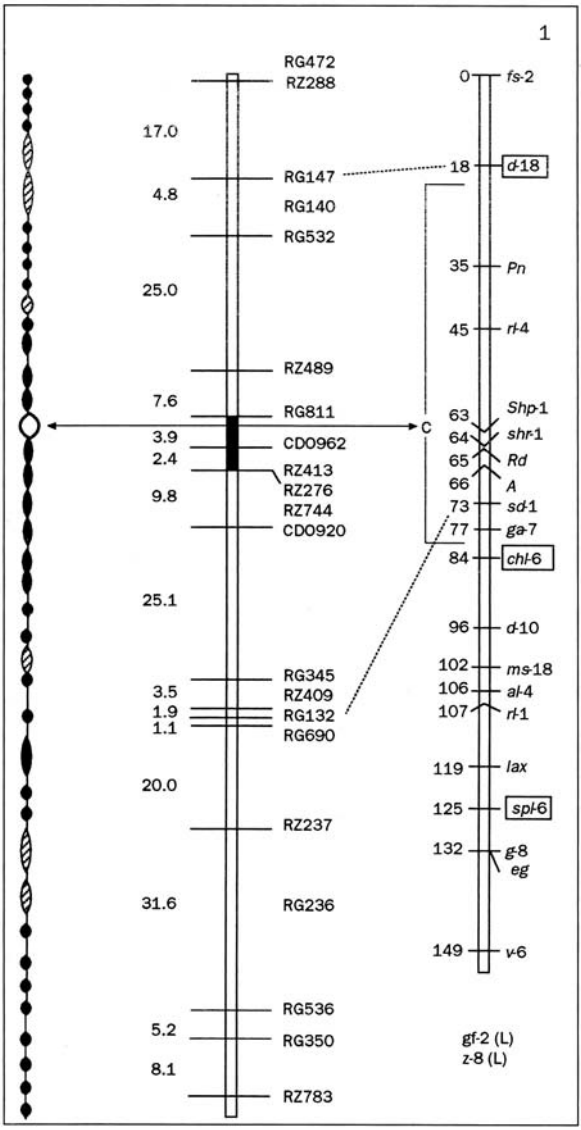
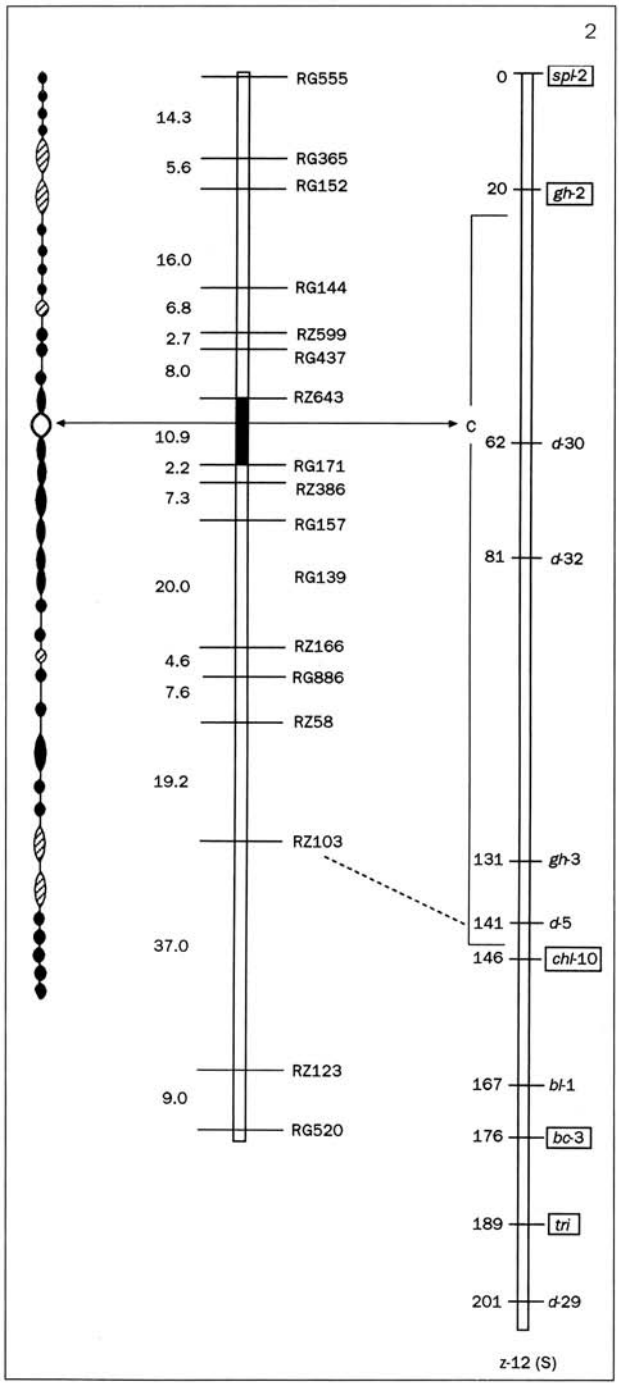


Fig. 1. Relationships between (from left to right) the pachytene ideogram of rice (Khush and Kinoshita 1991), the molecular linkage map (Causse et al 1994), and the classical map (Singh et al 1996a). Positions of the centromeres are indicated by Os on the ideogram, dark areas on the molecular linkage map, and by C on the classical map. Relationships between the molecular and morphological markers, where known, are indicated by dashed lines.


2
RG555
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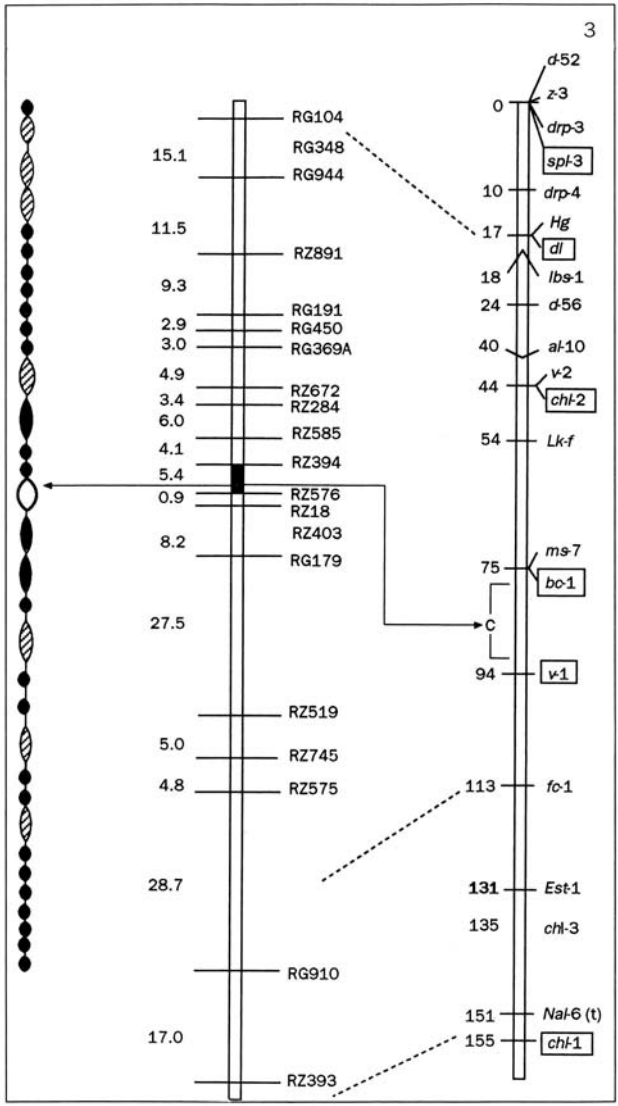
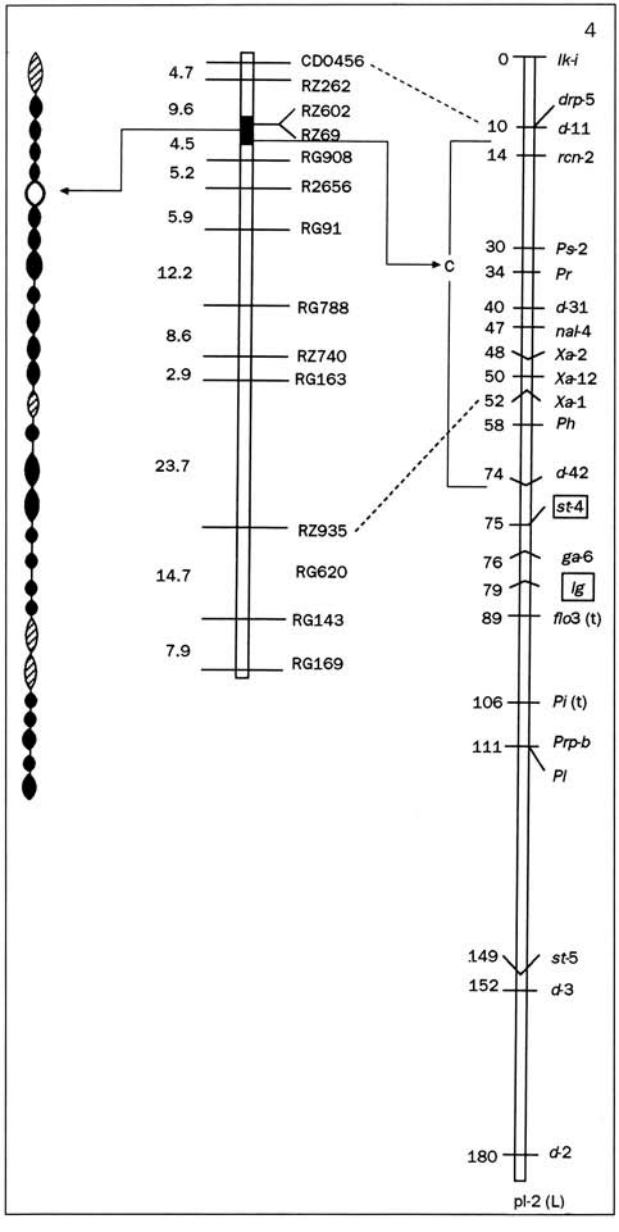
RG365
5.6
RG152
16.0
RG144
6.8
RZ599
2.7
RG437
8.0
RZ643
10.9
RG171
2.2
RZ386
7.3
RG157
RG139
20.0
RZ166
4.6
RG886
7.6
RZ58
19.2
RZ103
37.0
RZ123
9.0
RG520
0
spl-2
20
gh-2
C
62
d-30
81
d-32
131
gh-3
141
d-5
146
chl-10
167
bl-1
176
bc-3
189
tri
201
d-29
z-12 (S)

Figure 1 continued




4
CDO456
RZ262
RZ602
RZ69
RG908
R2656
RG91
RG788
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RG163
RZ935
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RG143
RG169
4.7
9.6
4.5
5.2
5.9
12.2
8.6
2.9
23.7
14.7
7.9
C
0
10
14
30
34
40
47
48
50
52
58
74
75
76
79
89
106
111
149
152
180
lk-i
drp-5
d-11
rcn-2
Ps-2
Pr
d-31
nal-4
Xa-2
Xa-12
Xa-1
Ph
d-42
st-4
ga-6
lg
flo3 (t)
Pi (t)
Prp-b
Pl
st-5
d-3
d-2
pl-2 (L)

Figure 1 continued

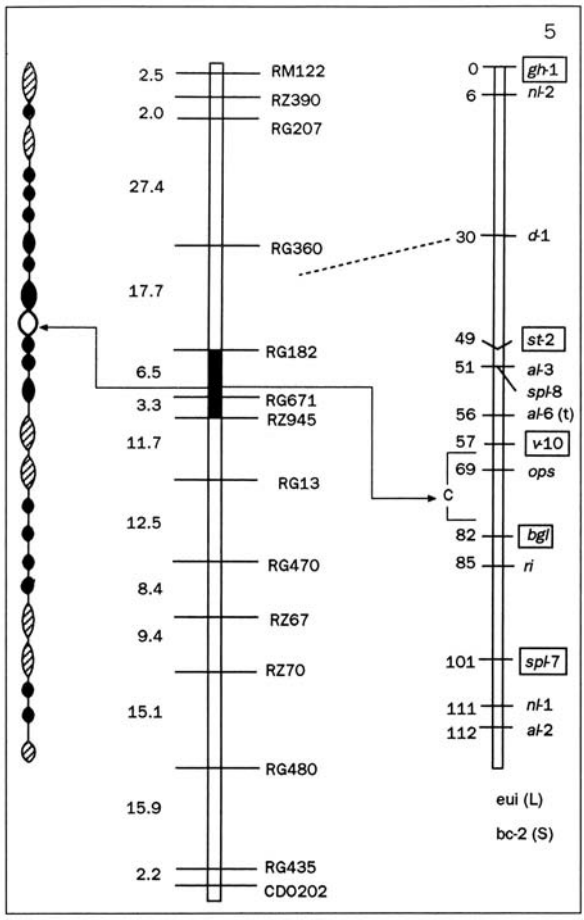
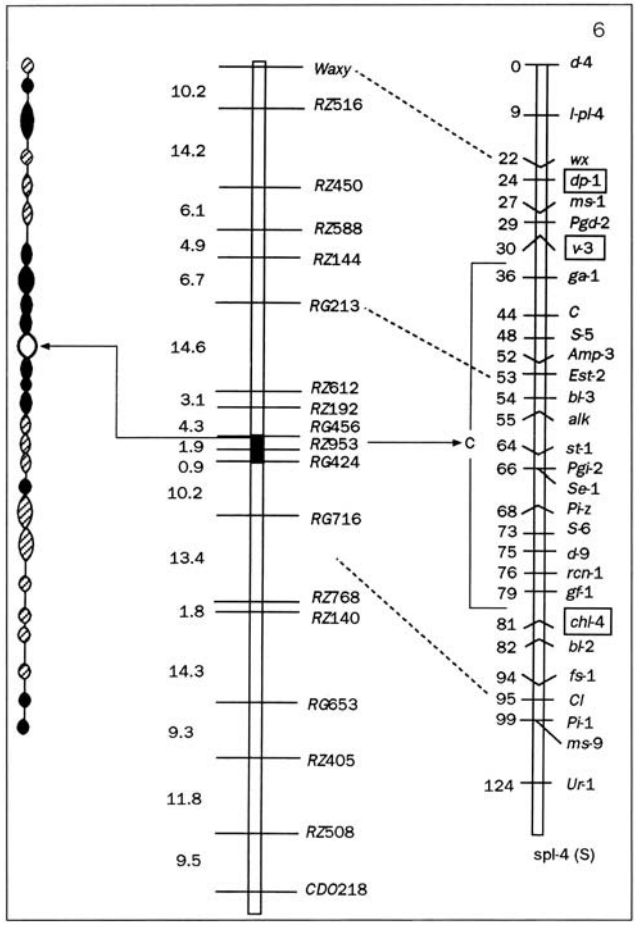
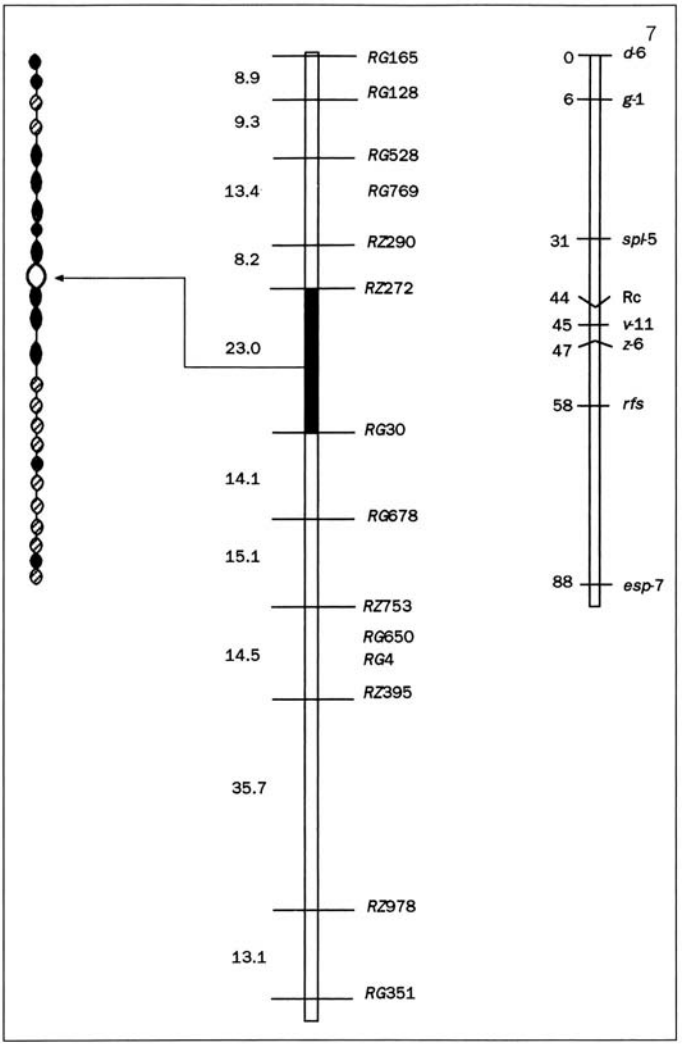


Figure 1 continued




7
RG165
8.9
RG128
9.3
RG528
13.4
RG769
RZ290
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RZ272
23.0
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14.1
RG678
15.1
RZ753
RG650
14.5
RG4
RZ395
35.7
RZ978
13.1
RG351
0
d-6
6
g-1
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spl-5
44
Rc
45
v-11
47
z-6
58
rfs
88
esp-7

Figure 1 continued

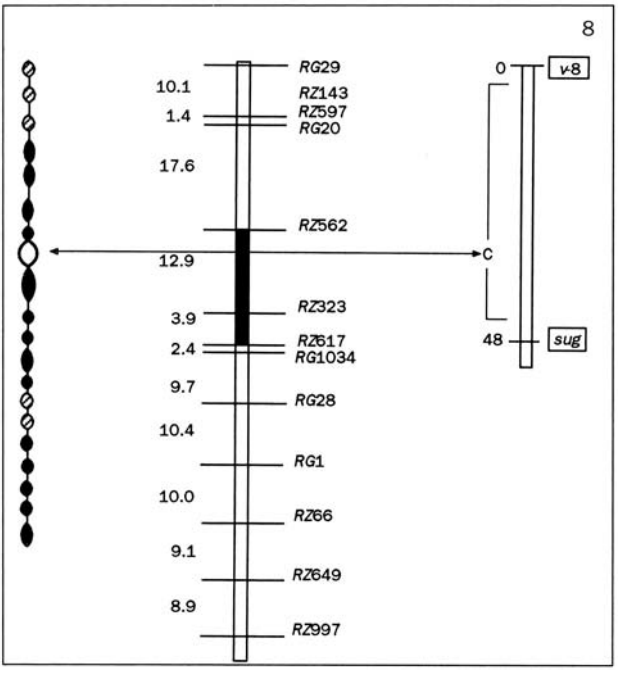
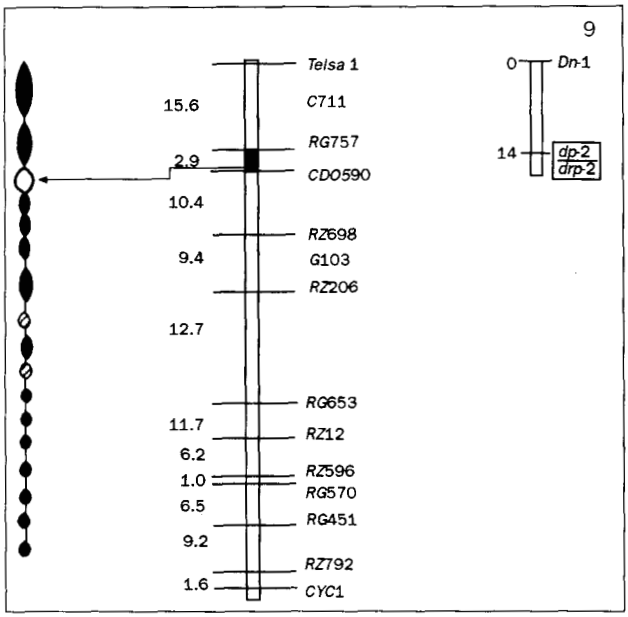


Figure 1 continued



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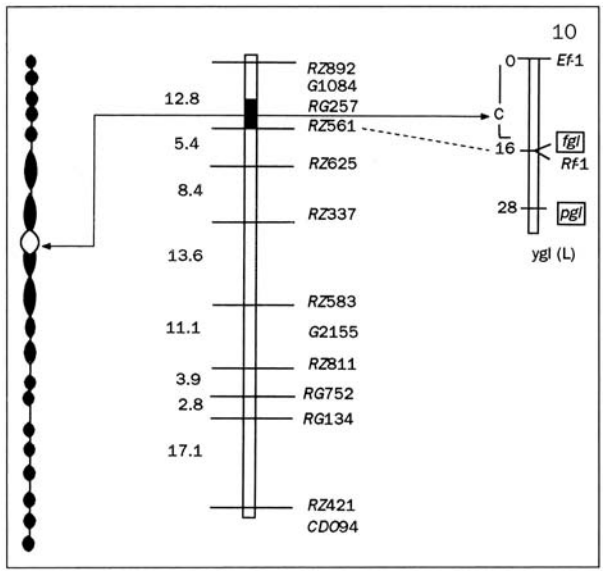

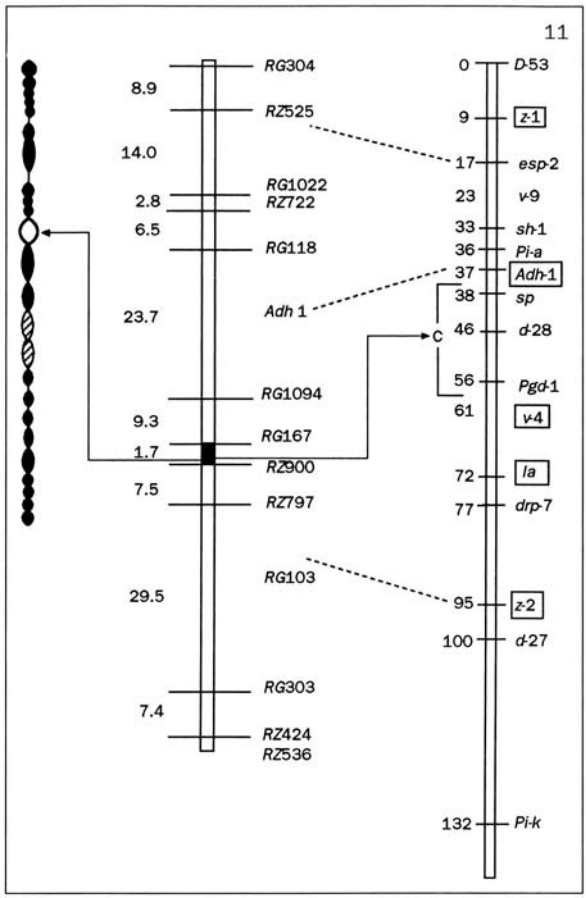
11
RG304
8.9
RZ525
14.0
RG1022
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RZ722
6.5
RG118
23.7
Adh 1
RG1094
9.3
RG167
1.7
RZ900
7.5
RZ797
RG103
29.5
RG303
7.4
RZ424
RZ536
0
D-53
9
z-1
17
esp-2
23
v-9
33
sh-1
36
Pi-a
37
Adh-1
38
sp
C
46
d-28
56
Pgd-1
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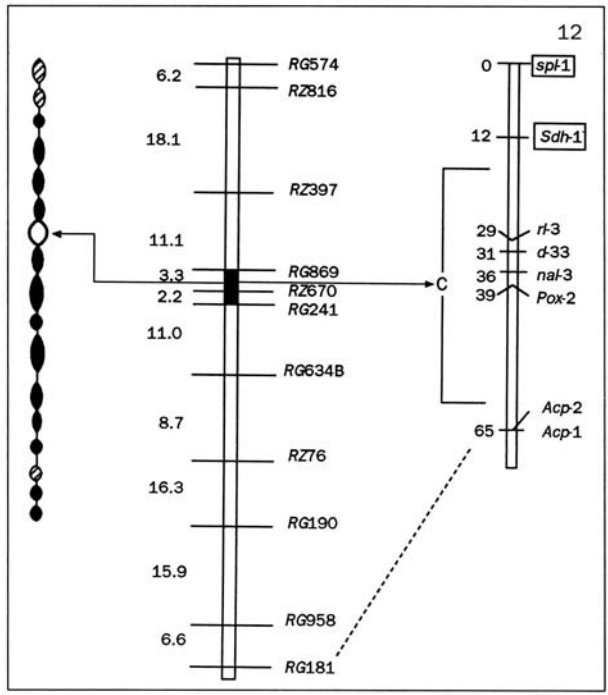
v-4
72
la
77
drp-7
95
z-2
100
d-27
132
Pi-k

Figure 1 continued



Arm location of morphological markers

From each of the 12 linkage groups (Khush and Kinoshita 1991, Kinoshita 1993), a few well-defined recessive marker stocks were crossed with corresponding secondary trisomics or telotrisomics. Trisomics were used as the female. The F_1 secondary trisomics or telotrisomics were confirmed cytologically and either selfed or backcrossed to the recessive mutants to produce F_2 or backcross progeny. The F_2 or backcross populations were grown and classified as disomic or trisomic and as normal or mutant. Based on the segregation data, a gene was assigned to a specific chromosome arm. In the F_2 or backcross populations of these trisomics, if the disomic portion segregates in normal 3:1 or 1:1 fashion and trisomic portion segregates in all:0 fashion, then the gene in question is located on the arm that is present as an extra isochromosome or a telocentric chromosome. By contrast, if both disomic and trisomic portions segregate in a 3:1 fashion, then the gene in question is located on the other arm of the chromosome

Table 1. Marker genes belonging to 11 linkage groups and assigned to specific chromosome arms.

Linkage group	Marker genes	
	Short arm	Long arm
1	*d18*	*chl6, spl6, z8, gf2*
2	*spl2, gh2, z12*	*chl10, tri, bc3*
3	*spl3, dl, ch12, bc1*	*v1, chl1*
4		*pl2, st4, lg*
5	*bc2, gh1, st2, v10*	*bgl, spl7, eui*
6	*dp1, spl4, v3*	*chl4*
8	*v8*	*sug*
9		*dp2, drp2*
10		*pgl, flg, ygl*
11	*z1*	*v4, la, z2*
12	*spl1*	

(Khush 1973). Forty-three marker genes belonging to 11 linkage groups (Table 1) were thus studied and their arm locations were determined (Singh et al 1996a).

Mapping positions of centromeres and orientation of the classical linkage map

When the arm locations of the marker genes became known, the positions of the centromeres could be approximated on linkage groups corresponding to chromosomes 1, 2, 3, 4, 5, 6, 8, 11, and 12 (Fig. 1). The centromere positions of linkage groups 9 and 10 could not be defined because these linkage groups have only a few markers and all the markers mapped on one arm only. In the absence of a dosage effect, the dominant marker genes *Dn* (chromosome 9) and *Efl* (chromosome 10) could not be mapped to specific arms using secondary trisomics or telotrisomics. The secondary trisomic for the long arm of chromosome 7 was very weak and did not set any seed. The secondary trisomic for the short arm of chromosome 7 was isolated only recently and segregation data for the mutants of this chromosome are not yet available.

By convention, the short arm of the chromosome is shown on the top and the most distal marker on the short arm is assigned the zero centiMorgan (0 cM) position. Thus, in linkage group 1, *fs2* occupies the 0 cM position (Fig. 1), although it was assigned the 149 cM position on the previous map. The corrected orientations of all the linkage groups except linkage groups 7, 9, and 10 are presented in Figure 1. The orientations of linkage groups 1, 2, 3, 4, 8, 10, 11, and 12, as shown in Khush and Kinoshita (1991), were reversed. Orientations of linkage groups 9 and 10 could not be determined because of a lack of marker genes on this linkage group.

Arm location of RFLP markers

A majority of the restriction fragment length polymorphism (RFLP) markers used for arm location in this study are from the Cornell University map (Causse et al 1994);

however, a few markers from the Rice Genome Research Program (RGP) map (Kurata et al 1994b) were also used. Secondary trisomics and telotrisomics representing all 12 chromosomes in the background of indica cultivar IR36 were crossed as the female to the tropical japonica line, MaHae. Similarly, the primary trisomics in the background of IR36 were also crossed with MaHae. The F_1 primary trisomics and F_1 secondary trisomics or telotrisomics were confirmed cytologically and these along with F_1 disomic sibs and parents formed a set of plant materials for each of the 12 chromosomes and were used for arm location of RFLP markers (Singh et al 1996b).

The rationale for arm location of RFLP markers using secondary trisomics and telotrisomics is as follows:

An F_1 disomic plant has one copy each of the IR36 and MaHae alleles and the intensities of both the autoradiographic bands are the same. An F_1 primary trisomic has two copies of the IR36 allele and one copy of the MaHae allele and thus the intensity of the IR36 band is twice that of the MaHae band. An F_1 secondary trisomic has three copies of the IR36 allele located on the arm for which it is secondary and one copy of the MaHae allele and accordingly the intensity of the IR36 band for a given marker present on that arm is three times that of MaHae (Singh et al 1996b). By contrast, if the marker in question is not located on the arm for which it is a secondary trisomic, then the F_1 secondary trisomic has intensities similar to those of the IR36 and MaHae bands. The telotrisomics behave in the same way as the secondary trisomics except that the intensity of the IR36 band is twice the intensity of the MaHae band (Singh et al 1996b). Based on this principle, RFLP markers could be assigned to specific chromosome arms.

More than 200 probes belonging to 12 linkage groups were surveyed for polymorphism using 24-31 restriction endonucleases and about 170 of those were assigned to specific chromosome arms on the basis of dosage vs absence of dosage effects in F_1 secondary trisomics and telotrisomics. Arm locations of about 170 markers determined in this manner are shown in Figure 1.

Centromere mapping and orientation of molecular linkage groups

Centromere position in a linkage group is mapped between the two nearest markers located on opposite arms of the chromosome. Thus, in chromosome 1, the centromere is mapped between RG811 and RZ413 with RG811 located on the short arm of the chromosome and RZ413 on the long arm of the chromosome (Fig. 1). Centromere positions were defined exactly between two RFLP markers in linkage groups 2, 3, 4, 7, 9, 10, and 11. However, one marker on each of linkage groups 1, 5, 6, 8, and 12 situated in the centromeric region could not be mapped to a specific chromosome arm due to a lack of polymorphism with the 31 restriction endonucleases used. Centromere positions thus have been mapped in all 12 linkage groups. The precision of mapping varies among chromosomes. The centromere in chromosome 4 is mapped between RZ602 and RZ69, which show cosegregation (Fig. 1) (Causse et al 1994). In linkage group 7, on the other hand, the centromere is mapped between RZ272 and RG30, which are 23.0 cM apart.

As mentioned earlier, the short arm of the chromosome is shown, by convention, on the top and the distal most marker on short arm of the map is assigned the 0 cM position. Thus, in linkage group 1, RZ288, which was shown at the bottom most position of the map of Causse et al (1994) is now assigned the 0 cM position (Fig. 1), and the orientation of the linkage group 1 is reversed. Likewise, orientations of linkage groups 2, 3, 4, 7, 11, and 13 of Causse et al (1994) were reversed and those of linkage groups 5, 6, 8, 9, and 10 remained the same.

Integration of the RGP and Cornell University molecular linkage maps

The RGP and Cornell University maps have been oriented with respect to each other (Xiao et al 1992). Efforts are under way to further integrate the two maps by exchanging additional molecular markers between the two groups and mapping them on their own maps. The centromere positions on the RGP molecular linkage groups are being approximated by mapping the centromere-flanking markers of the Cornell University map identified by Singh et al (1996b) on the RGP map.

Integration of the classical and molecular linkage maps

In an effort to integrate the classical and molecular linkage maps, Ideta et al (1992, 1993, 1994) studied 19 F_2 populations segregating for morphological and molecular markers of Saito et al (1991) map. Linkage relations between 39 morphological markers and 82 RFLP markers belonging to all 12 chromosomes were investigated. Similarly, Yu et al (1995) mapped 17 morphological markers on the Cornell University map. These studies established linear relationship between the classical and molecular linkage groups.

Correspondence between the cytological, classical, and molecular linkage maps

In the pachytene chromosome map, the centromere positions are well defined. Similarly, centromeres have been approximated on the classical and molecular linkage maps and the arm locations of key markers of the classical linkage map and of almost all the molecular markers have been determined. Linkages between selected morphological markers and molecular markers have been determined. On the basis of these results, the linear correspondence of the cytological, classical, and molecular markers has been established (Fig. 1). The Cornell University molecular linkage map (Causse et al 1994) seems to correspond well with the pachytene chromosome map for all chromosomes except for chromosomes 10 and 11. The molecular linkage maps for the short arm of chromosome 10 and the long arm of chromosome 11 are shorter than expected on the basis of the length of arms of the pachytene chromosomes. The RGP molecular linkage map for the short arm of chromosome 10, on the other hand, seems to correspond well with the pachytene chromosome arm length. Since a linear

correspondence between the classical and molecular maps is now established, further integration of the two maps, if intended, can be speeded up. Also, a linear correspondence between the Cornell University linkage map and the RGP linkage map is known and efforts are now under way to integrate the two maps and develop a more comprehensive molecular linkage map of rice.

These advances in the integration of various maps are helpful in further establishing synteny and collinearity between the rice genome and genomes of other cereals. This information is useful in breeding for improved varieties of rice and other cereals.

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Rice as the pivotal genome in the new era of grass comparative genetics

M.D. Gale, K.M. Devos, and G. Moore

Comparisons of the molecular maps of a number of grass species, including the major cereals rice, wheat, and maize, indicate that gene linkage relationships have been remarkably maintained ever since isolation by speciation—as long as 60 million yr ago. Collinearity between the maps of six grass crop species is such that a single consensus genetic map can be described by only 19 rice linkage blocks. These findings will be translated into the use of rice as a tool for cross genome map-based gene isolation, transfer and synthesis of information concerning syntenous genes and biochemical, developmental, or physiological pathways over species, and the availability of a wide range of alternative homoeogenes for the transgenic cereal breeder of the next century.

Comparative genetics, like molecular mapping, has its roots in mammalian studies stemming from the work with man-mouse hybrids in the late 1970s. The description and application of the information concerning the syntenous relationships between increasingly distantly related species have, however, progressed more rapidly in plants than in animals even though the work had to await the development of molecular maps, with the advent of restriction fragment length polymorphism (RFLP) technology in the mid-1980s. At that time, cereal geneticists were, in general, blinkered to developments outside their own crop and the concept of cross comparisons between even closely related genomes was slow to emerge. Researchers working with allopolyploids, such as wheat, had the best chance of observing synteny between genomes. However, these crops were precisely those that had the least developed classical maps because recessive mutants—widely studied and mapped in rice and barley—are, in the main, phenotypically masked by the buffering provided by homoeologous genes in polyploids.

Although the genetic maps of wheat were sparsely populated with markers prior to RFLPs, we did have many clues to the existence of extensive synteny. We were

aware that the homoeologous chromosomes of the A, B, and D genomes could compensate for one another (Sears 1954), and that homoeologous chromosomes contained similar genes, particularly from studies of storage proteins and isozymes in the 1970s and early 1980s (e.g., Hart and Langston 1977). Moreover, we were aware that this synteny extended to close relatives such as rye and barley. However, we did not make the last step—i.e., that of realizing not only were the same genes present on homoeologues but they were present in the same map order, until the first RFLP maps began to emerge.

Early steps—comparisons within tribes

Even as the group at the Plant Breeding Institute in Cambridge began the task of making an RFLP map of wheat in 1986, we imagined we were setting out to describe 21 different linkage groups. Possibly, we had one stroke of fortune in that, initially, we chose cDNA clones as RFLP probes. We now know that synteny and collinearity are restricted mainly to the genes, and thus the early publications (e.g., Chao et al 1988) showed an overly simplified view of the similarity between wheat homoeologues.

Present-day maps, e.g., the wheat homoloeogous group 3 chromosomes in Figure 1, show the presence of two sorts of markers: 1) cDNA probes with predictable locations over the three genomes and 2) nonhomoeologous loci. These latter RFLP loci are detected with genomic clones or, occasionally, represent odd, probably nonfunctional, cDNA copies. The presence of many nonhomoeologous loci confirms that chromosomes are evolving rapidly and independently in the intergenic regions. One consequence of these differences is that some polymerase chain reaction (PCR)-based markers, e.g., microsatellites, are almost all nonhomoeologous and thus not transferable between species in the way that many RFLP loci are (Devos et al 1995).

The studies on collinearity rapidly extended beyond the three diploid donor genomes of hexaploid wheat to rye (Devos et al 1993) and barley (Laurie et al 1993). The comparative picture among these five genomes within the tribe Triticeae revealed several large chromosomal rearrangements, mostly reciprocal translocations. However, within contiguous chromosome segments in the different genomes, complete collinearity is maintained. Moreover, within the usual errors associated with mapping, linkage distances between homoeogenes were also more or less similar in the different genomes.

Elsewhere, work in the Andropogoneae tribe, first comparing sorghum and maize (Hulbert et al 1990) and later sugarcane, maize, and sorghum (Grivet et al 1994) showed similar levels of genome conservation. Also in *Oryza,* Kochert and Jena (1993) demonstrated that the A genome of the *O. sativa* genome was reproduced faithfully, albeit with a few rearrangements, in the C genome of *O. officinalis.*

These studies, particularly in rice and wheat, will assume importance for consideration of further transfers of useful genes from wild to cultivated species by induced or homoeologous recombination (for discussion, see Devos et al 1993), and will usefully be extended to more potential donor wild relatives.

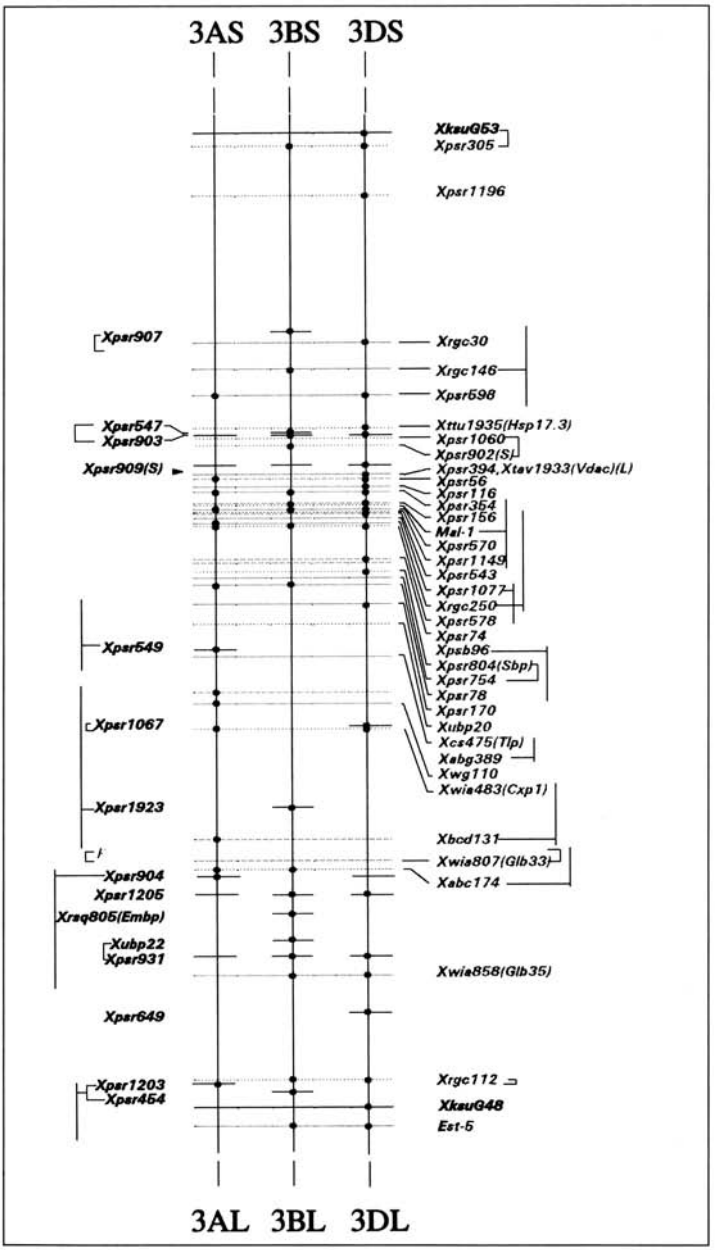

Fig. 1. A consensus map of homoeologous chromosomes 3A, 3B, and 3D of wheat (extended from Devos et al 1993). The loci on the RHS are "homoeologous" and probably represent mostly active genes. The DNA probes that recognize these genes also detect homoeoloci in barley, rye, and beyond. The LHS loci, which include some PCR-based markers, tend to be specific to one or two of the wheat genomes and have limited value in comparative mapping. Vertical bars indicate confidence limits to best-fit locations.

Wider comparisons within the Poaceae

As the technology improved, it became possible to answer a question that we could not have posed 10 yr ago—how do the genomes of the three major cereal relate to one another? These comparisons were aided by the development of particularly dense genetic maps of rice from the Rockefeller Rice Biotechnology Program at Cornell (Causse et al 1994) and the Japanese Rice Genome Program (RGP) at Tsukuba (Kurata et al 1994b). Ahn and Tanksley (1993) demonstrated that rice and maize were closely aligned, and the UK team, now relocated to the John Innes Centre at Norwich, working with RGP and University of Columbia, Missouri, showed that the wheat maps were closely related to those of maize and rice (Devos et al 1994, Kurata et al 1994a). More detail is now available in recent papers comparing the Triticeae, rice, maize, and the diploid oat genomes (Van Deynze et al 1995a,b).

Fine mapping collinearity studies

One of the immediate applications of these discoveries will be to employ map-based cloning tools available in rice, e.g., ordered yeast artificial chromosomes (YACs; Umehara et al 1995), to isolate genes from wheat, barley, maize, and other grasses. The hope was that fine maps in the target genome could be transferred to rice so that chromosome walks could be carried out in the smaller and better described rice genome. Recent studies employing the RGP YACs have indeed confirmed that collinearity is maintained down to the megabase level (Dunford et al 1995, Kilian et al 1995). Interestingly, recombination distances appear again to be maintained, even though the physical distances differed considerably, with the wheat and barley chromosomes containing about 30 times as much DNA as those of rice. This may lend weight to the studies of Civardi et al (1994), which indicate that recombination is restricted mainly to within-genes, thus making the intergenic regions irrelevant in comparative maps of gene loci. There will be interesting exceptions to the equivalence of genetic distances over syntenous regions in different genomes. For example, rice chromosome 10, with a normal whole chromosome genetic length, is inserted into rice chromosome 5 to form the Triticeae homoeologous group 1 chromosomes. In the centromeric region of wheat chromosomes, the genetic length of the segment corresponding to rice chromosome 10 is reduced to about zero in the Triticeae because there is very little recombination in the proximal regions.

A single grass genome?

A further breakthrough was reported by Moore et al (1995a) who, using the maize-rice comparisons of Ahn and Tanksley (1993) and Devos et al (1994), found that a single unique order of some 19 rice linkage blocks described entirely the two ancestral maize genomes. Moreover, when that chromosome order is circularized, as shown in Figure 2, the genomes of foxtail millet (n = 9, Z. Wang, unpubl. data), sugarcane (n = 9 or 10), sorghum (n = 10), maize (which now appears as an ancestral tetraploid

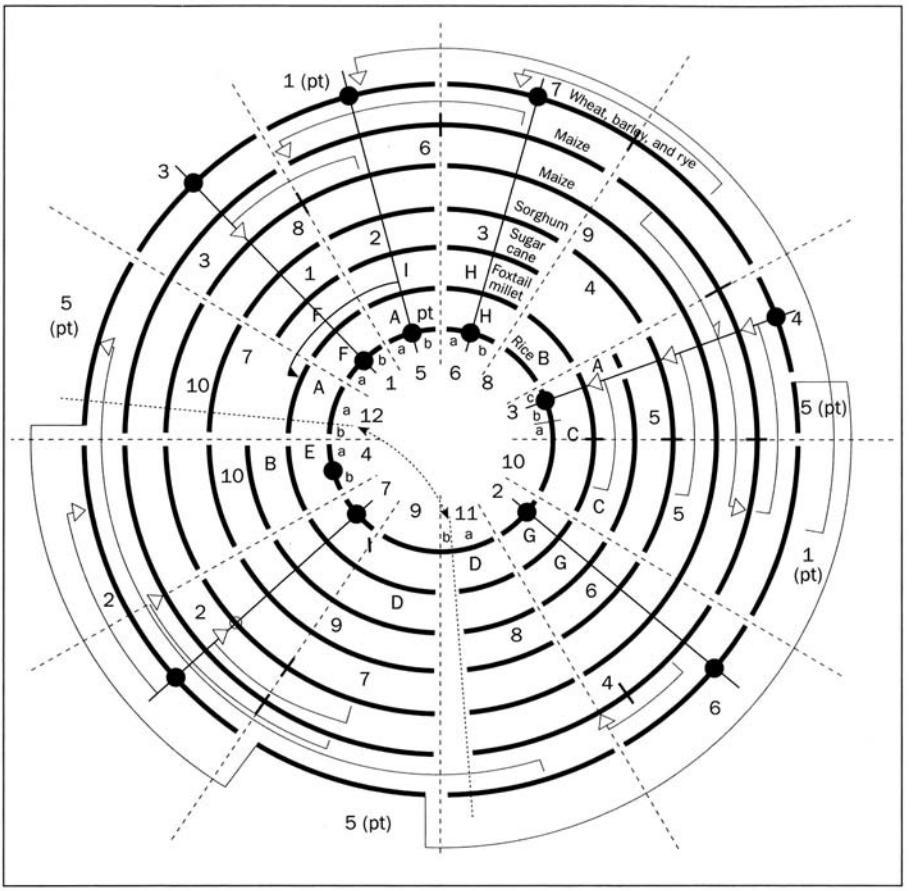

Fig. 2. A grass genome synthesis, extended from Moore et al (1995b). The arrows indicate major rearrangements necessary to reconstitute the present-day genomes of the different crop species, relative to rice. The solid markers indicate the relative positions of some rice and Triticeae centromeres. A number of further inversions and duplications within genomes are known but not indicated on the map.

with n = 5), and the Triticeae cereals (wheat, barley, and rye, n=7), can all be relatively simply aligned (Moore et al 1995b), and still be described by just 19 rice linkage blocks.

Recent information, provided at this symposium, allows us to begin to answer one of the conundrums raised by this comparative analysis. What happened to the centromeres? If rice, for example, has 12 and the Triticeae genomes only 7, they cannot all be syntenous. The wheat centromeres have been precisely located on the genetic maps through the application of ditelocentric genetic stocks and now a similar technique, using tertiary trisomic lines, has been used to map the rice centromeres (Khush et al 1996). Alignment of a few centromeres has been possible with the data at

hand (Fig. 2), and it is clear that these have been the points in the genome where many of the rearrangements—breaks and insertions—have occurred. If this pattern extends to the other grass species, then the rationalization of the centromeres of the different chromosome complements will leave few problems.

A final comment should be made concerning evolution. The pattern shown in Figure 2 simply represents the most parsimonious fit of the available data and does not imply that the smallest genome, rice with $1C = 0.4 \times 10^9$ bp, is the most primitive. On the other hand, similar rearrangements over genomes—for example, the insertion of rice linkage block 10 between 3a and 3b in each of the maize, sorghum, and foxtail millet genomes—almost certainly reflects single evolutionary events.

The future

It is clear that these results will radically change our perspective of cereal genetics. If we consider that a cereal genome contains 20,000 or 30,000 genes, then that number of radii can be drawn on the map and will pass through homoeoalleles of the same genes in the different grass species. This will certainly facilitate gene isolation experiments and, in prospect, will provide breeders with an almost unlimited supply of alternative alleles of any isolated gene to incorporate in their own transgenic breeding program. Similarly, our knowledge of specific gene action and the role of specific genes in biochemical and physiological pathways to produce the agronomic phenotype will take a quantum leap forward as the maps become integrated precisely enough to identify homoeoalleles among mapped genes and genes recognized only as quantitative trait loci. All of the information available on those traits for all grass species will be able to be combined. In short, we are all going to have to become grass geneticists in the broadest sense if we are going to be able to exploit all of the information available for the improvement of our own cereal crop species.

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Construction of a first-generation physical map of the rice genome

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We aim to construct a physical map of rice consisting of overlapping yeast artificial chromosome (YAC) clones that span all of the rice chromosomes. Using 1,383 restriction fragment length polymorphism markers and sequence-tagged sites established on our rice linkage map, we have started to screen our rice YAC library, which contains 6,932 rice YAC clones and represents six genome equivalents. As of August 1995, we have used 1,126 markers for the screening. Of these, 1,004 markers hybridized with a total of 4,645 YAC clones in the library. Out of these YACs, 2,369 were independent. We matched the locations of the isolated YACs to corresponding positions on the rice linkage map, detected overlaps between the YACs, and assembled all YACs to contigs. About half of chromosome 6 has now been covered with YAC contigs. We expect that such a combined genetic/physical map will provide a basis for analyzing the genome structure and for map-based cloning of agronomically important genes.

A complete physical map, which consists of ordered recombinant genomic clones spanning an entire genome, is essential for investigating the genome structure of any organism. A physical map has high resolution at the level of deoxyribonucleic acid (DNA) length and directly provides the DNA clones located at distinct positions of the genome for further study. In crop plants, such a map is indispensable for isolating and characterizing genes for agronomically important traits based on their known map position by chromosome walking and/or chromosome landing with DNA clones.

With the advent of yeast artificial chromosome (YAC) vectors capable of cloning DNA fragments of 100 to more than 1000 kb, it has become feasible to assemble DNA clones to construct a physical map in higher eukaryotes. For the human genome, physical maps with ordered YAC clones have been constructed for the long arm of chromosome 21 (Chumakov et al 1992) and chromosome Y (Foote et al 1992). In

Arabidopsis, one-third of the genome was reported to have been covered with YACs (Hwang et al 1991), and these YACs have already been used for map-based cloning of genes (Arondel et al 1992, Bent et al 1994).

We started to use YACs as a cloning vector for the construction of a physical map in rice. We constructed a rice YAC library (Umehara et al 1995) and subsequently screened it for more than 1,100 markers that had been located on a rice linkage map (Kurata et al 1994). By using these markers in the screening, we aim to integrate the physical map with the established molecular genetic map. Such a combined genetic/physical map will serve as a tool in a map-based cloning strategy by facilitating the progress from the genetic locus to the cloned gene.

Constructing and characterizing the rice yac library

High molecular weight genomic DNA was prepared from agarose-embedded protoplasts from cultured suspension cells of rice (*Oryza sativa* L. cv Nipponbare). The DNA was digested with *Not*I or partially digested with *Eco*RI. The digested DNA was size-fractionated, ligated to pYAC4 (for DNA digested with *Eco*RI) and pYAC55 (for DNA digested with *Not*I), and used to transform *S. cerevisiae* AB1380. A total of 6,932 clones with an average insert size of 350 kb was obtained. This library represents approximately six genome equivalents. About 40% of the YACs were estimated to be chimeric. Most of the chimeric YACs were longer than 500 kb; YACs shorter than 400 kb were colinear. The transformants were stocked in 96-well microtiter plates at -80 °C.

In screening this library, 89% of the restriction fragment length polymorphism (RFLP) markers used identified at least one YAC clone. This suggests that YACs in the library distribute fairly evenly over the entire genome. All our results indicate that this YAC library is well-suited for physical map construction and map-based cloning.

Methods for assembling yac contigs on the rice linkage map

Several different methods have been developed and used to construct YAC contig maps (Cohen et al 1993). All of these techniques aim to establish overlaps between YACs that allow the reconstitution of the original genome order of the cloned DNA fragments. Of these techniques, we have mainly employed a strategy of screening the library with our RFLP and sequence-tagged site (STS) markers. We have already constructed a high-density genetic RFLP map of rice (Kurata et al 1994) where 1,236 RFLP markers and 147 STSs were established, on average, at every 300 kb over the entire rice genome. With the average insert size of a YAC being 350 kb, picking up YACs with these mapped DNA markers could assemble the YACs into contigs located at particular positions on the chromosomes. This strategy has been successfully applied to cover the long arm of human chromosome 21 (Chumakov et al 1992) and chromosome Y (Foote et al 1992) with YACs.

To screen the library for the RFLP markers, we made high-density replica filters on which 6,932 DNAs from each YAC clone were gridded. YACs corresponding to

each RFLP marker in the genetic RFLP map were identified by colony hybridization using a set of these high-density filters. YACs recognized as positive by this hybridization were further characterized by Southern hybridization, and this confirmed whether the candidate YACs were derived from the marker loci.

An example of this screening is presented (Fig. 1). The high-density replica blots were hybridized with the probe R1553. Four YACs (indicated by the arrowheads) appeared to be positive in this colony hybridization. DNAs were isolated from these positive YACs, digested with endonucleases, electrophoresed, and hybridized with

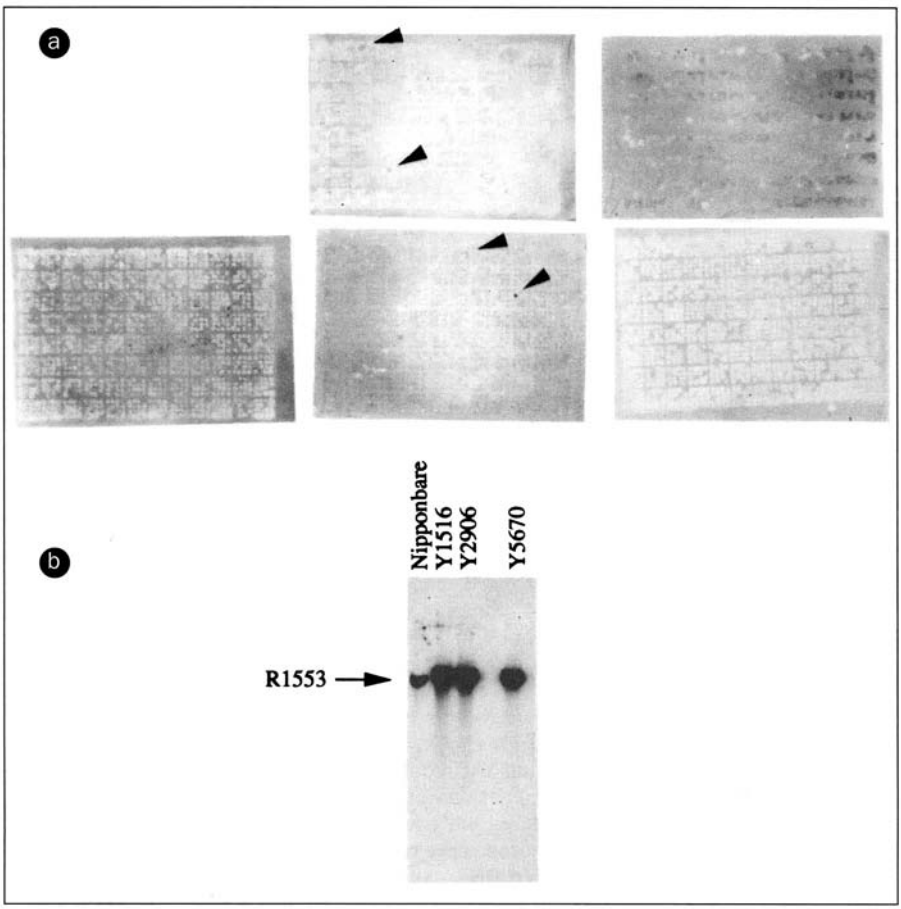


Fig. 1. A result of YAC screening by colony hybridization and Southern blot analysis. a) An autoradiogram image of a set of YAC high-density replica filters hybridized with R1553 probe. Colony hybridization was performed with the ECL system (Amersham). b) Southern blot analysis of candidate YAC DNAs as well as Nipponbare DNA with R1553 probe. The arrow points to the band mapped on the R1553 locus of chromosome 5. From the hybridization pattern, we assigned YACs Y1516, Y2906, and Y5670 to the locus R1553.

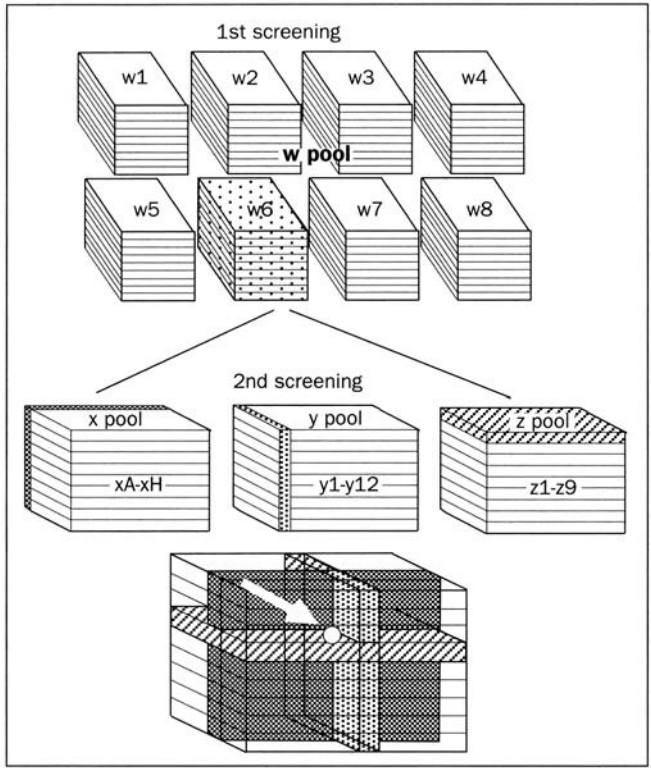
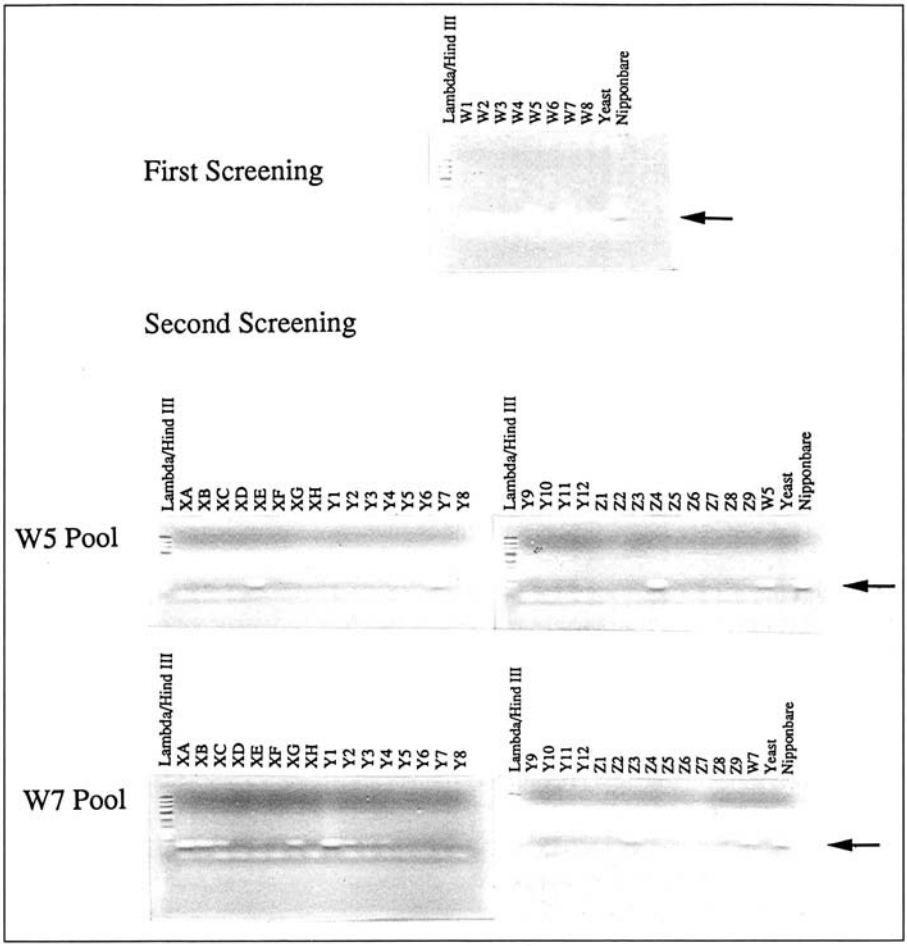


Fig. 2. a) Strategy for screening YACs with rice STSs by PCR amplification. DNAs for all YACs in every 7 or 8 plates (96-well microtiter) were pooled to create a W pool (with 672 or 768 clones in each W pool), and DNAs for YACs in 12 columns (YA-YH: 56 or 64 clones in each Y pool) and in 8 rows (X1-X12: 84 or 96 clones in each X pool) in every 7 or 8 plates were pooled to create a Y pool and an X pool, respectively. DNAs in all YACs in each plate were pooled to make a Z pool (96 clones). Two rounds of PCR, the first for the W pools and the second for the X, Y, and Z pools, identified coordinates of individual YACs containing a given STS. b) A result of YAC screening with a rice STS using the strategy in Fig. 2a. In this screening, we used a set of primers for the locus P130. These figures represent results of PCR amplification using the pooled DNAs as templates and the set of primers. The amplified products are indicated by the arrows. The results of the first screening indicate that the sequence corresponding to the locus P130 was contained in YACs in the W5 and W7 pools. From the results of the subsequent second screenings, we identified a YAC in the W5, XE, Y7, and Z4 well and another one in the W7, XA, Y1, and Z3 wells to be positive.

Figure 2 continued



R1553. Out of four candidate YACs, three (Y1516, Y2906, and Y5670) contained the band corresponding to the locus R1553, which assigned this YAC to locus R1553.

Polymerase chain reaction (PCR) amplification was used to identify YACs with rice STSs. In this process, it is not feasible to check amplification for each YAC DNA in the library. We instead created several pools of total YAC DNAs (Fig. 2a). Two rounds of PCR using the pooled DNAs as templates identified coordinates of individual YACs containing a given STS. DNA was then prepared from a single colony and analyzed by PCR to confirm the presence of the STS used to select the clone. An example of YAC identification with the P130 STS marker is given (Fig. 2b). Two YAC clones (Y3955 and Y5601) were identified in this screening.

Results of the screening were stored in a data base that runs on the relational data base system of 4th DIMENSION software. We use this data base to assist in managing and analyzing the YAC screening data on all chromosomes.

Constructing ordered yac clone libraries on all 12 chromosomes

Current status of assembly of rice YACs on all chromosomes

As of Aug 1995, we had screened the library for 1,126 markers (Table 1). Of these, 1,004 markers identified at least one YAC clone. On average, 4.1 YACs were isolated per probe; this number seems smaller than that expected from the size of this library (six haploid equivalents). There may be several regions for which the DNAs are difficult to clone into YACs because of the nature of their physical structure.

In this screening, the 1,004 markers identified 4,645 YACs, of which 2,369 YACs were independent. Through Southern hybridization of the positive YACs with the markers, 1,892 YACs were located at their specific positions on the chromosomes. The remaining 477 (2369-1892) YACs have not yet been located at particular positions because of no RFLP for the detected hybridization bands in thoseYACs. Among 2,369 YACs, 262 were assigned to more than two separate positions on one or two chromosomes, and these are considered to be chimeric.

We aligned each YAC identified in the screening at a corresponding position on the linkage map, detected overlaps—if any—between neighboring YACs, and integrated these YACs into contigs on the genetic map.

YAC assembly on chromosome 6

The most progress in chromosome assembling ofYACs has occurred on chromosome 6, where 257 uniqueYACs were identified by the 157 markers, with a total of 710 hits to YACs. The YAC locations were identified at particular positions on the chromosome, and they were assembled into 43 contigs. The minimal path of YACs in the contigs comprises 52YAC clones. Based on the average size of these YACs (350 kb), we estimate the contigs now encompass about 18 Mb or about 50% of this chromosome.

Table 1. Number of isolated YAC clones on different rice chromosomes.[a]

Chromosome	Probes	Clones[b]	Clones assigned[c]
1	230	814	341
2	113	446	236
3	162	711	285
4	71	369	212
5	104	595	346
6	157	710	257
7	107	480	193
8	16	71	45
9	21	21	27
10	31	106	85
11	85	257	108
12	47	120	59

[a]Data of Aug 1995. [b]Number of YAC clones isolated (simple sums of the number of clones identified with each probe). [c]Number of Independent clones assigned to a particular position on a chromosome.

We are presently refining the preliminary YAC contig maps of regions 1 and 2 on chromosome 6 (Fig. 3a,b). Complete maps of these regions will be published elsewhere. In these regions, most of the markers identified 1 to 11 YACs, confirming that most DNAs of this chromosome were incorporated in our YAC library and could be screened. The largest contig spans more than 6 centiMorgans (cM) (from markers C1003B to C952), and other contigs cover 1-3 cM on the chromosome linkage map. However, in the region of the markers from C1368 to R2749 (indicated by the arrow in Figure 3a), we could not isolate any YACs in our screening. DNA in this region might comprise sequences that are difficult to clone in yeast AB1380.

Physical mapping of DNA markers using assembled YAC clones

The resolution of this physical map appears to exceed the accuracy of our high-resolution genetic map. Many markers, which were not resolvable on the genetic map and were mapped at the same position, could be resolved on the physical map. One example illustrates the YAC contigs in the *eg* (extra glume gene) region on chromosome 1 (Fig. 4). There is one position where six markers (R2018, C346, R2414, G2200, L1082, and R2657A) were located together with no resolution at the level of this linkage map. Close to this position, another clustering of the markers G1133, R2625, and L543 at one position was also observed. These markers were used for screening and each one isolated YACs; some isolated YACs were commonly identified by several independent markers, and other YAC were uniquely isolated by one marker. The YACs isolated were arranged on the linkage map so that their alignment was consistent with the screening results. These arrangements of multiple YAC clones could consequently show us the order of the markers on the linkage map (Fig. 4).

Deducing locations of multiple-copy genes on the chromosomes

Copies of a multiple-copy gene are thought to be distributed at multiple positions on chromosomes. A hybridization pattern of genomic DNA probed with such a gene copy consists of many or several hybridizing bands. However, it is usually fairly difficult to assign the location of each copy corresponding to one of the bands on the chromosomes through genetic mapping. Even in this case, a physical map could give us information on the location or distribution of each copy of a multiple gene on chromosomes.

An example of the possible assignment on rice chromosomes of copies derived from one multiple-copy gene is given (Fig. 5). Southern hybridization patterns of Nipponbare genomic DNA and DNAs from 11 YACs probed with cDNA clone C425 are shown. Although the hybridization pattern of Nipponbare DNA consists of more than eight distinct bands, only one copy corresponding to the band at 3.6 kb (indicated by the arrow) could be assigned to a position on chromosome 6, at locus C425A, through genetic mapping. The hybridization pattern reveals that only YAC Y3685 contains this copy but not that of other copies of the gene. This indicates that the other copies are not near this locus. The copies revealed at 15 kb, 6.5 kb, and 5 kb are contained together in Y1061, Y4089, Y5223, and Y6828, and should thus map closely to each other on the chromosome. On the other hand, the copy corresponding to the

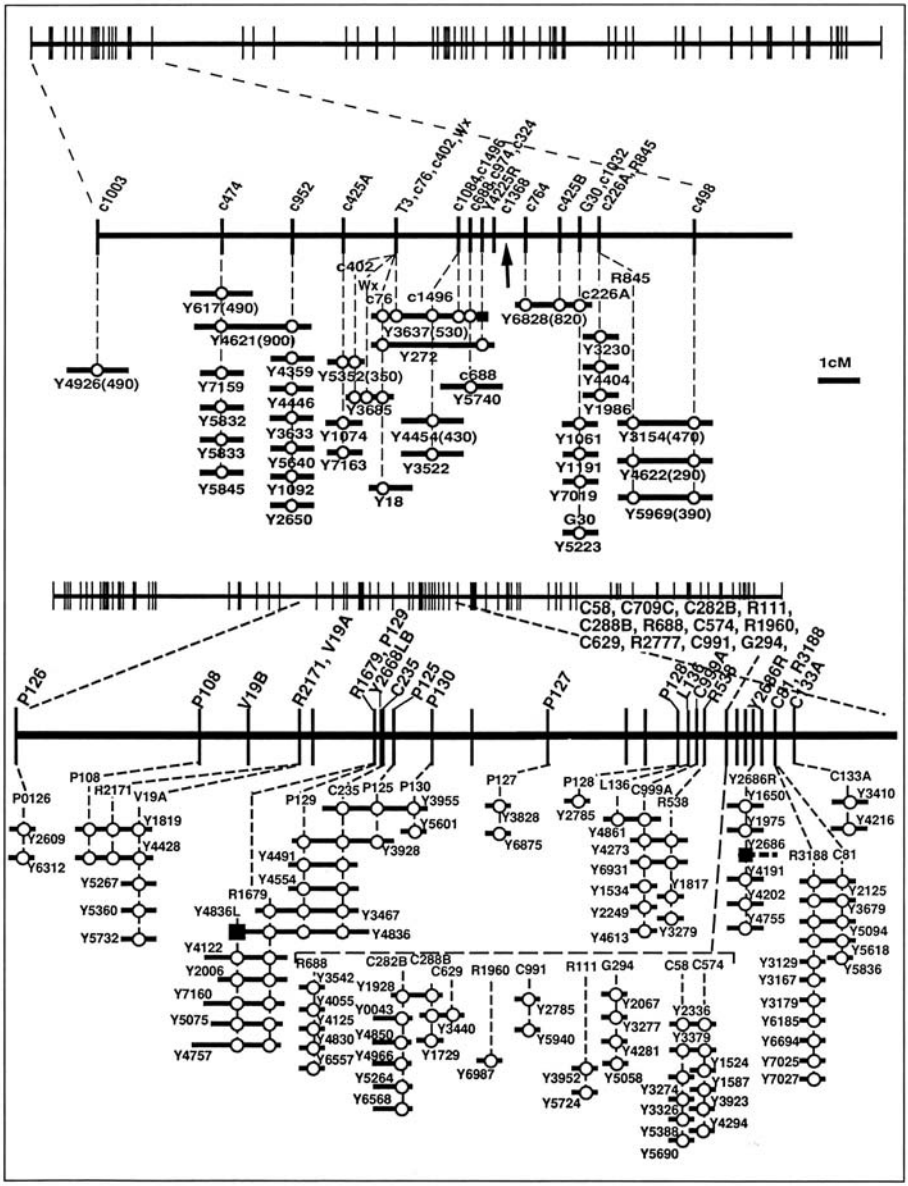


Fig. 3. Ordered YAC clones covering about half of chromosome 6 [a) region 1 and b) region 2]. YAC clones are shown by horizontal bars and their names under the genetic maps. Numbers in parentheses following YAC numbers represent lengths of the YAC inserts.

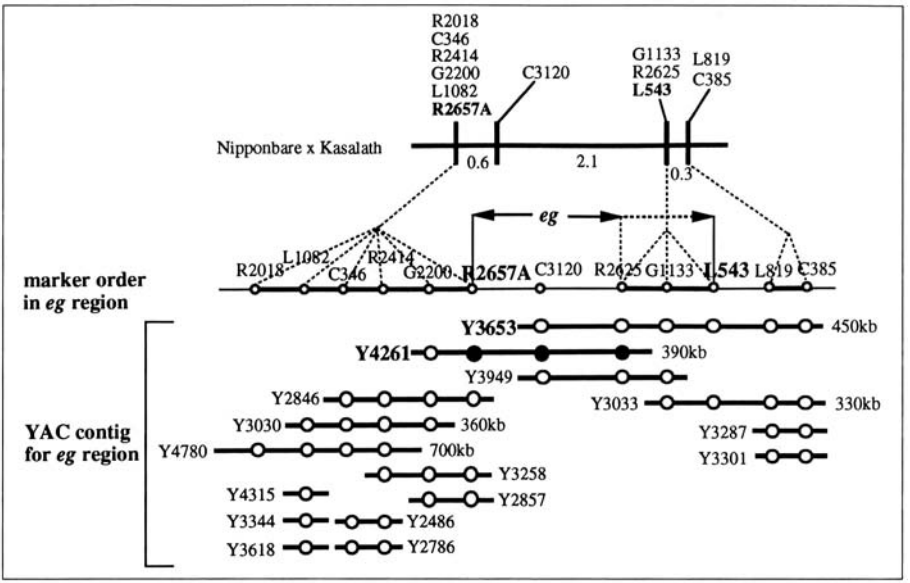


Fig. 4. Genetic map and YAC contig map in the *eg* (extra glume gene) region on rice chromosome 1.

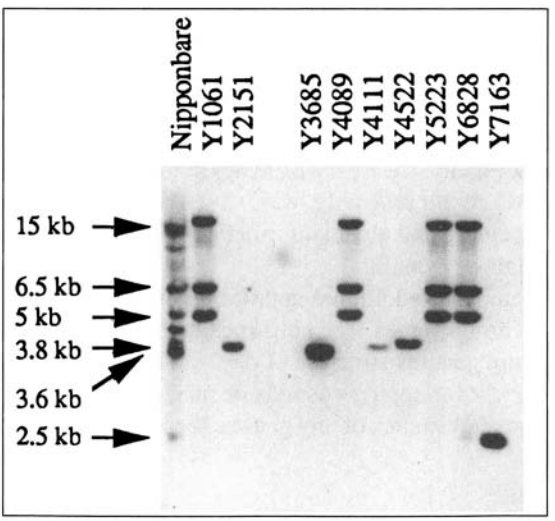


Fig. 5. Southern hybridization patterns of DNAs from 11 YACs together with Nipponbare DNA probed with C425 marker. The hybridizing bands at 3.6 kb, which were detected in Nipponbare DNA and Y3685, have been mapped to the locus C425A. Locations of copies corresponding to other bands have not yet been assigned on any rice chromosomes.

band appearing at 3.8 kb in Y2152, Y4111, and Y4522 and the copy at 2.5 kb in Y7163 should map to different positions on rice chromosomes.

Estimating physical distances on the map

Rate of recombination varies greatly from one region of a chromosome to another (Tanksley et al 1992). Now that we have integrated YAC contigs onto the linkage map, we can investigate this point quantitatively. An average physical distance per cM in the rice genome is calculated to be 273 kb (genome size [430 Mb]/total length of the linkage map [1575 cM]). In the case of chromosome 6, this value varied from 115 kb to more than 2 Mb in various regions, suggesting a very heterogeneous rate of recombination.

Toward refining the physical map

After using more than 1,100 DNA markers for YAC assembly, many gaps between YAC contigs remain on the map. To fill these gaps and to construct a more complete map, we are introducing the following additional methods.

First, after we complete the screening for all 1,383 markers on the genetic RFLP map, another 600 markers—which have been additionally located on our map—will be used for further screening of YACs. Using these additional markers will allow us to isolate YACs that might fill the gaps in the current physical map. Second, after we have used all available DNA markers, we intend to determine the locations of the remaining YAC clones (those that have not been picked up by any DNA marker) by genetic mapping using YAC end-clones or subclones. These YAC subclones are expected to be located in the gap regions. In addition, chromosome walking using YAC end-clones located at the most distal positions of each contig will help fill in the gaps of the physical map. Finally, we are trying to apply a restriction fragment fingerprinting approach to YAC contig assembly, with which we could identify YAC overlaps among neighboring clones and obtain information on the alignment of YACs, including overlapping length in a contig.

Using these combined additional approaches will facilitate assembly of YAC contigs on all rice chromosomes. The constructed physical map will be most useful for studying the entire genome structure of rice. Such a physical map can be used to isolate a collection of overlapping cosmids or phage clones in the area of interest, thereby enabling detailed studies of any genetic locus.

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Notes

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Availability of the rice YAC library: Sets of the high-density replica filters of the YAC library reported here are available for screening by hybridization-based method.

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Application of cDNA sequence information for characterization of the rice genome

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We have sequenced more than 20,000 clones from rice cDNA libraries up to Apr 1995. The libraries were made from shoot, root, and callus treated with various hormones or with a heat shock. Functions of the predicted gene products were deduced by similarity search against the NBRF-PIR data base using the FASTA algorithm. Profiles of gene expression (judged by frequencies of cDNAs coding for various genes) in these libraries closely reflected the physiological state of the rice tissue examined. The majority of proteins identified from green shoot cDNAs were photosynthesis-related, totaling more than 10% of the total number of clones. In etiolated shoots, it was rare to find these cDNA clones. In the normal callus library, the most often observed homology hits were translation-related proteins, histone proteins, etc. In the heat shock callus library, the most common hit clones had nucleotide sequences similar to heat shock proteins. From our genomic nucleotide sequence data of 1,220 clones and cDNA sequence data, we calculated frequencies of all decanucleotide sequences. In rice cDNA, after poly-A, the most abundant repetitive sequence is the CCG repeat. Repeats of AGG, AGC, AAG, ACC, ACG, and AG also appeared with high frequencies. We chose decamer nucleotide sequences for reliable RAPD primers using our decamer frequency data. Primers that have low frequency sequences could amplify several DNA fragments to detect clear polymorphisms.

Rice breeders have already produced many rice cultivars that have an excellent genetic background for good yield, taste, and resistance to various biotic and abiotic stresses. In the Japanese Rice Genome Research Program (RGP), we have been working to isolate genes with good characteristics for breeding superior rice. To isolate these genes, we have embarked on systematic cDNA cataloguing and large-scale cDNA sequence analysis (Sasaki et al 1994).

Our approach is based on DNA sequencing of many cDNA clones. The size of the rice genome, estimated to be about 430 Mbp (Arumuganathan and Earle 1991), is

too large to sequence completely at the present level of technology. So, we have decided to sequence only expressed genes. In this large-scale method, complementary DNA clones obtained from various life stages and types of rice tissue are analyzed by single-run sequencing. These nucleotide sequence stretches are then analyzed by similarity searches against public data bases to find similarities that already characterize sequences with known functions.

From our nucleotide sequence data, we found that some cDNAs from rice had repetitive sequences with two or three nucleotides. The number of repeats is sometimes different between japonica and indica rice and can be used for detecting polymorphisms. These cDNA clones are also used as RFLP mapping probes. In rice, cDNA probes tend to detect polymorphisms more frequently than genomic DNA probes. Moreover, when using cDNA clones as probes, we can easily determine linkage map positions of genes with a known function (Kurata et al 1994b). These map positions can be used as landmarks of the physical map constructed with yeast artificial chromosome (YAC) clones (Umehara et al 1995). When the targeted phenotypic locus is mapped on the linkage map, it is possible to obtain genomic DNA in a YAC clone containing this gene, which is then used as a probe to screen cDNA clones that encode some of the genomic regions corresponding to the YAC clone. Hence, the cycle of cDNA clone analysis, linkage mapping, and physical mapping, i.e., map-based cloning, will accelerate not only the isolation of new important genes, but also the characterization of the functions and physical ordering of genes in the rice genome.

Materials and methods

cDNA libraries

All cDNA libraries were made from the japonica rice Nipponbare. To cover almost all expressed genes, we constructed cDNA libraries from shoot, root, and callus. All RNAs were extracted by the guanidine thiocyanate method. Poly-A RNA was isolated by oligo-dT latex beads and then reverse-transcribed to cDNA. These cDNAs were ligated unidirectionally into plasmid vector pBluescript SK+. The recombinant plasmids were transformed into *Escherichia coli* NM522 and then stored at -80 °C.

For frequency analysis of genomic sequence, 1,300 clones of rice genomic DNA were made with pBluescript SK+ as a vector and 300-700-bp-long sonicated rice genomic DNA as inserts.

DNA sequencing

Isolation of single-strand DNA for sequencing was done using two methods: 1) the conventional manual method by Messing (1983) and 2) use of magnetic beads (FMP beads sequencing system, Amersham). The magnetic bead method is used in the full automatic DNA sequence reaction robot made by Amersham that can purify DNA templates from *E. coli* liquid culture or supernatant and make Sanger method reactions (Sanger et al 1977). Each sample was analyzed by DNA sequencer 373A (Applied Biosystems).

Similarity search and sequence frequency analysis

Vector sequences from nucleotide sequence data were removed from raw data and analyzed using two similarity search algorithms: 1) FASTA (Pearson and Lipman 1988) and 2) BLASTN (Altschul et al 1990). In FASTA analysis, the modified sequences are translated to amino acid sequences and searched for similarities against the NBRF-PIR data base. The BLASTN algorithm is used to detect redundancy of clones in each library.

All modified sequences were processed to a FASTA format file. Specially created programs were used to calculate the frequencies of all combinations of decamer sequences and short tandem repeat sequences from the FASTA format file.

PCR analysis

PCR conditions were as follows: preheating 94 °C, 1 min; 45 cycles at 94 °C, 1 min; 35 °C, 2 min; and 72 °C, 3 min; postheating 72 °C, 7 min. Amplified fragments were separated with 2% agarose gel. Primer sequences used in this study were RAC01(CGCCGCCGCC), RAC02(AGAGAGAGAG), RAC35(GCGGCTTTCC), and RAC51(CGCTATCGGA).

Results and discussion

Similarity search

We have sequenced more than 20,000 clones. Nucleotide sequences were converted to three reading frames of amino acid sequences and then the amino acid sequences were searched for similarity against the NBRF-PIR data base. This strategy depends strongly on the coverage of public data bases. At present, the "hit" rate (clones with the higher than the 200 optimized score) of PIR amino acids data base using FASTA algorithm is usually from 20 to 30% in each tissue. The gene products of the remaining "no-hit'' clones are so far of unknown function. Figure 1 shows the hit percentages of the most abundant clones in each library. For example, in the growing callus library, cDNA clones encoding protein synthesis genes, e.g., ribosomal proteins, were frequently found. In the heat shock callus library, clones of heat shock proteins were most abundant. In the redifferentiating callus, clones for protein synthesis and alpha-amylase gene clones were frequent. In the gibberellin-treated callus, the pattern of hit clones is almost the same as for growing callus, except for so-called arrest-defective proteins, which act in the cell cycle, and pectin esterase genes. In the benzyladenine-treated callus, we found chitinase III. In the root library, we found many isozymes of peroxidase. In the green shoot library, many photosynthesis-related genes (e.g., chlorophyll a/b binding protein) were detected. More than 10% of the total clones in this library encoded photosynthesis-related gene products and these sequences did not appear in clones from the other libraries. In the etiolated shoot library, expression of photosynthesis-related genes was very low, and the pattern of hit clones was otherwise similar to the patterns in callus and root libraries. We found a specific abundantly expressed gene in etiolated shoots that is similar to viscotoxin, with an unknown function. This gene may be expressed specifically in a dark environment.

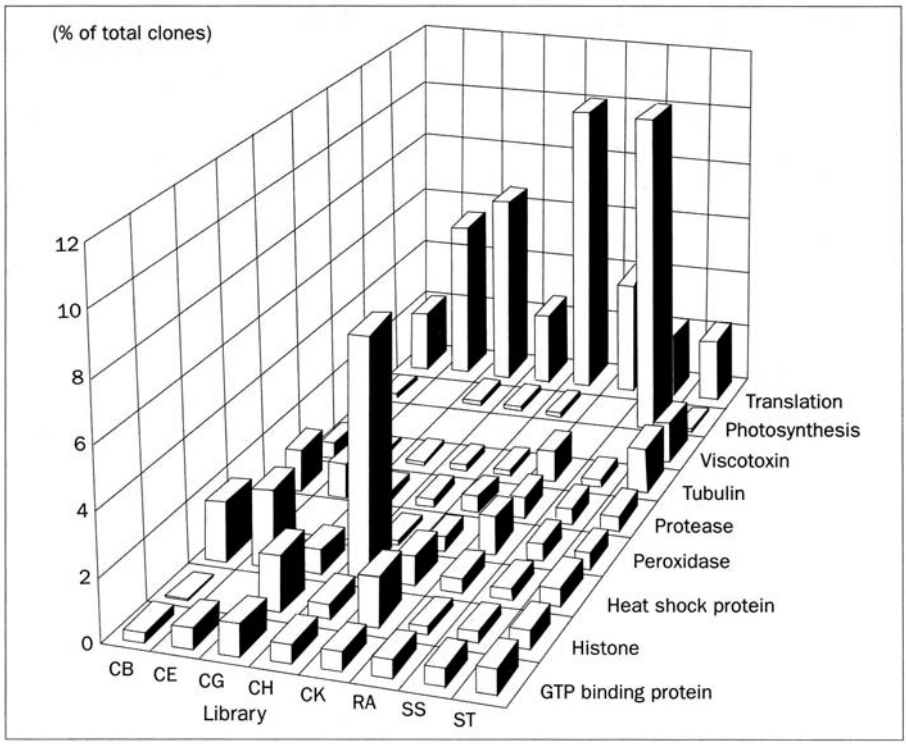

Fig. 1. Frequency of cDNA sequences in different gene classes in various cDNA libraries. CB, benzyladenine-treated callus; CE, redifferentiating callus; CG, gibberellin-treated callus; CH, heat shock callus; CK, growing callus; RA, roots; SS, green shoot; ST, etiolated shoot.

As of Mar 1995, 10,990 of our cDNA sequences were available in the DDBJ data base—and also available from GenBank and EMBL. Clones corresponding to these sequence data are all available from us via the DNA Bank of the National Institute of Agrobiological Resources.

Redundancy of the cDNA sequences was analyzed by BLASTN algorithm. The definition of an identical sequence is a match of more than 90% in a 50-bp stretch or a 100% match in a 30-bp stretch between two clones. We searched for redundant sequences among our approximately 20,000 sequences. About 10,000 kinds of unique sequences were detected. As the estimated total gene number in rice is around 30,000, partial sequences of about 30% of expressed genes are already identified.

Oligomer frequency analysis

To characterize the nucleotide sequence patterns in the rice genome, we analyzed frequencies of all combinations of decamer sequences and short tandem repeat (STR) sequences from both cDNA and genomic DNA. Total lengths of cDNA and genomic DNA sequences were 8,079,446 and 333,383 bp, respectively. Table 1 lists the 20

Table 1. The 20 most frequently appearing decamer sequences.

cDNA	Count	Genomic	Count
CGCCGCCGCC	5727	CGCCGCCGCC	65
CCGCCGCCGC	5319	CCGCCGCCGC	57
CGGCGGCGGC	5008	AAAAAAAAAA	56
AAAAAAAAAA	4962	GAGAGAGAGA	51
CCTCCTCCTC	2943	CGGCGGCGGC	49
CTCCTCCTCC	2783	AGAGAGAGAG	49
AGGAGGAGGA	2389	GAAAAAAAAA	38
GAGAGAGAGA	2354	AAAAAAAAAT	37
AGAGAGAGAG	2281	AACCCTAAAC	36
AGCAGCAGCA	1580	AAACCCTAAA	36
CTGCTGCTGC	1470	TAAACCCTAA	34
CTTCTTCTTC	1455	CCCTAAACCC	34
CAGCAGCAGC	1455	ACCCTAAACC	34
CACCACCACC	1335	CTAAACCCTA	33
AAGAAGAAGA	1333	AGGGTTTAGG	33
AGAAGAAGAA	1189	AAAAGAAAAA	33
CGTCGTCGTC	1186	AAAAAGAAAA	30
CGACGACGAC	1176	TTAAAAAAAA	27
GCGGCGGCGA	1140	CAAAAAAAAA	26
CCACCACCAC	1047	AGAAAAAAAA	26

most frequently appearing decamer sequences. Decamer sequences with a high GC content appeared more frequently in cDNA sequences than in genomic sequences. This result indicates that expressed regions in the rice genome have GC-rich sequences. From genomic sequence data, we chose some frequently appearing and some unusual decamer sequences. From these decamer sequences, primers for RAPD detection were designed. Figure 2 shows a result of agarose gel electrophoresis that separated PCR products amplified using these decamer primers. Primer RAC01, whose sequence is the most frequent among our genomic sequences, produced a smear pattern because its annealing sites in rice genomic DNA are abundant. Reaction of primer RAC51, whose sequence was not found at all in our analyzed 333,383-bp genomic sequence, shows several clear DNA bands. The GC content in the decamer primer also influenced the quality of RAPD-PCR. If GC content is less than 50%, primers cannot anneal to the template at normal temperature (35 °C). At lower temperatures (e.g., 30 or 25 °C), product yields were increased, but a lot of nonspecific bands appeared on the gel. When primers with a high GC content were used, clearly separated bands were detected. At more than 80% GC content, variations of decamer sequences are limited. Usually, we chose decamer sequences with a GC content of about 70% for RAPD detection.

Interestingly, in rice cDNA sequences, we detected several repetitive sequences. Table 2 shows a number of clones that encoded repetitive sequences repeated more than four times. Except for poly-A, the most abundant repetitive sequence is the CCG repeat. AGG, AGC, AAG, ACC, ACG, and AG repeats also appeared with high frequencies. However, the AC repeat sequence, which is the most abundant repetitive

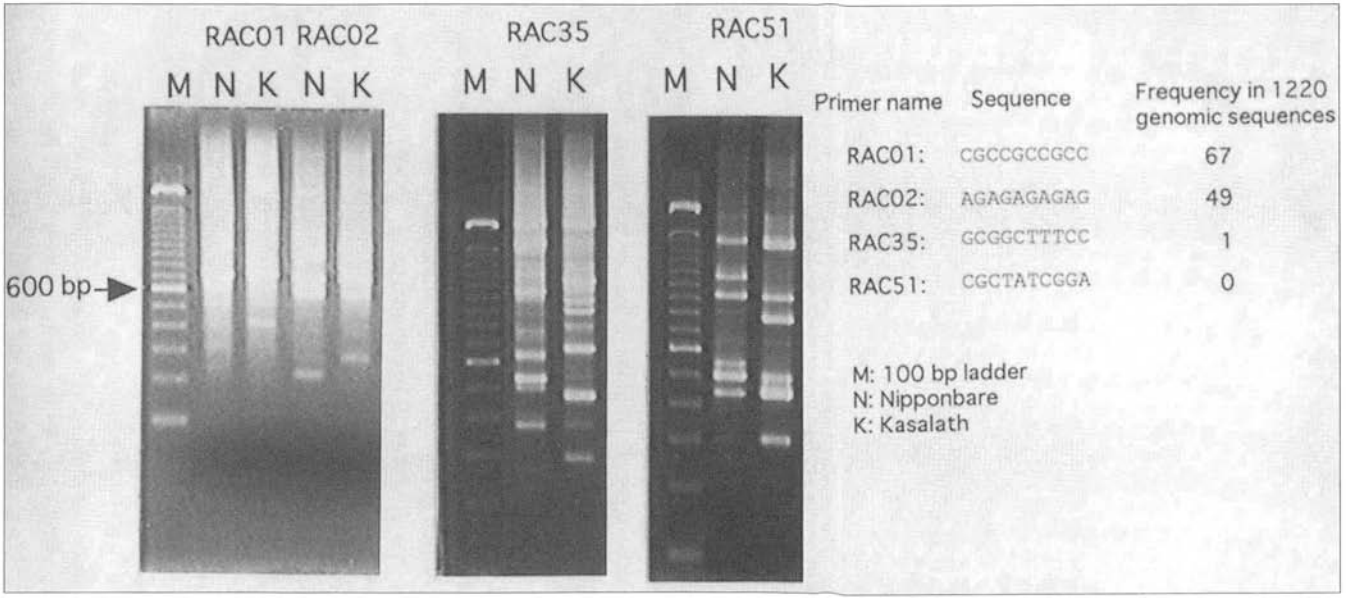

Fig. 2. Gel patterns of separated PCR products using the decanucleotide primers. 'Frequency' means the number of decamers found among 1,220 random genomic sequences (total 333,383 bp).

Table 2. Number of clones encoding repetitive sequences repeated more than four times. A total of 22,709 cDNA clones was analyzed. Repeat sequences are shown in alphabetic order (only the nonredundant repeats are shown).

Dinucleotide	Clones	Trinucleotide	Clones
AA	1488	AAA	330
AC	423	AAC	66
AG	1743	AAG	451
AT	296	AAT	20
CC	180	ACC	439
CG	970	ACG	434
		ACT	65
		AGC	665
		AGG	1016
		ATG	94
		CCC	39
		CCG	2003

sequence in human DNA, was low in frequency in rice cDNA and genomic DNA. We constructed specific primers that amplify the CCG and AG repeat regions. Some fragments amplifying these primer sets showed fragment length polymorphisms between templates from Nipponbare and Kasalath. Several loci of polymorphic fragments were mapped on our RFLP map, with segregation data from 186 F_2 progenies between Nipponbare and Kasalath (data not shown). Hence, we can easily obtain STS markers from cDNA clones that have repetitive sequences. These STS markers can be used in PCR and electrophoresis analysis to screen YAC clones containing target loci.

Our large-scale cDNA analysis resulted in not only many expressed gene clones, whose functions were assigned by similarity searches, but also in useful information about the expression level of many genes. Also, because these DNAs and sequence information were used as probes for RFLP mapping and as primers for PCR mapping, we can easily map genes with known functions into our linkage map. Recently, synteny of gene location between wheat and rice chromosomes was studied using our RFLP markers from rice cDNA and wheat RFLP markers (Kurata et al 1994a). It was found that gene order is conserved between rice and wheat, although the genome of the wheat is 10 times larger than that of rice. This result suggests that the minimum set of genes required for plant growth is very similar between these two crop plants. Hence, data from rice cDNA analysis will be of wide application, not only for rice genetics and molecular biology, but also for studies of other plants.

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Notes

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Structure and gene expression of the mitochondrial genome of rice

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We examined the structure and organization of the mitochondrial genome and the expression of the mitochondrial genes of rice (*Oryza sativa* cv Nipponbare). In particular, the entire set of the transferred chloroplast DNA sequences and a family of the palindromic repeated sequences that mainly exist in the intergenic spacer regions were identified. Furthermore, we determined the transcription initiation sites of rice mitochondrial genes by in vitro capping and ribonuclease protection assays and primer extension analysis. A consensus sequence was observed around the sites of initiation of transcription, indicating that the consensus motif is recognized as promoters of rice mitochondrial genes.

It has been known that the mitochondrial genome of higher plants is the largest and most complex among those of the eukaryotes. However, the plant mitochondrial genome does not contain many genes. Noncoding regions, the intergenic spacer regions, occupy a majority of the mitochondrial genome. There are a lot of pseudogenes derived from both mitochondrial and chloroplast genes, many small repeated sequences, and regulatory elements such as promoter and terminator elements in the intergenic spacer regions. In the present study, we investigated the structure and gene expression of the mitochondrial genome of rice, placing priority on the features of the intergenic spacer regions.

Identification of the entire set of transferred chloroplast DNA sequences in the mitochondrial genome of rice (Nakazono and Hirai 1993)

ctDNA sequences in the rice mitochondrial genome

The mitochondrial genomes of higher plants are much larger and more complex than those of other eukaryotic organisms (Newton 1988). It has been suggested that one of the reasons for such complexity might be the presence of many chloroplast sequences in the mtDNA of higher plants. Although there are many reports of chloroplast-like sequences in the mtDNA of higher plant, there are still few comprehensive surveys of these sequences. However, Stern and Palmer (1986) identified all locations of chloroplast-like sequences in the mtDNA of spinach by Southern hybridization.

We have carried out hybridization analysis using clone banks of chloroplast DNA (ctDNA) and mtDNA from rice (*Oryza sativa* cv Nipponbare) to examine the chloroplast sequences in the mtDNA of rice. Nucleotide sequences of hybridized fragments were determined and compared with the reported chloroplast sequences. We describe the entire set of chloroplast sequences in the rice mitochondrial genome with exact lengths of transferred fragments.

We found 16 chloroplast fragments in rice mtDNA that ranged from 32 bp to about 6.8 kb in length. As shown in Figure 1, the transfer of ctDNA occurred from

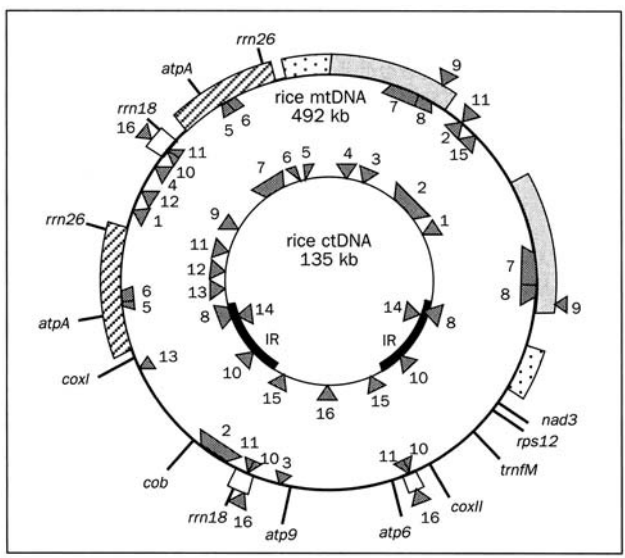

Fig. 1. The chloroplast DNA sequences in the rice mitochondrial genome. The inner circle indicates the rice chloroplast genome (Hirai et al 1985). Thick lines indicate the inverted repeat (IR) sequence. The outer circle is a master circle of the rice mitochondrial genome (Iwahashi et al 1992). The repeated sequences are indicated by boxes outside the master circle. The sequences homologous to ctDNA are indicated by shaded triangles with numbers. The numbers correspond to those in Table 1.

Table 1. Chloroplast DNA sequences in the mitochondrial genome of rice.

No.	Size (bp) in ctDNA[a]	Position	Gene(s)	Sequences around the borders mt|*ct*[c] *ct*|mt[c]
1	570	18752-18425	*trnC*	ATCAATTCAA|*ATTAATAAAA—CCATTAACTA*|TATTAATTAA
2	~6,800	18985-25732	*rpoB,rpoC1,rpoC2*	TTTATATACC|*CATTTTCATT—GAGCCAGGGA*|CAATTGATAC
2'	371	18985-19356	*rpoB*	TTTATATACC|*CATTTTCATT— ATCACGGAAT*|GGATACGAAA
3	463	32051-32533	*atpH*	CATTCCACTA|*ATTGCTGCTG—CTTTTTCCTT*|CTCCCGTGCT
4	1,090	34853-35963	*atpA,trnR*	CAAGAAGAAA|*AAGAGGGGGC —TCTTTCATTT*|TGTAAACAAC
5	1,530	43974-45573	*trnS,rps4*	ATATGGGCTT|*TCTGCJCAAA—TCCAATGCT*|GCAAGCAAGC
6	2,536	46932-49319	*trnL*[b]*,trnF,ORF159,ndhK*	
7	~5,500	50595-55954	*trnV*[b]*,trnM,atpE,atpB,rbcL*	AATGATGCAG|*GAGCAATACC—CAGATCGTAT*|ACTAGTATTC
8	2,094	132158-134393	*rp123,rp12*[b]*,trnH,rps19*	
9	457	64009-64458	*trnW,trnP*	TTTTTGATC|*CGACATAACA—ACCCAACCTA*|ACGTATAAAG
10	32	90916-90947	*spacer region (ORF85-trnV)*	TGATTGGTCG|*AGCCCGGAGG—GCTTCTTCAT*|TTCTCTAAAC
11	48	68198-68245	*clpP*	
12	821	71837-72654	*petB*[b]	AGTTGAATGC|*TGAGATTTTT—GCCCTTTCTA*|TGAAGCAATG
13	358	77238-77609	*rp114*	ATAGTCTCTA|*CTACTAGTAT—AGGGTCTGAG*|TGAGATGGAT
14	32	131904-131935	*trnI*	TCACTCGCTA|*AAGCATCCAT—AAAGCGCCCA*|CCAACCTAGA
15	86	99203-99288	*trnN*	TTCGAATTCT|*GAATGAATCA—TACTGAGGAA*|GAACGGACTT
16	57	109079-109131	spacer region *(ndhE-ndhG)*	AACAGGTACG|*AATCACTAGA—GGTATTTGTG*|AAAAGAGAAA

[a]Numbers are taken from Hiratsuka et al (1989). [b]Intron-containing genes. [c]mt: mitochondrion. *ct:* chloroplast.

widely separated regions of ctDNA to widely separated sites in the mtDNA. Transferred chloroplast sequences are summarized in Table 1. The results show that about 6% (22 kb) of rice mtDNA, excluding repeated sequences, is made up of chloroplast sequences. Thus, about 19% of the rice ctDNA, omitting one of the inverted repeats, must have been transferred to the rice mtDNA. We did not find any common sequences or structures around the termini of chloroplast sequences that might explain the insertion of the ctDNA into the mtDNA.

Rearranged ctDNA sequences in rice mtDNA

It was previously reported that the mitochondrial genome of rice (*O. sativa* cv Labelle) contains a rearranged cluster of chloroplast genes, namely, *rp12* plus *yrp123-rbcL-atpB-atpE-trnM-trnV* (Moon et al 1988). As shown in Figure 2, we also detected *rp12/yrp123-rbcL-atpB-atpE-trnM-trnV* in the mitochondrial genome of the cultivar Nipponbare. In the previous report, the terminus on the *rp12* side was not reported. However, this terminus was found to be located within *rps19* in our analysis. We compared rice mitochondrion-specific sequences at the border with available sequences

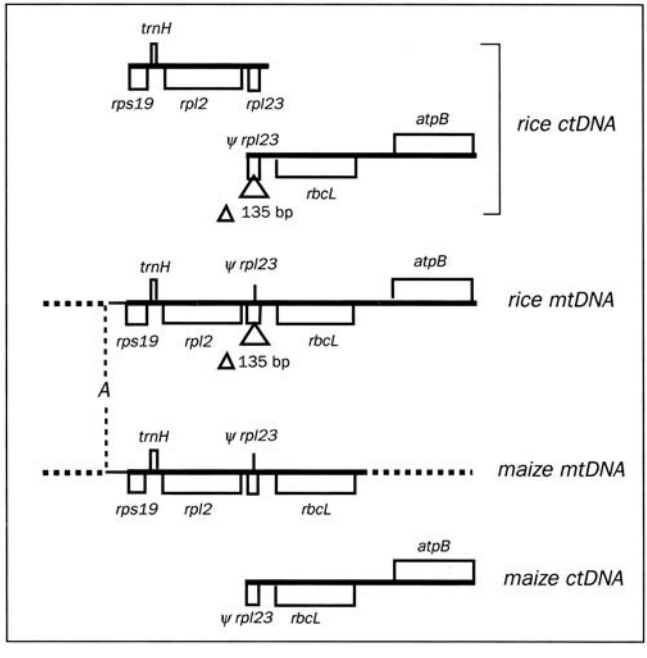

Fig. 2. Comparison between chloroplast sequences in the mitochondrial genomes of rice and maize. Thick lines indicate chloroplast sequences. Regions of high homology between rice and maize mitochondrion-specific sequences at the border of chloroplast sequences are shown by thin lines. The sequence upstream from position A in maize mitochondrial DNA was not available at the time of writing.

from maize (Iams et al 1985), and found 93% homology among sequences of 84 bases. The result indicates that the transfer of this region occurred before the divergence of rice and maize. By contrast, rice *yrp123* in ctDNA and mtDNA had a 135-bp deletion. A maize mitochondrial clone, pLSH20, containing *yrp123* was sequenced, and we found that neither maize gene had a deletion, as illustrated in Figure 2 (Bowman et al 1988). These facts reveal that this region was transferred from ctDNA after the divergence of rice and maize. Thus, it is suggested that *rps19-trnH-rp12-rp123* must have been transferred to the mtDNA before the divergence of rice and maize, and the region corresponding to *yrp123* must have been transferred after the divergence of rice and maize. Then the two regions must have become connected as a result of at least two recombination events, one in rice and the other in maize. This sequence must be a "hot spot" for recombination in mitochondria.

Insertion of palindromic repeated sequences (PRSs) after divergence of genus *Oryza* from other Gramineae (Nakazono et al 1994)

Number and location of PRSs in the rice mitochondrial genome

When analyzing transferred sequences of chloroplast DNA in the mitochondrial genome of rice (*O. sativa* cv. Nipponbare), we found three small repeated sequences, which were about 60 bp in length, in the flanking regions of chloroplast-like (ct-like) sequences. To determine whether these repeats are widely distributed in the mitochondrial genome of rice, we performed plaque hybridization to lambda clones in a clone bank of rice mtDNA using a mixture of two oligonucleotides, which corresponded to sequences on the right (35 bases; IS-R, 5'-AAGAAAACGGATGCGCGTGCTAACGTTTCGCGCTA-3' and the left (36 bases; IS-L, 5'-, CCAAACAAGCAACGGATTGAGCGCACTAGCGCGAAA-3') sides of the small repeated sequence (Iwahashi et al 1992). It was found that rice mtDNA contained at least 10 copies of the small repeated sequence. We carried out subcloning and sequencing of the hybridizing regions. These small repeated sequences were distributed throughout the mitochondrial genome of rice (Nakazono et al 1994). It was predicted by computer-assisted analysis that the small repeated sequences had the potential ability to form stem-and-loop structures. Therefore, these repeats were designated the *PRSs* (palindromic repeated sequences) of rice mtDNA (Fig. 3a). Sequencing data show that these repeats are from 60 to 66 bp in length and exhibit a high degree of homology to one another (Fig. 3b).

A PRS is located in the intron of the gene for ribosomal protein S3 (*rps3*) of rice mtDNA

We identified genes for ribosomal proteins S3 (*rps3*) and L16 (*rpl16*) in rice mtDNA. Both genes were not only closely linked but overlapped, as they do in maize mtDNA (Fig. 4; Hunt and Newton 1991, Nakazono et al 1995a). One of the PRSs (361-PRS; PRS included in clone No. 361) was located in the intron of rps3 in rice mtDNA, but not in maize mtDNA (Fig. 4). It seemed likely that either a PRS had been inserted in

Fig. 3. a. Possible secondary structure of the small repeated sequence designated 363. The stem-and-loop structure was generated using GENETYX Software (SDC, Tokyo, Japan). b. Alignment of 10 small repeated sequences in the mitochondrial genome of rice. Capital letters indicate conserved nucleotide sequences.

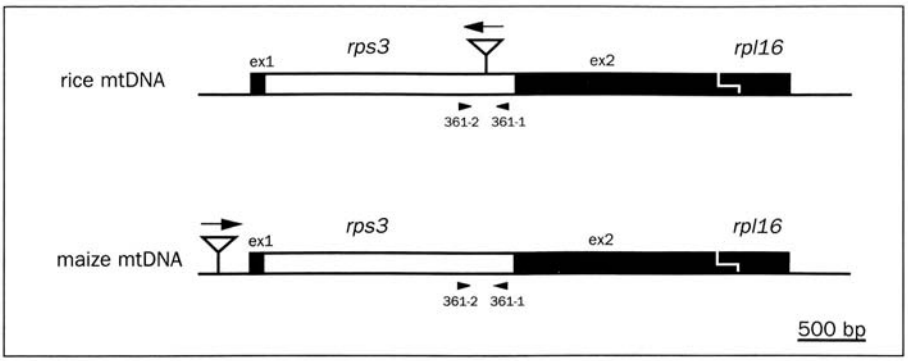

Fig. 4. Organization of *rps3* and *rpl16* in rice and maize mtDNAs. The closed boxes and the open boxes indicate the coding regions and the introns, respectively. ex1 and ex2 indicate exons 1 and 2 of *rps3.* The positions of each *PRS* are indicated by open triangles. Arrows indicate the directions of each *PRS.* Arrowheads indicate the positions of each primer (361-1, 361-2) used for amplification by PCR.

this region in rice or had been deleted from the corresponding location in maize. To examine these possibilities, we performed amplification by PCR using primers and total DNAs from various species of *Oryza* [*O. sativa* (Nipponbare), *O. rufipogon* (perennial type; W0120), *O. barthii* (W0822), *O. glaberrima* (W0025), *O. punctata* (W1515), *O. minuta* (W1319), *O. eichingeri* (W1521), *O. latifolia* (W0542), and *O. australiensis* (W0008)], as well as from maize, wheat, barley, and sorghum. The two primers [27 bases and 26 bases; designated 361-1 (5'-GAGGATCCTCTCTGAACCGTGCTAGAT-3') and 361-2 (5'-GCGGATCCAGAACAACTCTGAACGTT-3'), respectively] corresponded to sequences in the two flanking regions of 361-PRS.

The anticipated 323-bp fragments were amplified in all of the species of *Oryza,* as shown in Figure 5a. However, the fragments amplified in the case of the other Gramineae were about 60 bases shorter than those of *Oryza* species. Southern hybridization was performed with the probes described above (IS-R and IS-L; Fig. 5b). Hybridization signals were obtained only from species of *Oryza,* showing that only fragments amplified from *Oryza* DNAs contained PRSs. We cloned the individual PCR-generated fragments of the other Gramineae in the pUC119 vector and determined their sequences. Only rice mtDNA had a PRS in this region, but the flanking sequences around the PRS were much the same among the five members of the Gramineae (Nakazono et al 1994). These results indicate that a PRS had been inserted in the intron of *rps3* of *Oryza* mtDNA after the divergence of *Oryza* from other Gramineae. Both ends of this PRS had incomplete direct repeats of nine bases ($TCTT^{c}_{g}$ CTCC). This evidence indicates that some PRSs may be capable of movement within the mitochondrial genomes of *Oryza.*

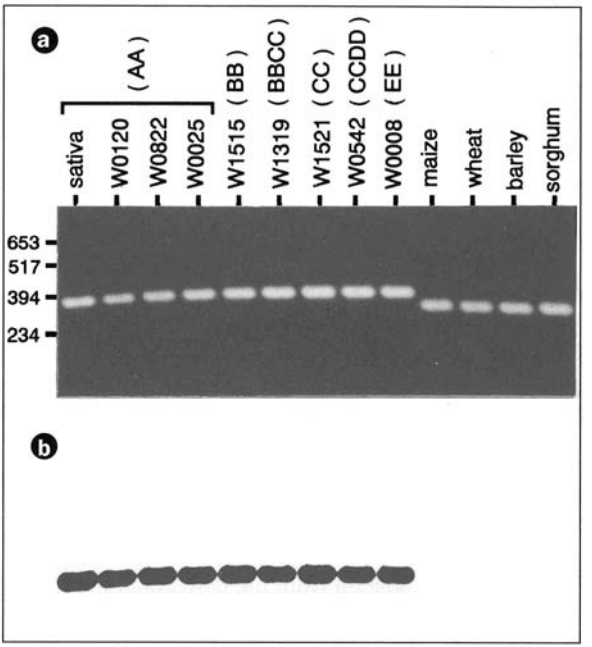

Fig. 5a and b. Ethidium bromide staining and Southern hybridization of PCR-generated fragments after electrophoresis. Amplifications by PCR were performed using primers 361-1 and 361-2. PCR-generated fragments were subjected to electrophoresis in a 1.2% agarose gel. AA, BB, BBCC, CC, CCDD, and EE indicate each nuclear genometype of Oryza. The numbers on the left indicate sizes of fragments in basepairs (a). Southern hybridization was performed using a mixture of oligonucleotides IS-R and IS-L as probes (b).

Identification of transcription initiation sites on the mitochondrial genome of rice (Nakazono et al 1995b)

The 5' end of a primary transcript, synthesized from the site of initiation of transcription in a plant organelle, can be specifically labeled in vitro by the capping enzyme guanylyltransferase (Auchincloss and Brown 1989, Vera and Sugiura 1992). The precise 5'-ends of primary transcripts of five genes *(orf483, rrn18, atp9, rrn26,* and *trnfM* and one gene cluster *(rps3-rpll6-nad3-rps12)* were investigated using in vitro cappinghibonuclease protection experiments and primer extension analysis (Nakazono et al 1995a,b,c). The transcription initiation site of the *cob* gene has been established by Kaleikau et al (1992). As shown in Figure 6, we aligned sequences around the initiation sites of these monocistronic and polycistronic transcripts *(orf483, rrn18, atp9, rrn26, trnjfM, rps3-rp116-nad3-rps12,* and *cob).* The alignment revealed a high

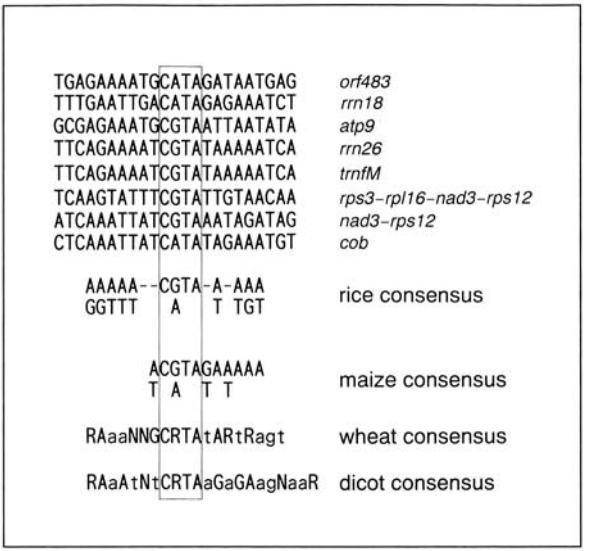

Fig. 6. Alignment of the nucleotide sequences around the transcription initiation sites of rice mitochondrial genes and a gene cluster (*orf483, rrn18, atp9, rrn26, trnfM, rps3-rp116-nad3-rps12,* and *cob*). The consensus motif of the promoters of rice mitochondrial genes was deduced from the alignment. Consensus promoter sequences of the mitochondrial genes of maize, wheat, and dicots are taken from the reports by Mulligan et al (1991), Covello and Gray (1991), and Binder and Brennicke (1993), respectively. The box indicates the CRTA motif that is highly conserved in the promoter regions of monocots and dicots.

degree of similarity among sites, suggesting that the conserved motif [(A/G) (A/G) (A/T) (A/T) (A/T) NNC (G/A) TAN (A/T) N (A/T) (A/G) (A/T)] is recognized as the promoter of rice mitochondrial genes. This consensus sequence of rice exhibits a high degree of homology to those of other gramineous plants, i.e., maize (Mulligan et al 1991) and wheat (Covello and Gray 1991). In particular, the sequence C (G/A) TA, known as the CRTA motif, was found to be highly conserved in the promoter of rice mitochondrial genes examined by the in vitro capping method, as it is in those of other plants [maize (Mulligan et al 1991), wheat (Covello and Gray 199l), soybean (Brown et al 1991), and *Oenothera* (Binder and Brennicke 1993)]. We propose that the CRTA motif is necessary for the active transcription of the mitochondrial genes of rice, as it appears to be the case in the other plants examined.

To determine the number of promoters in the mitochondrial genome of rice, rice mtDNA digested by *Xba*I, *Sal*I, and *Bam*HI, respectively, was allowed to hybridize with in vitro capped mtRNA and was then treated with ribonucleases T1 and A. Thirteen to 16 signals (*Xba*I, 13 signals; *Sal*I and *Bam*HI, 16 signals each) were detected

(Nakazono et al 1995b). If one transcription initiation site is present in each detected fragment, this observation indicates that the mitochondrial genome of rice includes at least 16 promoters. As described by Binder and Brennicke (1993), transcription initiation sites of primary transcripts that are rapidly processed and/or degraded may be not detectable with this method. However, it is possible that signals that originate from different promoters might be duplicated in restriction fragments of a single size. Furthermore, we cannot rule out the possibility that some primary transcripts, transcribed from promoters in repeated sequences, might hybridize to several fragments. The locations of the individual promoters on the mitochondrial genome of rice should now be investigated.

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Advances in rice chromosome research, 1990-95

K. Fukui

After providing a historical perspective on rice chromosome research, this paper reviews and summarizes advances made during the 1990-95 period. The most significant research accomplishments during the last 5 yr are the complete and objective identification of all the somatic rice chromosomes (80 yr after the first accurate report of the number of rice chromosomes) and the development of a quantitative rice chromosome map. Also, in 1991, the long discussion on unifying the various numbering systems for the rice chromosomes ended with the implementation of a new numbering system. New technologies recently developed include 1) imaging methods that make it possible to identify rice chromosomes automatically using personal computers, 2) fluorescence in situ hybridization, 3) genomic in situ hybridization for differential painting of the rice genomes, and 4) the laser microdissection method used in dissecting specific regions of individual rice chromosomes.

Historical perspective

Chromosome analysis in rice

Kuwada (1910) first reported the chromosome number of cultivated rice, 2n=24, using both mitotic and meiotic cells. Many cytological studies followed to elucidate the characteristics of the rice chromosomes. For example, Rau (1929) classified rice chromosomes into three types and Nandi (1936) reported that chromosome size ranged from 0.7 to 2.8 μm at mitotic metaphase.

Hu (1964) identified seven or eight different rice chromosome types at mitotic metaphase and karyotypic similarity between *Oryza sativa* and *O. glaberrima,* which later was unequivocally proven by imaging methods (Ohmido and Fukui 1995). The effectiveness of the prometaphase stage in identifying rice chromosomes was also

demonstrated by Hu (1964). Kurata and Omura (1978) and Wu et al (1985) reported the respective karyotypes of japonica and indica rices. Also. Kurata and Omura (1978) used an elegant enzymatic maceration and flame-drying method for preparation of chromosomes, which gives an excellent spread.

Shastry et al (1960) examined pachytene chromosomes, which are suitable for detailed observation of rice chromosomes. Nishimura (1961) developed 12 reciprocal translocation lines in a japonica variety, Kinmaze. Khush et al (1984) and Iwata and Omura (1984) developed 12 primary trisomic series using indica and japonica rices, respectively. In 1991, a new chromosome numbering system was proposed to integrate the somatic karyotype, pachytene karyotype, Nishimura's numbering system, trisomics,

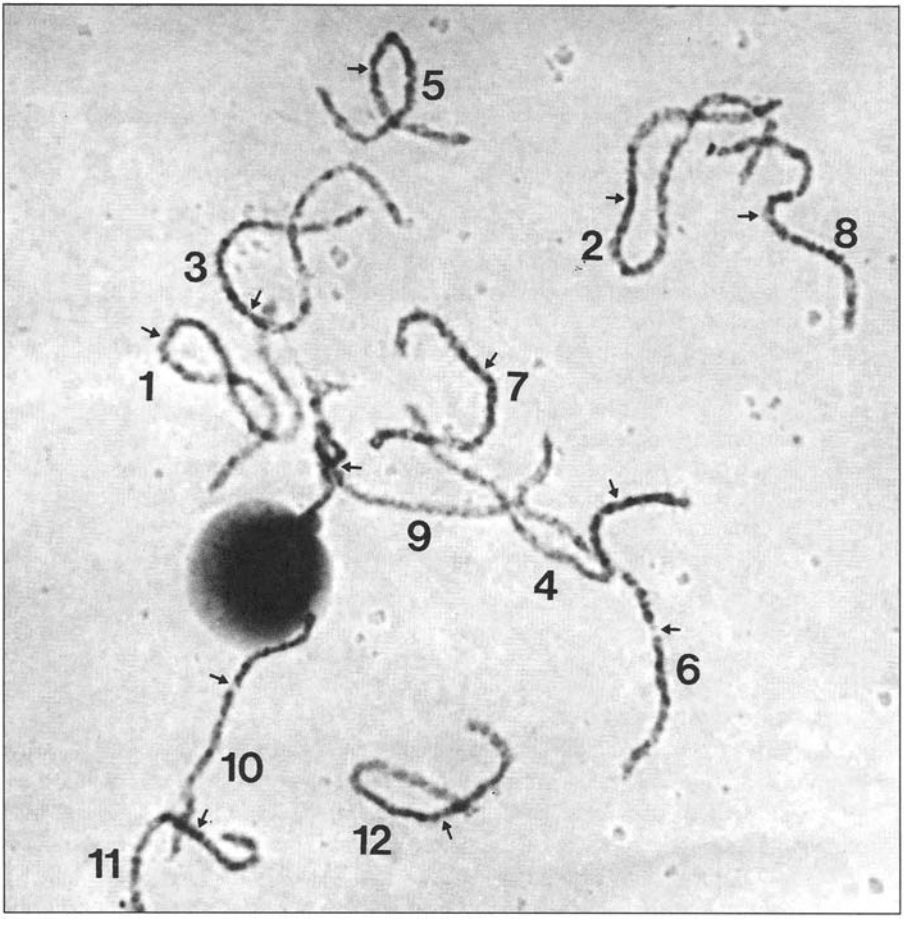

Fig. 1. Pachytene chromosomes of an indica (IR36) haploid plant. Arrows and numbers show centromeres and "new" chromosome numbers, respectively. Chromosomes 9 and 10 are attached to a nucleolus, clearly indicating that the two chromosomes have the nucleolar organizing regions (Khush and Kinoshita 1991).

and linkage groups based on the length of pachytene chromosomes (Khush and Kinoshita 1991). Figure 1 shows the spread of the pachytene chromosomes. Dark proximal chromomeres and faint distal chromomeres are both clear in all the chromosomes.

Genome analysis in rice

Morinaga (1937, 1939) first identified different genomes in the rice species and proposed five different genomes from A to E based on the results obtained. The differently proposed genome formulas of rice species were integrated by a committee at the Symposium for Rice Genetics and Cytogenetics in 1963. To date, six different genomes from A to F and corresponding diploid species except the D genome are identified in the genus *Oryza* (Vaughan 1989). The D genome is recognized only in combination with the C genome in amphidiploid CCDD species.

Identification of rice chromosomes and a quantitative chromosome map

Identification of rice chromosomes

Banding methods are often useful to identify plant chromosomes. No banding method has so far been successful in rice chromosomes in spite of many trials. Although there are some preliminary reports on G-bands in rice, they are not stable and reproducible for identifying rice chromosomes and for developing a chromosome map.

Small plant chromosomes, such as those in rice, often show uneven condensation at mitotic prometaphase as shown in Figure 2 (Hu 1964, Kurata and Omura 1978, Fukui 1986a, 1989). Thus, the condensation pattern (CP) appearing at prometaphase was only a morphological landmark to divide the rice chromosomes into subregions (Fukui and Mukai 1988, Fukui 1989). Characteristics of each rice chromosome with uneven condensation have quantitatively been analyzed by using image analysis methods (Fukui 1985, 1986b, 1988; Iijima and Fukui 1991).

Figure 3 shows the steps in conducting an image analysis of rice chromosomes at prometaphase. First, a photographic or analog image of the part of a rice prometaphase chromosomal spread was digitally captured (Fig. 3a) and the contrast of the image was digitally enhanced (Fig. 3b). After determining the chromosomal regions by binalization or generation of a black and white image (Fig. 3c), the contour line of each chromosome was extracted and superimposed onto the chromosome image to check whether the extraction of the chromosomal region was adequate (Fig. 3d). Then the chromosomal images were pseudo-colored to make the minute differences of the chromatin condensation distinct (Fig. 3e). The CP or density profile along a midrib line of the chromatid was measured by using the enlarged image (Fig. 3f). By observing the condensation patterns of the chromosomes, all the rice chromosomes could be identified.

Characterization of rice chromosomes

Figure 4 shows the characteristics of 12 japonica chromosomes demonstrated by a Giemsa-stained image (upper panel), a pseudo-color image (middle panel), and a pseudo-three-dimensional image (lower panel). The average condensation pattern, based on 30 haploid prometaphase spreads, is represented graphically (Fukui and Iijima 1991). Pseudo-color and pseudo-three dimensional representation dramatically improve the ability of identifying the fine structures of each rice chromosome.

Heavy condensation of the short arm of chromosome 6(12) and the long arm of chromosome 10(7) is clearly demonstrated both by pseudo-colors and their differential coloring steps. The numbers in parentheses are the new chromosome numbers decided upon in 1991. Chromosomes 1(1), 2(3), and 3(2) are large chromosomes with long dispersed ends on both arms and lightly condensed proximal regions. Chromosome 11(9) is a subtelocentric, satellite chromosome with tandem-repeated 45S ribosomal DNAs (nucleolar organizing region) at the end of the short arm in the case of japonica. The number of rDNA sites is different among different rice species. Chromosomes 4(4) and 12(10) are of the submetacentric type without visually dispersed regions at the end of the short arm. There are the other satellite chromosomes in *O. sativa* indica (chromosomes 11[9], 12), and *O. officinalis* (chromosomes 4, 9[11], 11). Chromosome 9 is the only chromosome with the tertiary constriction at the long arm. Chromosomes 5(6) and 8(5) are middle-sized metacentric types. Chromosome 7(8) is also a middle-sized metacentric type with a longer long arm. Numerical data and detailed characteristics of each rice chromosome were given without (Iijima et al 1991) and with the appearing frequencies (Fukui and Iijima 1992).

Although the characteristics of the rice chromosomes are based on those in japonica rice, the basic features of each chromosome are similar in the other rice species as suggested by Hu (1964). Major differences in chromosome morphology among the species are found in the condensation at the subterminal regions, the number of satellites, and the total size of the chromosomes.

Discrimination chart for rice chromosomes

Iijima et al (1991) developed a method for identifying rice chromosomes based on a flow chart that consists of 11 discriminants, which classify specific chromosome groups. All rice chromosomes can be identified and numbered by comparing the categories given by the discriminants, one after another. The chromosomal spread is worth analyzing if chromosomes 4, 11, and 12 are distinguishable by visual inspection and if chromosomes 1, 2, and 3 can be completely recognized. If these six chromosomes can be identified using discriminants 1 through 6 in order, then there is a great possibility of identifying all 12 chromosomes within the particular spread. Note that the steps for discriminant no. 9 have been updated from those originally provided by Iijima et al (1991) as follows: “Discard the two submetacentric chromosomes. The remaining two chromosomes are either chromosome 5(6) or 8(5).”

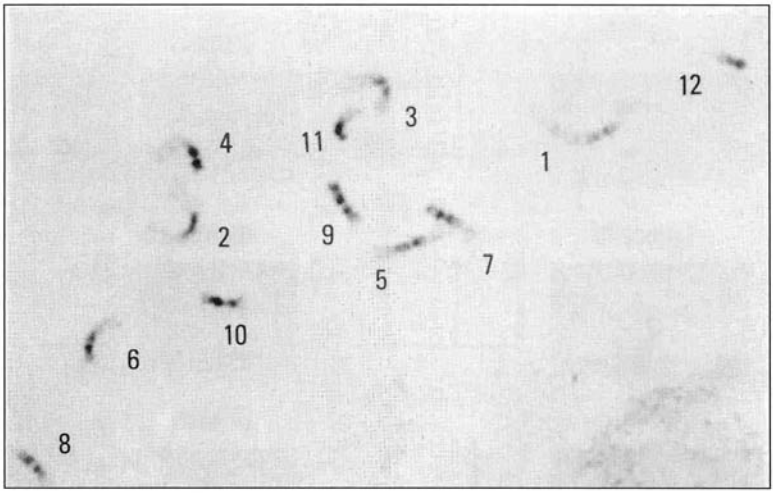

Fig. 2. Typical somatic chromosomes of a japonica (Koshihikari) haploid plant. The chromosome sample was prepared using the EMA method (Fukui and Iijima 1991). The bar in the lower right indicates 5 pm. (Prof. Hiroo Niizeki supplied the haploid plants.)

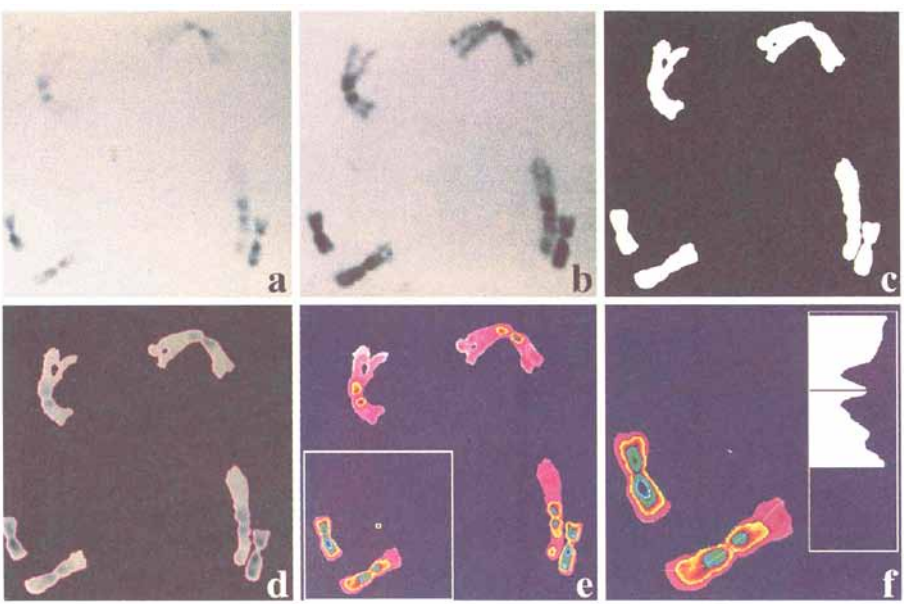

Fig. 3. Six steps in conducting an image analysis of rice chromosomes at prometaphase. Good rice chromosomal spread was microphotographed and a part of the photograph was captured by a high-resolution TV camera and subjected to digital image analysis. (See text for more detailed discussion.)

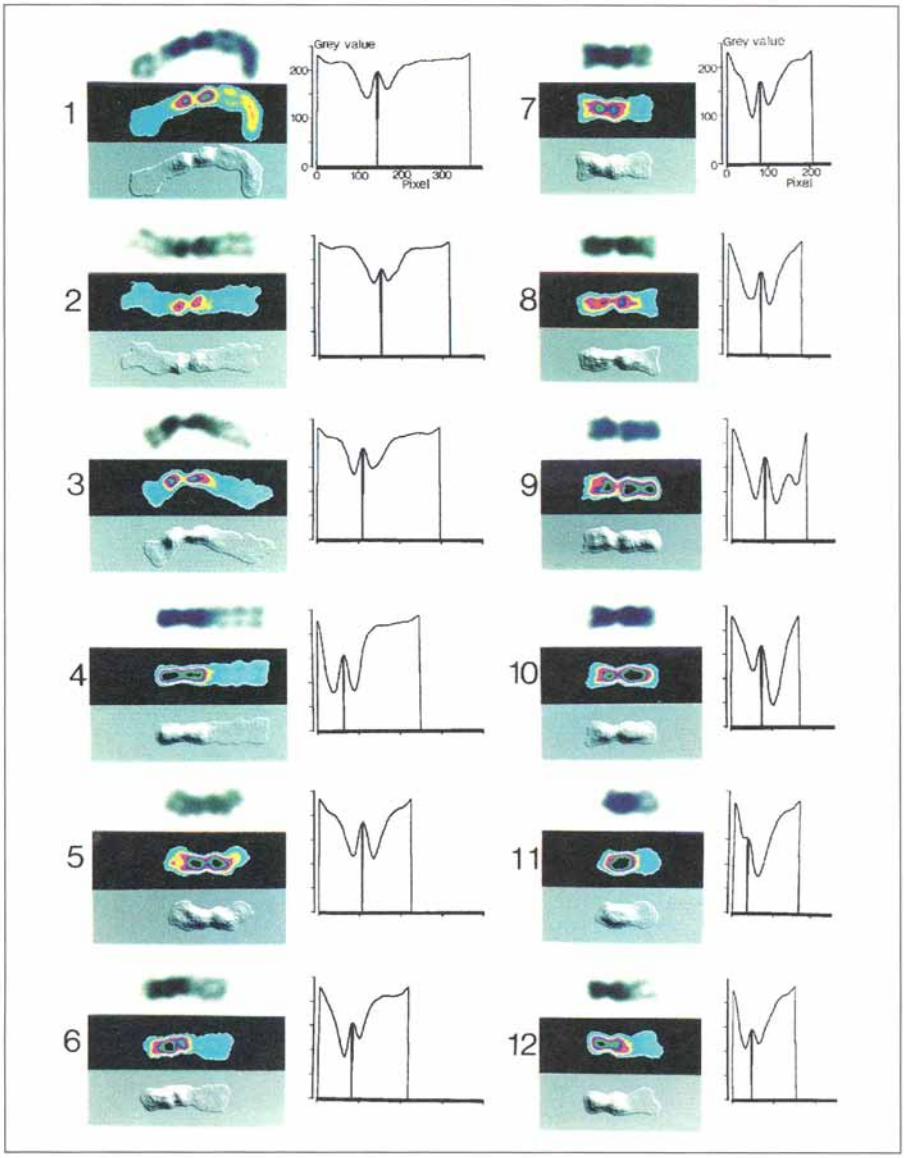

Fig. 4. Characteristics of 12 japonica chromosomes demonstrated by a Giemsa-stained image (upper panel), a pseudo-color image (middle panel), and a pseudo-three-dimensional image (lower panel). The average condensation pattern is represented graphically on the right (Fukui and Iijima 1991). (See text for more detailed discussion.)

Quantitative chromosome map of rice

Figure 5 shows a quantitative chromosome map using the new chromosome numbering system. Black bars show the most condensed regions, gray bars represent moderately condensed regions, and white bars depict dispersed regions. Visual borders between the condensed and dispersed regions appearing at prometaphase correspond to borders between the gray and white regions. As the quantitative rice chromosome map has been developed, several nucleotide sequences and genes are now being physically located on the map by in situ hybridization.

New technologies in rice chromosome research

Automatic identification of rice chromosomes using imaging methods

Two questions arose after the development of the quantitative rice chromosome map based on CP. First, how stable and reproducible is the map? Second, to what extent does the map represent the morphological features of the chromosomes? To answer these questions, the chromosome number visually determined under the microscope and the chromosome number automatically determined by a computer using CP numerical data were compared. All morphological information on the rice chromosomes appearing under a microscope were taken into account by experienced researchers to determine the chromosome number with the aid of image manipulation of contrast enhancement, enlargement, and psuedo-coloration as shown in Figure 4. The same chromosomal spreads were utilized for automatic identification of rice chromosomal number using the CP numerical data, which were analyzed by three computerized statistical methods—discrimination chart, linear discrimination, and minimum distance classifier.

Kamisugi et al (1993) reported results on automatic identification of rice chromosomes based on CP data. They extracted 31 image parameters from each of the CPs, such as the position of a condensation region, depth of a condensation region, etc. Then they used statistical methods mentioned above to determine automatically the chromosome numbers.

The minimum distance classifier, discrimination chart, and linear discrimination methods correctly identified 92.2, 91.1, and 84.4% of the rice chromosomes, respectively. This clearly demonstrates that CPs contain sufficient information to determine the chromosome number and are stable parameters that represent the morphology of rice chromosomes at prometaphase.

In situ hybridization and physical mapping of rice genes

In situ hybridization of rice was first accomplished by using tritiated rbcS probes (Wu et al 1986) and ^{125}iodine-labeled ribosomal DNA (Fukui et al 1987). Two signals at the end of the short arm of chromosome 11 (new numbering system used throughout the rest of this paper) were clearly detected in the latter case. However, a main obstacle in using in situ hybridization was the size of rice chromosomes, which were too small to localize the radioactive probes on the defined chromosomal regions. So, in situ

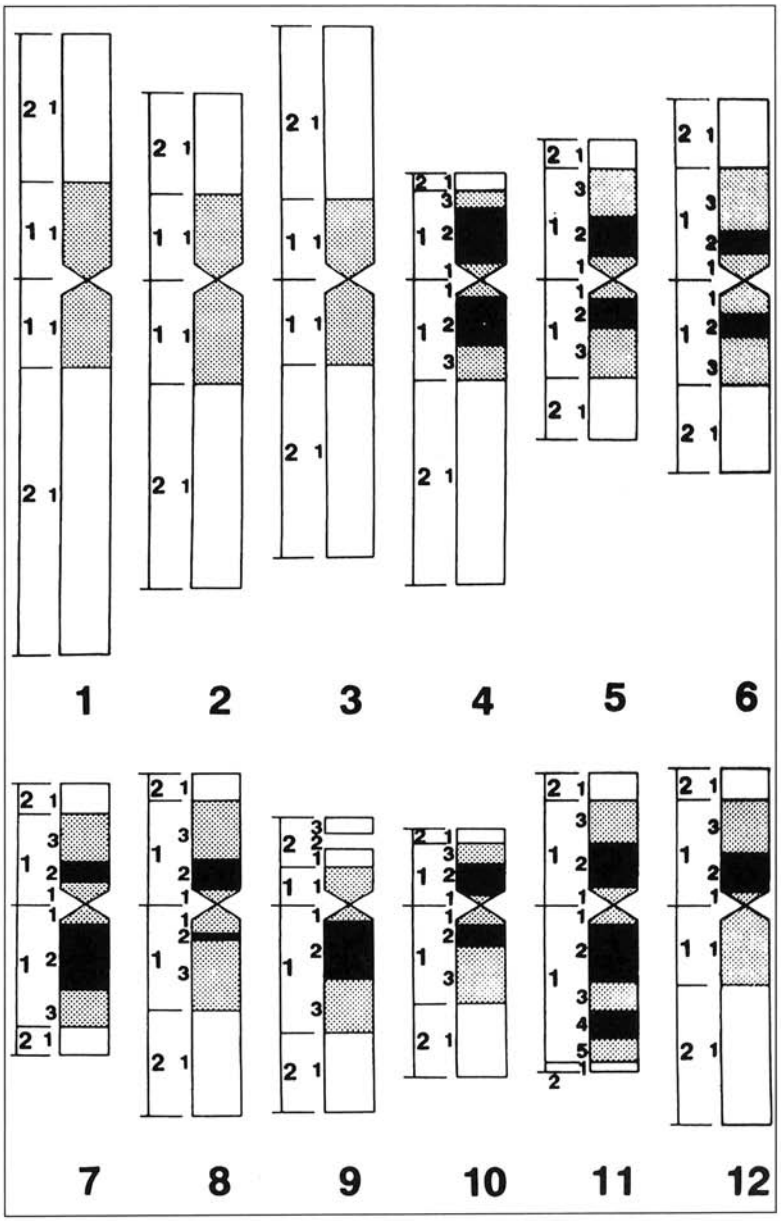

Fig. 5. This quantitative somatic rice chromosome map, based on the condensation pattern (Fukui and Iijima 1991), allows researchers to physically locate the chromosomal addresses (Fukui and Mukai 1988). Black bars show the most condensed regions; gray bars represent moderately condensed regions; and white bars depict dispersed regions.

hybridization did not become truly practical until a biotin labeling method was introduced.

In situ hybridization with biotinylated probes was used to detect rDNA loci both in japonicas (Fukui 1990) and indicas (Islam-Faridi et al 1990). The number of rDNA loci revealed was consistent with the number of the satellite chromosomes. Shortly after this, researchers detected a few copy genes for storage protein and restriction fragment length polymorphism (RFLP) markers by using either radioactive or biotinylated probes (Suzuki et al 1991, Gustafson and Dillé 1992).

Introduction of the fluorescent in situ hybridization (FISH) technique improved detection efficiency and sensitivity. Fukui et al (1994) reported the variability of 45S rDNA sites among rice species. They detected one rDNA locus in *O. sativa* (japonica), *O. rufipogon, and O. brachyantha;* two loci in *O. sativa* (indica [Fig. 6] and javanica), *O. eichingeri, O. rufipogon,* and *O. australiensis;* and three loci in *O. officinalis.* It seems that there is no species-dependent tendency. but there is a geographical variation. It is interesting that the same variation in rDNA loci detected in cultivated rice also exists in the ancestral species, *O. rufipogon.*

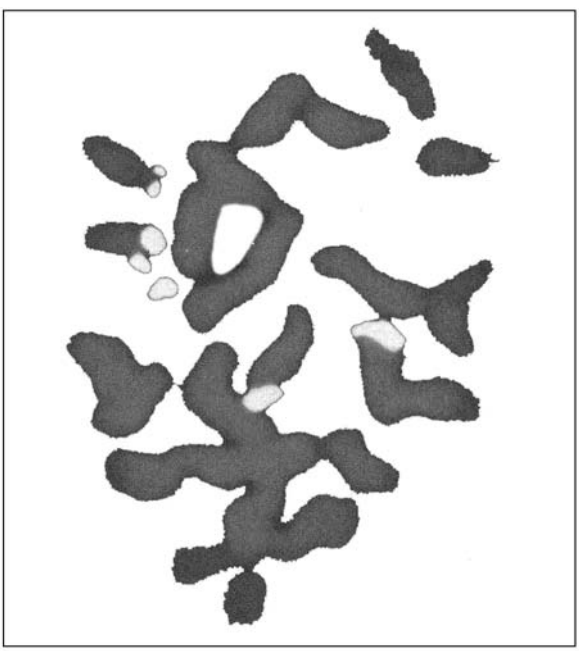

Fig. 6. Fluorescent in situ hybridization in IR36 clearly shows four fluorescein-isothiocyanate fluorescent signals on propidium iodide counterstained rice chromosomes. This photograph is the result of an improved FISH methodology designed especially for working with rice chromosomes (Fukui et al 1994). (See the cover of this volume for a color rendition.)

Another ribosomal DNA species of 5S rDNA has also physically been mapped by FISH on chromosome 9 of indica rice (Song and Gustafson 1993) and on chromosome 11 of japonica rice (Kamisugi et al 1994). The 5S rDNA locus lies at the position 4% away from the centromere toward the distal end on the short arm in japonica rice. Further research on localization of 5S rDNA on several rice species using FISH revealed that rice has only one such locus and it is located on chromosome 11 (Shishido et al 1994). Ohmido and Fukui (1995) developed multicolor FISH (McFISH) using the two probes of 45S and 5S rDNAs simultaneously in African cultivated *O. glaberrima* and detected the identical localization of the rDNAs on the chromosomes as in japonica rice.

Several repeated sequences of rice such as TrsA, TrsB, TrsC, and RIRE1 (Ohtsubo et al 1991; H. Ohtsubo, University of Tokyo, Japan, 1994, unpubl. data) have successfully been mapped on the rice chromosomes (Ohmido et al 1993). In most cases, TrsA and TrsC were localized at the subtelomeric regions of the chromosomes. There is variability in the number of the TrsA sites even within *O. sativa.* Japonicas have the fewest number of TrsA sites, two per haploid genome, whereas indicas have six sites, which are all different from the japonica sites. TrsA's locations at the subtelomeric regions suggest that it has a certain biological role.

Jiang et al (1995) reported mapping bacterial artificial chromosome (BAC) clones on rice chromosomes using FISH. The successful mapping of BACs, yeast artificial chromosomes (YACs), cosmid clones, and RFLP markers on rice chromosomes have also been obtained (N. Ohmido and K. Fukui, Hokuriku National Agricultural Experiment Station, unpubl. data). Therefore, it is anticipated that reproducible mapping of single-copy genes on rice chromosomes will be accomplished in the very near future.

Genomic in situ hybridization (GISH)

Genomic in situ hybridization (GISH)—where total genomic DNA is used as the probe—has been used for phylogenetic studies and identification of alien chromosome(s) from different genomes. Since the probe is a mixture of unique and repetitive sequences, the signal is uniformly obtained throughout the chromosome(s).

Shihido et al (1993) extracted total genomic DNA from *O. officinalis,* a diploid C genome species, and used GISH as the probe to paint the chromosomes originating from the C genome. Two amphidiploid species, *O. minuta* (BBCC) and *O. latifolia* (CCDD), were used in preparing the chromosome samples. Twenty-four chromosomes, derived from the ancestral diploid C genome species, were clearly painted in both *O. minuta* and *O. latifolia.* This work identified chromosomes originating from a D genome species for the first time. The differences in the strength of the fluorescent signals between the B and C genome chromosomes being clearer than those between the C and D genome chromosomes indicate that the C and D genomes are more closely related to each other than are the B and C genomes.

Optical manipulation of rice chromosomes

Recent advances in laser optics have made it possible to produce pores on protoplasts (optical poration), to "pinch" microscopic plant materials (optical tweezers), and even to dissect plant chromosomes (laser knife; Fukui et al 1995). Laser microdissection seems to be one of the most useful new technologies in chromosome research.

Figures 7a-d show the representative steps of laser dissection of rice chromosomes (Fukui et al 1992). Figure 7a shows the rice chromosomal spread prepared by the enzymatic maceration/air-drying (EMA) method (Fukui and Iijima 1992). To dissect two chromosomal regions of chromosome 4, scattered cytoplasmic debris was first eliminated and nontarget chromosomes in the peripheral regions were removed by laser ablation (Fig. 7b). Then, the remaining chromosomes within the spread, except for chromosome 4, were removed by laser ablation (Fig. 7c). A fine laser beam was then used to eliminate the condensed region to clarify the dispersed tail region of chromosome 4. For another chromosome 4, the dispersed region was laser-ablated to obtain the condensed region of the chromosome (Fig. 7d).

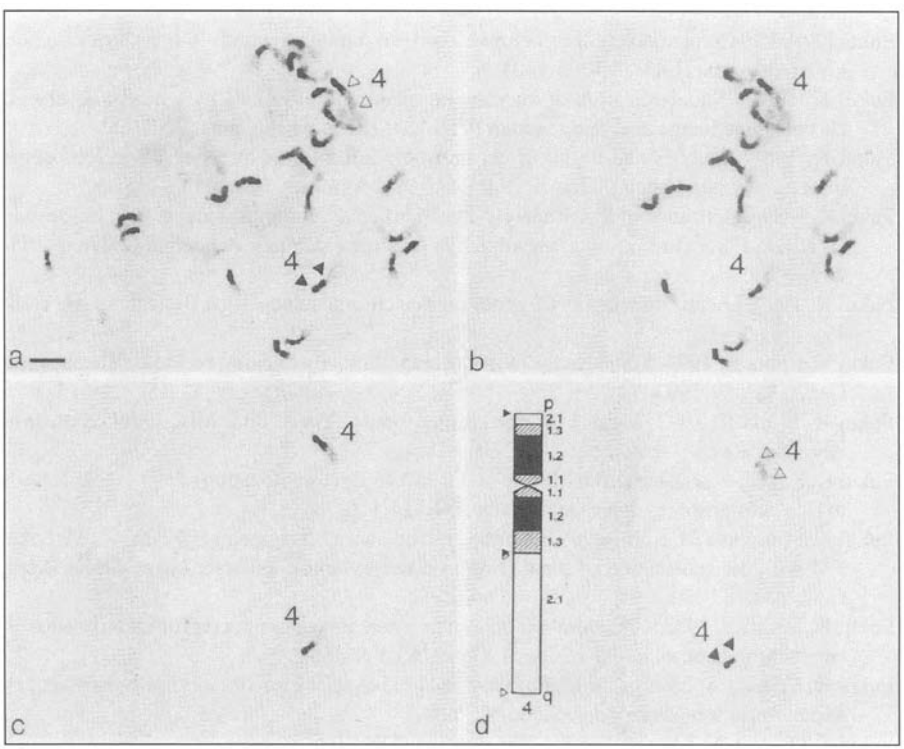

Fig. 7. Chronological steps for laser dissection of rice chromosome 4 using ACAS 470. The bar in 7a indicates 5 µm. The solid and open triangles in 7a and d indicate the chromosomal regions subjected to laser dissection (Fukui et al 1992). In 7d, p and q indicate the short and long arms, respectively. (See text for more detailed discussion.)

The rice chromosomal spread subjected to direct cloning of nucleotide sequences from a certain chromosomal fragment is prepared on the polyester membrane. Then, an octagon disk, on which the chromosome fragment is located at the center, is dissected by the much stronger laser beam and the disk is recovered with fine forceps. The disk itself is used as the DNA template for polymerase chain reaction (PCR) amplification. DNA amplified by PCR is simultaneously labeled with biotin or degoxigenin for further use of the labeled DNA in in situ hybridization.

The new techniques just described—identifying the rice chromosome and specifying the region using the imaging methods, dissecting the region of interest with a fine laser beam, direct cloning and direct labeling of the DNA, and confirming the origin of the amplified DNA using FISH—are now available to researchers. The next step is to integrate these procedures into one logical, complete system.

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Stable inheritance of transgenes in rice plants transformed by *Agrobacterium tumefaciens*

Y. Hiei and T. Komari

Inheritance of foreign genes in rice transformed by Agrobacterium tumefaciens has been investigated up to the R_4 progeny. Rice cultivar Tsukinohikari was transformed with A. *tumefaciens* strains LBA4404(pTOK233) and EHA101(plG121Hm). Cultivar Koshihikari was transformed with LBA4404(pTOK233). pTOK233 is a "super-binary" vector and LBA4404(pTOK233) had a greater transformation efficiency than EHA101(plG121Hm). The T-DNA of these strains carried a *gus* gene and a hygromycin resistance gene, both of which were driven by the 35S promoter. The *gus* gene contained an intron in the coding region. The selfed progeny of 20 independent plants from each of the three populations of transformants were evaluated for *gus* expression and resistance to hygromycin. The two markers were genetically linked and inherited in a Mendelian fashion. The segregation ratio of 3:1 (positive:negative for the transgenes) was observed in the progeny of 60-75% of the transformants in each of the groups of 20 plants. The R_2 progeny of six plants from each of the groups were scored for GUS expression and hygromycin resistance. Thus, the Mendelian transmission of the two markers was further confirmed and offspring homozygous for the two genes were identified in the R_2 lines. R_3 plants were obtained from the homozygous R_2 offspring, and consequently R_4 plants were produced. The two genes were expressed in the R_3 and R_4 generations and none of the plants in these generations lost the genes. The DNA from selected offspring was analyzed by Southern hybridization and a tight correlation between the phenotype and genotype was demonstrated.

Stable inheritance and expression of foreign genes are of critical importance in application of genetically engineered cereal crops in agriculture, but have not been extensively studied.

Transformants of cereals have been produced mainly via direct uptake of DNA by protoplasts or cells. Mendelian inheritance of transgenes in such transformants was observed on various occasions (Shimamoto et al 1989, Datta et al 1990, Christou et al 1991), but only R_1 progeny (selfed progeny of primary transformant [R_0]) were examined in most of the cases. Non-Mendelian types of segregation patterns of transgenes were also reported. For example, foreign genes (*gus* and *neo*) in three rice plants transformed by the polyethylene glycol (PEG)-mediated method were analyzed up to the R_3 generation (Peng et al 1995), and the progeny of the two plants did not show segregation of the genes in a Mendelian fashion. In another case, *bar* and *gus* genes integrated into a maize plant by particle bombardment were unstable and poorly transmitted to the progeny (Spencer et al 1992).

Non-Mendelian inheritance of transgenes was also reported in dicotyledonous transformants produced by direct transformation methods (Potrykus et al 1985). On the other hand, genes introduced into dicotyledons via *Agrobacterium*-mediated methods appear to be very stable and are usually inherited in Mendelian fashion (De Block et al 1984, Budar et al 1986, Chyi et al 1986, Feldmann and Marks 1987, Muller et al 1987).

We have previously reported the development of an efficient transformation method for japonica rice mediated by *A. tumefaciens*, and observed clear Mendelian transmission of transgenes in the R_1 and R_2 progeny (Hiei et al 1994). In this paper, we present further, large-scale analysis of inheritance. expression and stability of the foreign genes up to the R_4 progeny of the rice plants transformed by *A. tumefaciens.*

Materials and methods

Vectors and transformation

The procedure for transformation of rice and *A. tumefaciens* strains LBA4404(pTOK233) and EHA101(pIG121Hm) were described by Hiei et al (1994). pTOK233 is a "super-binary" vector (Komari 1990, Hiei et al 1994). The T-DNA of these strains carried a b-glucuronidase (*gus*) gene and a hygromycin resistance gene, both of which were driven by the CaMV35S promoter. The *gus* gene contained an intron in the coding region (Fig. 1).

Analysis of progeny

GUS activity in leaves was scored by the procedure described by Hiei et al (1994). Hygromycin resistance of seedlings was assayed by culturing root segments on 2N6 medium (Hiei et al 1994) containing hygromycin (50 mg L^{-1}). Root segments from sensitive seedlings did not show any response and root segments from resistant seedlings produced calli (Fig. 2a).

Southern blot analysis

DNA was extracted from leaf tissue using the procedure described by Komari et al (1989). Ten micrograms of DNA were digested with *Hin*dIII or *Xba*I and fractionated on a 0.7 % agarose gel by electrophoresis at 1.1 V cm^{-1} for 16 h. Southern hybridiza-

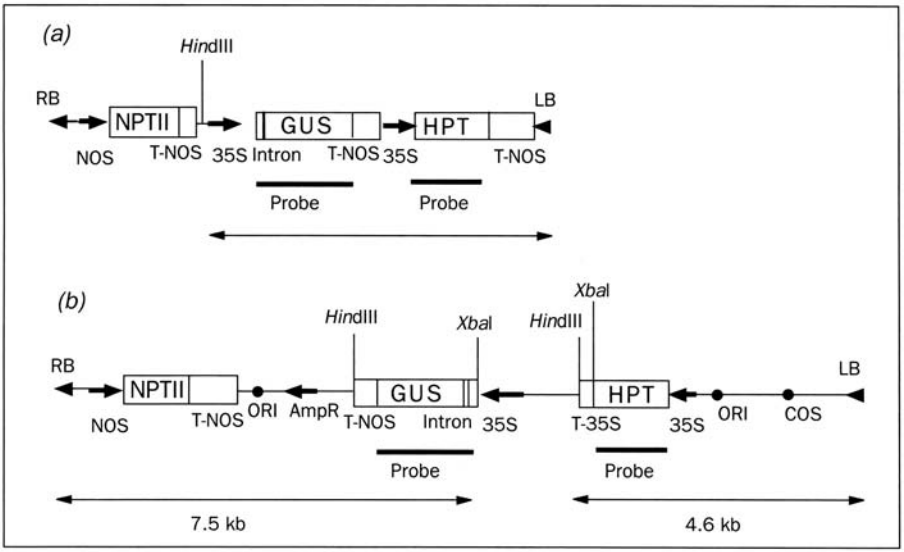

Fig. 1. T-DNA regions of plG121Hm(a) and pTOK233(b). RB; right border, LB; left border, NPTII; neomycin phosphotransferase, GUS; b-glucuronidase, HPT; hygromycin phosphotransferase, NOS; nopalin synthase promoter, 35S; 35S promoter, T-NOS; 3' signal of nopalin synthase, T-35S; 3' signal of 35S RNA, ORI; origin of replication of ColE1, AmpR; ampicillin resistance gene active in *Escherichia coli.*

tion was carried out as described by Sambrook et al (1989). The probe for *hpt* was the 1.1-kb *Bam*HI fragment from pGL2-IG (Hiei et al 1994) and the *Gus* probe was the 1.9-kb *Sal*I-*Sac*I fragment from pGL2-IG.

Results and discussion

Transformation

Japonica rice cv Tsukinohikari was transformed with LBA4404(pTOK233) (population T) and EHA101 (pIG121Hm) (population E), and cv Koshihikari was transformed with LBA4404(pTOK233) (population K). Twenty independent, GUS-positive, hygromycin-resistant transgenic plants (R_0) from each of the three populations of transformants were analyzed by Southern hybridization. The copy numbers of integrated genes varied from one to six (Tables 1 and 2), but the majority of the transformants contained one or two copies of the integrated genes. In several plants, the copy numbers of *hpt* and *gus* were different, probably due to rearrangement of DNA upon transformation.

R_1 generation

The selfed progeny were evaluated for resistance to hygromycin and GUS expression. The segregation patterns of offspring from the 20 independent R_0 plants (cv Koshihikari transformed by LBA4404[pTOK233], K lines) are shown in Table 1.

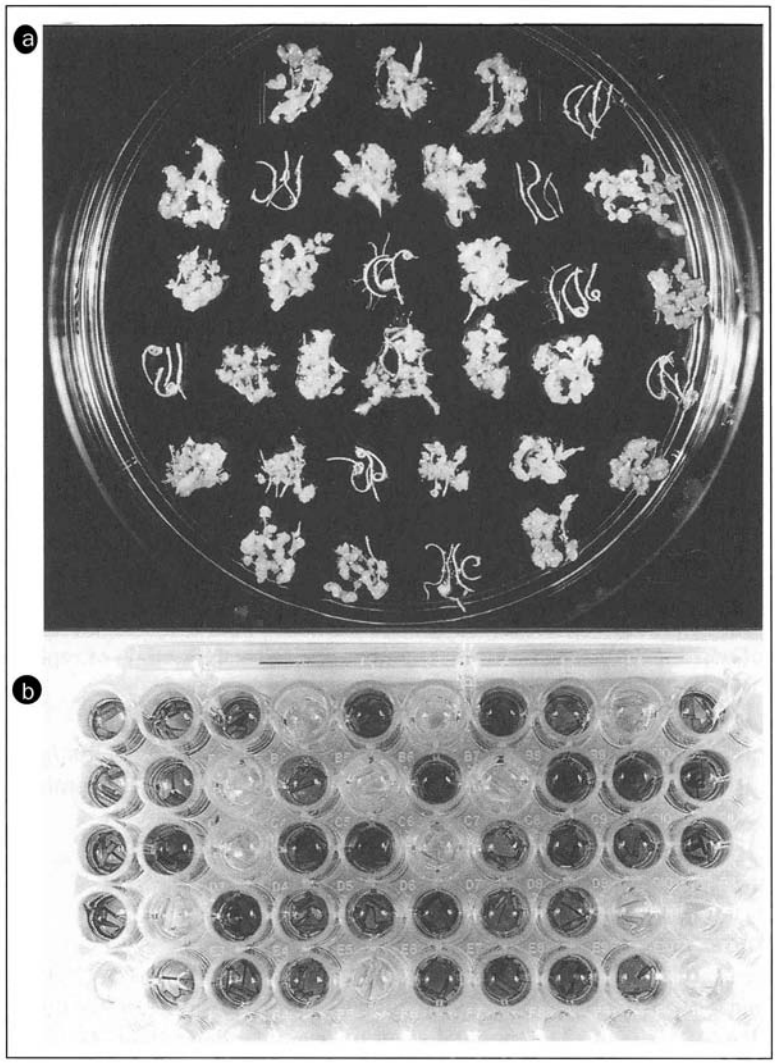

Fig. 2. Test of the R_1 progeny of cv Tsukinohikari transformed by LBA4404(pTOK233) for resistance to hygromycin and for expression of GUS. a) Root segments from R_1 seedlings were plated on selective medium and the photograph was taken 3 wk later. b) R_1 seedlings were cultured in darkness and their leaves were stained with 5-bromo-4-chloro-3-indolyl glucuronidase.

Table 1. Estimation of copy numbers of transgenes in the transformants of cv Koshihikari produced by LBA4404 (pTOK233), and segregation patterns for hygromycin resistance and expression of GUS in the R_1 progeny.

Line no. (R_0)	Copy number (R_0)		Number of R_1 plants		χ^{2c}	
	hpt[a]	*gus*[a]	GUS+ HygR[b]	GUS- HygS[b]	3:1	15:1
K1	3	4	45	8	2.774	7.075**
K2	1	2	38	12	0.027	26.885**
K3	2	2	31[d]	8	0.419	13.540**
K4	1	1	29	11	0.133	30.827**
K5	1	2	29	11	0.133	30.827**
K6	3	3	36	12	0.000	28.800**
K7	3	2	31	2	6.313*	0.002
K8	2	2	35	10	0.185	19.593**
K9	2	2	35	2	7.575**	0.045
K10	4	3	71	5	13.754**	0.014
K11	2	2	40	0	13.333	2.667
K12	1	2	32	8	0.533	12.907**
K13	2	2	65	14	2.232	17.743**
K14	3	2	36	2	7.895**	0.063
K15	1	1	52	15	0.244	29.780**
K16	2	3	35	12	0.007	29.823**
K17	2	2	38	1	10.470**	0.904
K18	2	2	65	14	2.232	17.743**
K19	2	1	37	13	0.027	33.285**
K20	1	1	58	15	0.772	25.469**

[a] *hpt:* hygromycin resistance gene, *gus:* GUS gene. [b] HygR: hygromycin-resistant. HygS: hygromycin-sensitive. [c] * and **: significant at 5 and 1% level, respectively. [d] Expressing GUS scattered and poor hygromycin-resistant calli.

Table 2. Copy numbers of transgenes in R_0 and number of genetic loci estimated by segregation patterns in the R_1 progeny.

Lines[a]	Lines analyzed (no.)	Transgene[b]	Copy numbers of transgenes			Genetic loci (no.)	
			1	2	3-6	1	2-
E	20	*hpt*	4	10	6	15	5
		gus	7	8	5	16	4
T	20	*hpt*	2	8	10	12	8
		gus	4	7	9	13	7
K	20	*hpt*	6	10	4	14	6
		gus	6	9	5	14	6

[a] E: cv Tsukinohikari transformed by EHA101 (plG121Hm), T: cv Tsukinohikari transformed by LEA4404 (pTOK233), K: cv Koshihikari transformed by LEA4404 (pTOK233). [b] *hpt:* hygromycin resistance gene, *gus:* GUS gene.

GUS expression and hygromycin resistance cosegregated clearly in Mendelian fashion, and 14 of the 20 R_1 lines showed a 3:1 ratio (positive:negative for the transgenes) of segregation.

The leaf segments from most of the GUS-positive R_1 plants uniformly expressed GUS activity, but scattered expression of GUS, which was associated with relatively low level of hygromycin resistance, was observed in line K3. This line still had a segregation ratio of 3:1. Such expression of *gus* and *hpt* was also observed in one of the R_1 lines of Tsukinohikari transformed with EHA101 (pIG121Hm).

Analysis of the R_1 progeny of the three populations of transformants is summarized in Table 2. The estimated number of loci was smaller than the copy number of genes measured by Southern blot analysis in some lines. It is likely that more than two copies of genes were integrated close to each other on a chromosome of such plants. The numbers of *gus* and *hpt* loci were different in a few cases, possibly due to rearrangements of T-DNA.

R_2 generation

Fifteen R_1 lines with a segregation ratio of 3: 1 and 3 R_1 lines with a segregation ratio of 15:1 were grown to maturity. The R_2 plants from these lines were evaluated.

The R_1 analysis of a 3:1 line, K4, is shown in Table 3. The R_2 progeny from GUS-positive, hygromycin-resistant R_1 plants either had a segregation ratio of 3:1 or

Table 3. Segregation patterns for hygromycin resistance and expression of GUS in the R_2 progeny of transgenic Koshihikari mediated by LBA4404(pTOK233), Line K4.

Line no. (R_1)	Phenotype of R_1		Number of of R_2 plants		c^2(3:1)	Apparent genotype of R_1[c]	
	GUS	Hyg	GUS+[a] HygR[b]	GUS-[a] HygS[b]			
K4-1	+	R	40	0		*gus/gus*	*hpt/hpt*
K4-2	+	R	32	8	0.533	*gus/-*	*hpt/-*
K4-3	+	R	40	0		*gus/gus*	*hpt/hpt*
K4-4	+	R	40	0		*gus/gus*	*hpt/hpt*
K4-5	+	R	32	8	0.533	*gus/-*	*hpt/-*
K4-6	+	R	30	10	0.000	*gus/-*	*hpt/-*
K4-7	+	R	39	0		*gus/gus*	*hpt/hpt*
K48	+	R	36	14	0.240	*gus/-*	*hpt/-*
K4-9	+	R	41	11	0.410	*gus/-*	*hpt/-*
K4-10	+	R	33	16	1.531	*gus/-*	*hpt/-*
K4-11	+	R	40	0		*gus/gus*	*hpt/hpt*
K4-12	+	R	40	0		*gus/gus*	*hpt/hpt*
K4-13	+	R	34	7	1.374	*gus/-*	*hpt/-*
K4-14	+	R	47	12	0.684	*gus/-*	*hpt/-*
K4-15	+	R	34	6	2.133	*gus/-*	*hpt/-*
K4-16	+	R	26	13	1.444	*gus/-*	*hpt/-*
K4-17	+	R	29	14	1.310	*gus/-*	*hpt/-*
K4-18	+	R	40	0		*gus/gus*	*hpt/hpt*
K419	-	S	0	40		–/–	–/–
K4-20	-	S	0	40		–/–	–/–

[a] GUS+: GUS positive, GUS- and -: GUS negative. [b] HygR: hygromycin-resistant, HygS: hygromycin-sensitive. [c] *Hpt:* hygromycin resistance gene, *gus:* GUS gene.

were all GUS-positive and hygromycin-resistant. The R_2 progeny from GUS-negative, hygromycin-sensitive R_1 plants were all GUS-negative and hygromycin-sensitive. The examination of the other 3:1 lines gave similar results. The scattered expression of GUS and low level of hygromycin resistance in line K3 were also inherited by the R_2 progeny as Mendelian loci.

The R_2 analysis of a 15:1 line, K7, is shown in Table 4. R_2 lines consisting solely of plants positive for the transgenes, 3:1 lines, 15:1 lines, and lines consisting solely of plants negative for the transgenes are expected in a ratio of 7:4:4:1. This expectation was confirmed and examination of the other 15:1 lines gave similar results.

Table 4. Segregation patterns for hygromycin resistance and expression of GUS in the R2 progeny of transgenic Koshihikari mediated by LBA4404(pTOK233), Line K7.

Line no. (R_1)	Phenotype of R_1		Number of R_2 plants		c^2c	
	GUS	Hyg	GUS+[a] HygR[b]	GUS-[a] HygS[b]	3:1	15:1
K7-1	+	R	39	0	13.000**	2.600
K7-2	+	R	28	9	0.009	20.628**
K7-3	+	R	36	0	12.000**	2.400
K7-4	+	R	35	1	9.481**	0.740
K7-5	+	R	40	0	13.333**	2.667
K7-6	+	R	38	0	12.667**	2.533
K7-7	+	R	38	0	12.667**	2.533
K7-8	+	R	40	0	13.333**	2.667
K7-9	+	R	31	2	6.313*	0.002
K7-10	+	R	38	2	8.533**	0.107
K7-11	+	R	40	0	13.333**	2.667
K7-12	+	R	39	1	10.800**	0.960
K7-13	+	R	42	0	14.000**	2.800
K7-14	+	R	43	0	14.333**	2.867
K7-15	+	R	40	0	13.333**	2.667
K7-16	+	R	30	7	0.730	10.135**
K7-17	+	R	39	0	13.000**	2.600
K7-18	+	R	38	2	8.533**	0.107
K7-19	+	R	39	0	13.000**	2.600
K7-20	+	R	39	9	1.000	12.800**
K7-21	+	R	39	0	13.000**	2.600
K7-22	+	R	39	0	13.000**	2.600
K7-23	+	R	35	14	0.333	41.667
K7-24	+	R	44	17	0.268	48.657**
K7-25	+	R	47	3	9.627**	0.005
K7-26	+	R	39	0	13.000**	2.600
K7-27	+	R	40	0	13.333**	2.667
K7-28	+	R	40	0	13.333**	2.667
K7-29	+	R	45	20	1.155	66.692**
K7-30	+	R	35	18	2.270	69.465**
K7-31	+	R	39	10	0.551	16.763**
K7-32		S	0	30	90.000**	450.000**
K7-33		S	0	30	90.000**	450.000**

[a]GUS+: GUS positive, GUS-: GUS negative. [b]HygR: hygromycin-resistant, HygS: hygromycin-sensitive. [c]* and **: significant at 5 and 1% level, respectively.

R_3 and R_4 generations

R_3 plants were obtained from R_2 plants homozygous for the transgenes, and then R_4 plants were produced. Thus, R_3 and R_4 progeny, which originated from six independent R_0 plants in each of the three populations of the initial transformants were characterized. All of the R_3 and R_4 plants were GUS-positive and hygromycin-resistant. A typical pattern (line E8) of transmission of the transgenes from the R_0 to the R_4 generation is shown in Table 5.

Plants showing scattered expression of GUS and low level of hygromycin resistance were newly identified in two independent R_3 lines. E9 and E10, and these traits were inherited by the R_4 progeny. Expression of the transgenes was normal in the R_1 and R_2 generations. The scattered expression of GUS and low level of hygromycin resistance in line K3 were stably inherited by the homozygous R_4 progeny.

R_3 plants homozygous for a single locus of the transgenes, originating from R_0 plants that contained two loci of the transgenes, were successfully identified in all of the three independent lines tested.

Southern blot analysis of progeny plants

The progeny plants were analyzed by Southern hybridization (parts of data are shown in Figures 3 and 4), and a tight correlation between phenotype and genotype was confirmed. For example, the R_0, R_1, R_2, R_3, and R_4 plants positive for the expression of the transgenes in line E8 showed an identical hybridization pattern (Fig. 3, Table

Table 5. Segregation patterns for hygromycin resistance and expression of GUS up to the R_4 progeny of transgenic Tsukinohikari mediated by EHA101(plG121Hm), Line ES.

Parent				Number of plants in the progeny				
Line no. (generation)		Phenotype		GUS+[a] HygR[b]	GUS-[a] HygS[b]	c^2 (3:1)	Apparent genotype of parent[c]	
		GUS	Hyg					
E8	(R_0)	+	R	45	13	0.207	*gus/-*	*hpt/-*
Ea-1	(R_1)	+	R	34	0		*gus/gus*	*hpt/hpt*
Ea-4	(R_1)	+	R	35	13	0.111	*gus/-*	*hpt/-*
Ea-9	(R_1)	+	R	26	9	0.010	*gus/-*	*hpt/-*
Ea-10	(R_1)	+	R	34	0		*gus/gus*	*hpt/hpt*
E8-15	(R_1)	+	R	25	10	0.238	*gus/-*	*hpt/-*
E8-16	(R_1)	+	R	39	17	0.857	*gus/-*	*hpt/-*
E8-2	(R_1)	–	S	0	35		*-/-*	*-/-*
E8-6	(R_1)	–	S	0	35		*-/-*	*-/-*
E8-1-1	(R_2)	+	R	30	0		*gus/gus*	*hpt/hpt*
E8-10-5	(R_2)	+	R	30	0		*gus/gus*	*hpt/hpt*
E8-1-1-2	(R_3)	+	R	30	0		*gwgus*	*hpt/hpt*
E8-1-1-4	(R_3)	+	R	30	0		*gus/gus*	*hpt/hpt*
E8-1-1-5	(R_3)	+	R	30	0		*gus/gus*	*hpt/hpt*

[a] GUS+: GUS positive, GUS-: GUS negative. [b] HygR: hygromycin-resistant, HygS: hygromycin-sensitive. [c] *hpt:* hygromycin resistance gene, *Gus:* GUS gene.

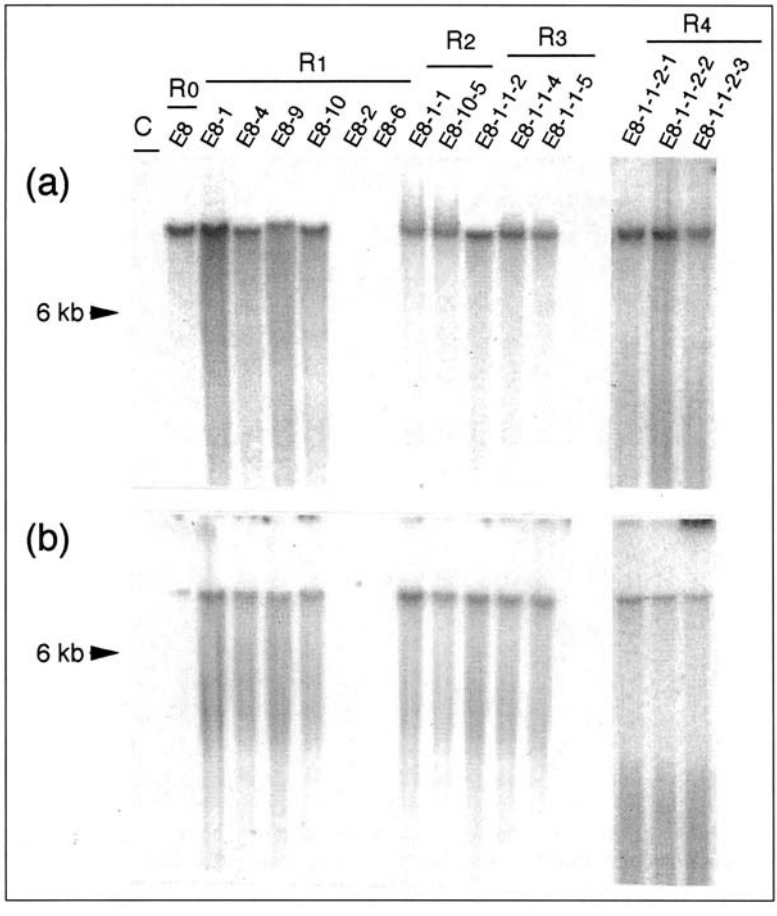

Fig. 3. Southern analysis of the R_1, R_2, R_3, and R_4 progeny of transformant E8. DNAs from R_0 transformant and the progeny were digested with *Hin*dIII, fractionated by electrophoresis, transferred to a nylon membrane, and allowed to hybridize to the *gus* (a) or the *hpt* (b) probe.

5). Independent transmission of the two copies of the foreign DNA fragments, which corresponded to the two loci of the transgenes, was verified in line K7 (Fig. 4, Table 4).

The plants with scattered expression of GUS and a low level of hygromycin resistance showed hybridization patterns identical to those of their parents, which expressed the transgenes normally.

Conclusion

The present study has demonstrated stable, Mendelian inheritance of two marker genes introduced into rice by *A. tumefaciens.* Progeny of 18 independent transgenic rice

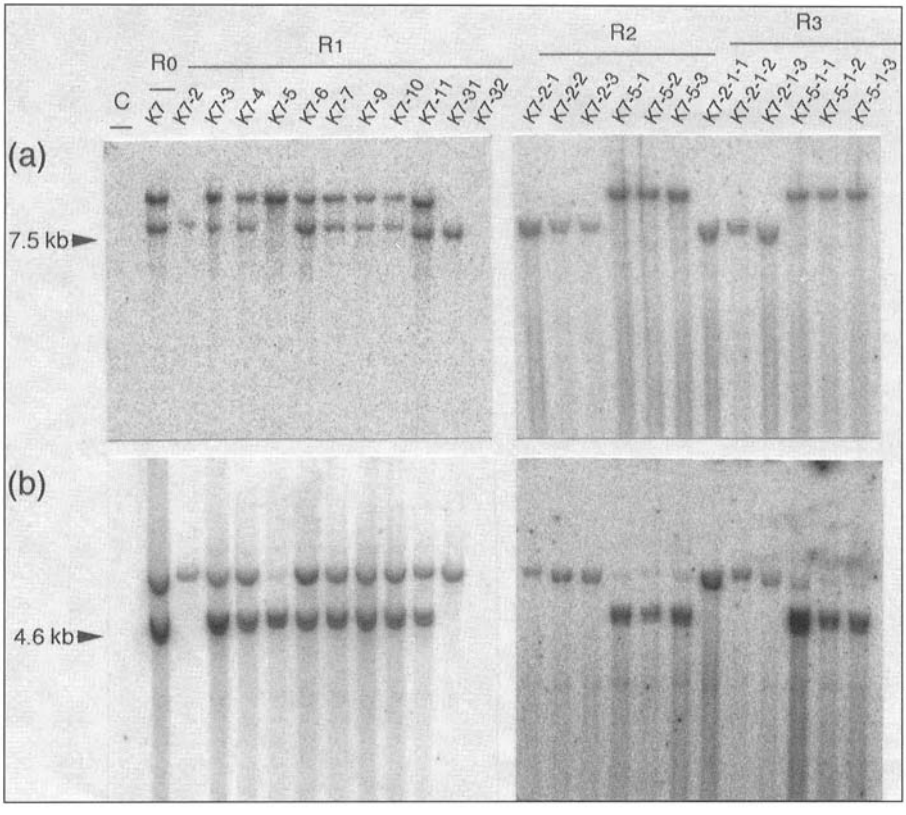

Fig. 4. Southern analysis of the R_1, R_2, and R_3 progeny of transformant K7. DNAs from R_0 transformant and the progeny were digested with *Xba*I (a) or *Hin*dIII (b), fractionated by electrophoresis, transferred to a nylon membrane, and allowed to hybridize to the *gus* (a) or the *hpt* (b) probe.

plants were analyzed up to the R_3 and R_4 generations. All lines showed clear Mendelian transmission of the transgenes, and Southern hybridization confirmed the genetic data. Therefore, the foreign genes integrated into rice in this study appeared to be as stable genetically as those in dicotyledonous transformants produced by *A. tumefaciens.*

Plants showing scattered expression of GUS and a low level of hygromycin resistance emerged in a few lines in the R_1 or R_3 generations. As expression of the foreign genes in their parents was normal and molecular analysis did not detect any flaw, the nature of such expression is not understood. This phenomenon may be related to "gene silencing" observed in various transformation systems.

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Agrobacterium-mediated transformation of an elite cultivar of Texas rice

J.J. Dong, W.M. Teng, W.G. Buchholz, and T.C. Hall

While useful advances have been made using biolistic and other naked DNA uptake methods for rice transformation, high copy numbers of rearranged sequences are common and lack of stable expression due to silencing or other causes is proving to be a major problem. Attempts to transform rice and other monocots using *Agrobacterium tumefaciens* based vectors have typically yielded results that were promising, but inconclusive. Recently, an *Agrobacterium*-based system starting from scutellar tissue has been described in the literature. Using only minor modifications of this system, we were able to repeat their transformation of callus derived from the cultivar Koshihikari. We have also extended the work to include Gulfmont, a javonica variety widely used for commercial cultivation in Texas, and Taipei 309, a japonica line that has been widely used for transformation by direct DNA transfer. Transformation using the *Agrobacterium* approach occurred at a reasonably high frequency and vigorous, phenotypically normal plants were recovered. Only one or two unrearranged copies of the T-DNA were present at a single locus; no silencing of the selectable marker or the gene of interest (*gus*) was observed and the introduced genes were transmitted in a normal Mendelian fashion.

Transformation of rice (*Oryza sativa*) and other agronomically important graminaceous monocot crops has frequently been reported using naked DNA uptake stimulated by polyethylene glycol or electroporation (Shimamoto et al 1989; Battraw and Hall 1990, 1992; Datta et al 1990; Li et al 1990; Davey et al 1991) or by biolistics (Christou et al 1991). Unfortunately, DNA introduced by these direct transfer methods is often incorporated into the genome as multiple fragmented or rearranged copies. Transformation events resulting in a single-copy, unrearranged sequence containing the gene of interest are rare. Presumably as a result of these complex integration events, the expression pattern of the gene of interest is often aberrant (Xu et al 1995) and/or unstable in R_0 and progeny plants. Furthermore, data obtained from promoter studies

are difficult to interpret due to the presence of multiple copies of rearranged promoter fragments, uncertainty in the number of functional copies. and potential competition for *trans*-acting factors.

In contrast to direct DNA uptake methods, *Agrobacterium*-mediated transformation usually results in a discrete, unrearranged segment of DNA being inserted in the recipient genome at a fairly low copy number. Though successful transformation of rice by *Agrobacterium* has been reported several times (Raineri et al 1990, Chan et al 1992), the data have been equivocal and direct confirmation has been lacking. Potrykus (1990) has provided a critical review describing several ways in which false positive results may have been obtained. In contrast to earlier reports, the data presented by Hiei et al (1994) of *Agrobacterium*-mediated rice transformation appeared persuasive. Since the impact of this technology would be so great, we felt it was very important that the results be independently confirmed. Therefore, we obtained the bacterial strains and plasmids used by Hiei et al and attempted to reproduce their results.

Here, we describe experiments in which we successfully reproduced the earlier observation that japonica lines of rice could be stably transformed using the pTOK233 vector (Hiei et al 1994). Using relatively minor modifications of the described procedure, we were able to extend their results to transformation of two additional rice cultivars.

Results

Derivation and transformation of scutellar calli

In the original report by Hiei et al (1994), four Japonica cultivars of rice were used, one of which was Koshihikari. In the studies reported here, we used the cultivars Koshihikari, Taipei 309, and Gulfmont, an elite javonica line used commercially in Texas.

Twelve- to fifteen-day-old immature rice embryos were isolated from greenhouse-grown plants of each cultivar, and placed scutellum side up on MS medium containing 2 mg L^{-1} of 2,4-D (MS2). After 7-10 d, callus began to develop from the scutellum. This was removed and placed on fresh MS2 medium for 3-4 wk.

The scutellum-derived calli were transformed essentially as described by Hiei et al (1994) by cocultivation with either of two disarmed *Agrobacterium tumefaciens* strains LBA 4404 (Hoekema et al 1983) or EHA 101 (Hood et al 1986) harboring binary vectors (pTOK233) or (pIG12lhm), respectively. *Agrobacterium* binary vector plasmids pTOK233 and pIG121hm have similar complements of genes encoded in their T-DNA regions; importantly, both encode an intron-containing b-glucuronidase (*gus*) gene and a hygromycin phosphotransferase (*hpt*) gene, each driven by the CaMV35S promoter. The calli were cocultivated for 10 min in AAM solution (Hiei et al 1994) containing around 5×10^9 *Agrobacteria* ml^{-1}, after which they were placed on MS medium supplemented with 2 mg L^{-1} of 2,4-D and 150 EM acetosyringone, pH 5.2. After 3 d, *Agrobacteria* that were overgrowing the calli were washed away using cefotaxime solution (250 mg L^{-1}) before transferring the calli to MS medium containing 2 mg L^{-1} of 2,4-D and 250 mg L^{-1} of cefotaxime. If no bacteria were

evident after 1 wk, the calli were ready for selection on hygromycin; if bacteria were present, a second round of cefotaxime treatment was performed and the calli were reevaluated 1 wk later.

Selection of resistant calli and plant regeneration

Cocultivated calli were placed on selective medium (MS2 supplemented with 50 mg L^{-1} of hygromycin B). After 3-4 wk, resistant calli were visible (Fig. 1A). Continuous selection on hygromycin-containing medium resulted in the appearance of proliferating, apparently resistant, calli from all three cultivars used. To confirm transformation, segments of the calli were subjected to histochemical staining for GUS activity (Jefferson et al 1987) and many regions of intense blue were observed (Fig. lB-D). Calli derived from Gulfmont (Fig. 1B) and Taipei 309 (Fig. 1D) appeared to be embryogenic, and transfer to regeneration medium (MSD4) resulted in the development of plantlets after 3-4 wk (Fig. 1E). Thus far, we have not been successful in regenerating plants from the Koshihikari calli (Fig. 1C).

The plantlets were first transferred to magenta boxes and subsequently transferred to soil and grown to maturity in a confined greenhouse. Tissue samples (Fig. 1F-H) confirmed that GUS expression was retained in the mature vegetative tissues. Intense expression of GUS was readily seen in roots (Fig. 1F), but initial experiments with leaves resulted in staining only at cut surfaces (Fig. 1G). We determined that this resulted from limited penetration of the X-gluc substrate, rather than from a wounding phenomenon, by solubilization of leaf membranes by brief (3 min) immersion in diethyl ether. Mature leaves treated in this way stained uniformly blue (Fig. 1H). The primary transformants were all vigorous and fertile.

DNA analysis of transgenic rice plants

A summary of experiments conducted using the three rice cultivars in combination with two strains of *Agrobacterium* is presented in Table 1. Thus far, 96 plants expressing *gus* as well as the selectable marker have been obtained. Of these, 29 were Gulfmont. Because of our special interest in this elite Texas cultivar, we have undertaken initial molecular analyses on these plants. Genomic DNA blot analyses of the Gulfmont transformants have shown that four independent lines were obtained in one experiment (Fig. 2) and three independent lines in two other, entirely separate, experiments (Table 1). Further analyses (not shown) of the four independent lines from experiment 1 revealed that two contained single-copy inserts of the T-DNA and two contained two inserts. No rearrangement of the transgene occurred and probing of plant genomic DNA with region failed to detect any bacterial DNA outside the T-DNA region.

Analysis of progeny plants

Vigorous, phenotypically normal and fertile plants were obtained (Fig. 1I) in about 3 mo after transfer from regeneration medium (Fig. 1E). Seeds from these plants showed deep blue GUS activity in less than 2 h after exposure to the X-gluc substrate (Fig. 1J). Histochemical staining of 7-d-old seedlings showed high levels of GUS activity in

Table 1. *Agrobacterium*-mediated transformation of rice.

Experiment		Number of calli				Number of plants	
Cultivar Strain (plasmid)		Initial calli	After 1st selection	After 2nd selection	Total	GUS+	Independent transformants
Gulfmont	1	70	23	4	16	14	4
LBA4404 (pTOK233)	2	77	16	3	8	7	3
	3	71	27	3	9	8	3
Gulfmont	1	70	10	0	0	0	0
EHA101 (plG121hm)	2	78	4	0	0	0	0
	3	74	7	0	0	0	0
Taipei 309	1	80	21	6	17	12	nd
LBA4404 (pTOK233)	2	51	17	5	21	15	nd
Taipei 309	1	75	30	11	27	21	nd
EHA101 (plG121hm)	2	59	23	9	28	20	nd
Koshihikari	1	45	8	4	5	0	0
LBA4404 (pTOK233)	2	32	11	1	2	0	0
	3	53	10	5	7	0	0
Koshihikari	1	48	6	2	5	0	0
EHA101 (plG121hm)	2	50	12	3	9	0	0
	3	25	3	1	4	0	0

Table 2. Segregation of Gulfmont R_1 progeny.[a]

Line	Hygromycin			GUS		
	Resistant	Sensitive	Ratio	Positive	Negative	Ratio
W1	149	50	3.0 : 1	72	21	3.4 : 1
W10	31	9	3.4 : 1	35	11	3.2 : 1
W17	28	9	3.1 : 1	25	8	3.1 : 1
W21	48	15	3.2 : 1	45	14	3.2 : 1

[a]Segregation tests of progeny of four independently transformed rice lines. Sterilized and dehusked seeds were germinated on hormone-free medium, with or without 50 mg L^{-1} of hygromycin B. Germination rates on hormone-free medium without hygromycin were more than 90% (data not shown). Segregation ratios were close to 3:1 for hygromycin resistant : sensitive or GUS positive:negative. These data Indicate that the transgenes were inserted at a single locus.

root tissues. As was the case for the leaves of the primary transformants, treatment of the leaves was required to allow penetration of the substrate. However, three cycles of freeze-thaw treatment (liquid nitrogen/room temperature) proved to be more effective for the young seedling leaves (Fig. 1L) than was immersion in diethyl ether.

Germination tests were conducted on progeny from each of the primary Gulfmont transformants of experiment 1 (Table 1). As anticipated for a single-locus insertion, segregation ratios close to 3:1 (hygromycin resistant:sensitive) were obtained for all four lines (Table 2). In a parallel experiment, seedlings were germinated and stained for GUS activity. In accord with the segregation of the selectable marker, we obtained 3:l ratios for GUS positive:negative seedlings (Table 2). We have established that progeny of each of these four Gulfmont lines retained the DNA restriction fragment profile of the parental, primary transformant (data not shown).

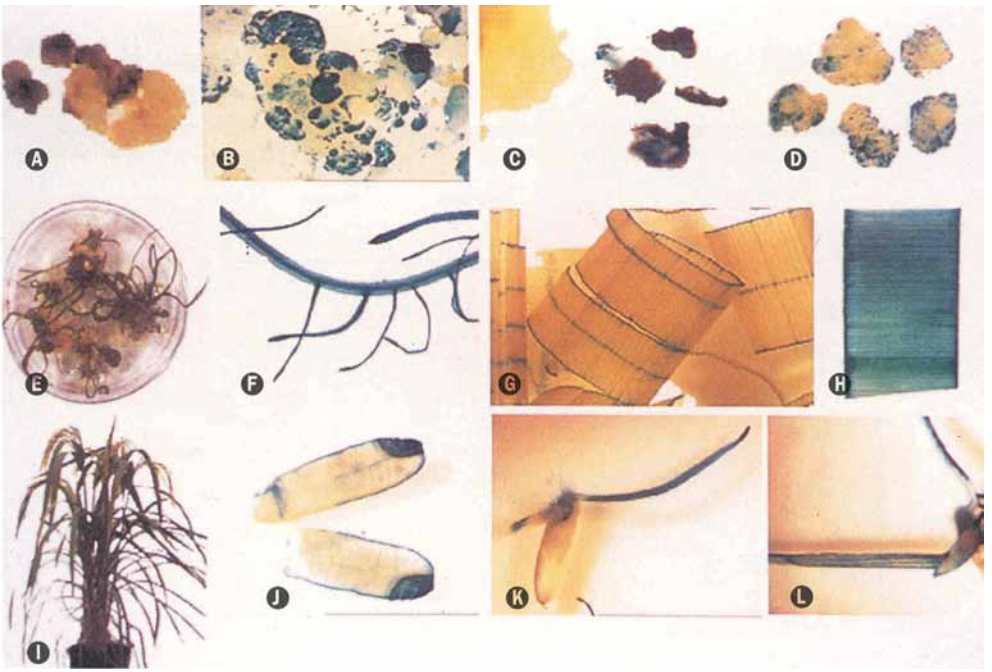
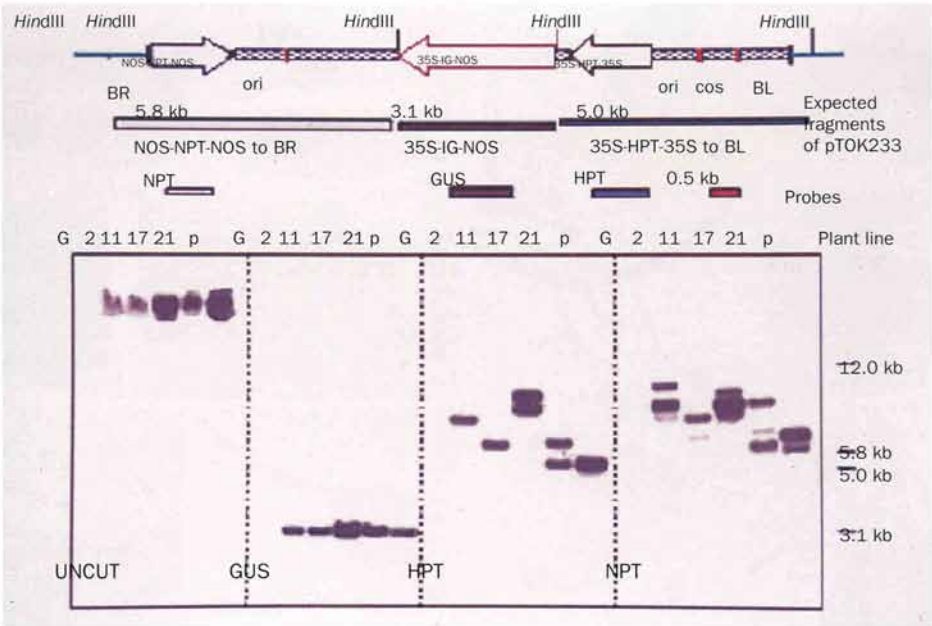


Fig. 1. Production of fertile transgenic rice plants by *Agrobacterium*-mediated transformation of scutellum-derived calli. A) Gulfmont calli on hygromycin selection (50 mgL^{-1}) following cocultivation. B-D) GUS expression in hygromycin-resistant calli from B) Gulfmont, C) Koshihikari, D) Taipei 309; bar q 1 mm. (E) Gulfmont plants after 3-4 wk on regeneration medium. F-H) GUS expression in tissues of a Gulfmont primary transformant. Roots show uniform blue staining (F), but mature leaf segments (G) only show staining at cut edges and wound sites unless penetration of substrate is assisted, e.g., by treatment with ether (H). I) Fertile plant. J) Longitudinal section of a seed showing intense staining of embryonic and maternal (testa) tissues but little staining in the endosperm, even after wounding. K-L) The roots of progeny seedlings stain readily, but penetration of leaf tissues by the X-Gluc substrate needs assistance, e.g., by several freeze-thaw cycles (L).


HindIII
HindIII
HindIII
HindIII
HindIII
NOS-NPT-NOS
35S-IG-NOS
35S-HPT-35S
BR
ori
ori
cos
BL
5.8 kb
3.1 kb
5.0 kb
Expected fragments of pTOK233
NOS-NPT-NOS to BR
35S-IG-NOS
35S-HPT-35S to BL
NPT
GUS
HPT
0.5 kb
Probes
G 2 11 17 21 p G 2 11 17 21 p G 2 11 17 21 p G 2 11 17 21 p
Plant line
12.0 kb
5.8 kb
5.0 kb
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UNCUT
GUS
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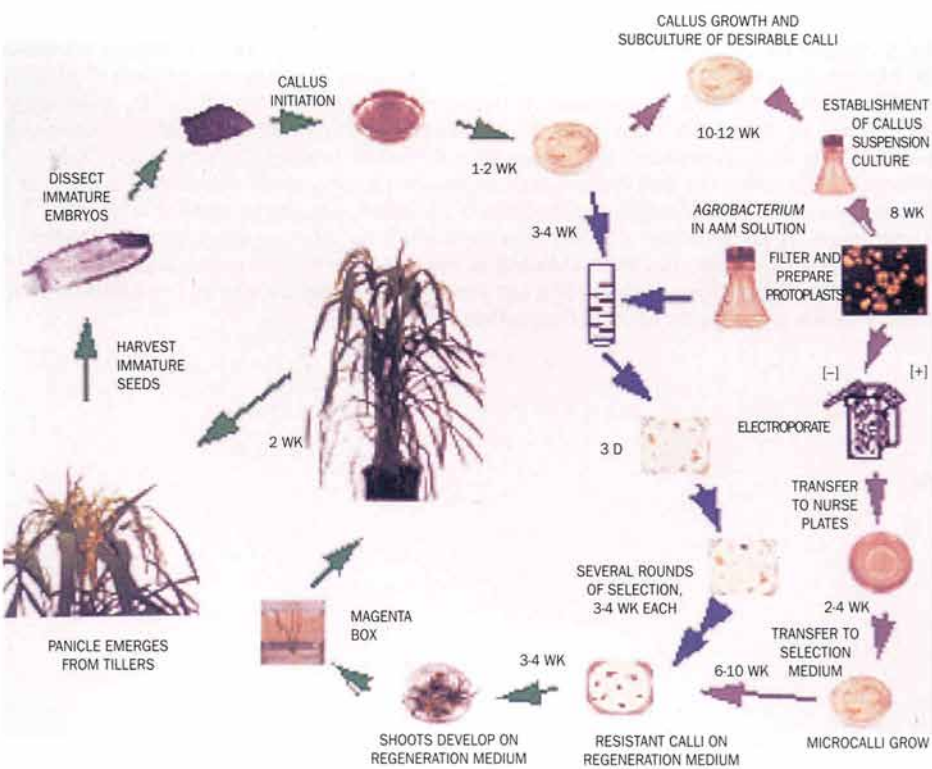
CALLUS GROWTH AND SUBCULTURE OF DESIRABLE CALLI
CALLUS INITIATION
10-12 WK
ESTABLISHMENT OF CALLUS SUSPENSION CULTURE
1-2 WK
DISSECT IMMATURE EMBRYOS
3-4 WK
AGROBACTERIUM IN AAM SOLUTION
8 WK
FILTER AND PREPARE PROTOPLASTS
HARVEST IMMATURE SEEDS
[-]
[+]
ELECTROPORATE
2 WK
3 D
TRANSFER TO NURSE PLATES
SEVERAL ROUNDS OF SELECTION, 3-4 WK EACH
2-4 WK
PANICLE EMERGES FROM TILLERS
MAGENTA BOX
TRANSFER TO SELECTION MEDIUM
3-4 WK
6-10 WK
SHOOTS DEVELOP ON REGENERATION MEDIUM
RESISTANT CALLI ON REGENERATION MEDIUM
MICROCALLI GROW

Fig. 2. Genomic DNA blot analysis of transgenic Gulfmont. The upper panel shows a diagram of the T-DNA of pT0K233, the probes used in the analysis and expected fragments following digestion with *Hin*dIII. The scale bar (red) indicates 0.5 kb. Genomic DNA from Gulfmont (G), four different independent transformants of Gulfmont (2, 11, 17, 21), and pT0K233 (p) was digested to completion with *Hin*dIII, fractionated by electrophoresis in an agarose gel (0.7%), blotted by standard pressure blot procedures, and probed with ^{32}P-labeled fragments of GUS or NPT or HPT. The Southern blots in the lower panel show the lack of signal from wild type Gulfmont probed for GUS or NPT or HPT, and four independent transformants: lines 2 and 11 appear to have a single complete insertion and another partial insertion; lines 17 and 21 have two inserted copies of T-DNA. The blot of uncut genomic DNA was probed using the ^{32}P-labeled GUS fragment.

Fig. 3. Procedures for *Agrobacterium*-mediated and electroporation-mediated transformation and regeneration of rice. Arrows indicating steps common to both systems are shown in green; those specific for the *Agrobacterium* approach are in blue and those for electroporation approach in gray. Immature embryos were dissected 10-12 d after pollination and placed on callus induction medium. After 4-6 wk of growth, with one subculture, the scutellum-derived calli were inoculated with *Agrobacterium* and cocultivated in the presence of acetosyringone. After washing with cefotaxime solution, the calli were put onto selection medium with cefotaxime and subcultured at least three times at 3 to 4-wk intervals. Actively growing calli were transferred to regeneration medium. Regenerated plants, confirmed to be transformed by molecular analysis (Fig. 2) were transferred to magenta boxes and then to soil in the greenhouse. As shown, it takes about 6 mo from initiation of callus to obtain mature seeds; in contrast, the electroporation approach requires at least 12 mo.

Discussion

The results presented here represent the confirmation of a report of *Agrobacterium*-mediated transformation of rice, and extend the initial observations by showing production of fertile, stably transformed plants from the japonica cultivar Taipei 309 and the javanica cultivar Gulfmont. Cotransformation of *hpt* and *gus* was essentially 100%, although this has yet to be rigorously established. Importantly, only a single, or two copies of the introduced genes were present and they segregated as expected for a single-locus insertion.

The plants obtained by the present procedure were phenotypically normal and much more vigorous than plants we have previously obtained using electroporation of protoplasts (Battraw and Hall 1990, 1992). In contrast to the protoplast system, which takes 1 yr to obtain fertile plants, we obtained progeny seed in 5-6 mo (Fig. 3). This is similar to the time taken for plants derived from biolistic transformation (Christou et al 1991), but the low copy number of inserted genes is a notable advantage. Additionally, in *Agrobacterium*-mediated transformation, only the DNA sequence between the T-DNA borders is transferred, unrearranged, to the recipient genome. In contrast, bombardment often results in the delivery of multiple, ill-defined plasmid fragments along with the desired genes.

As shown in Fig. 1C, we obtained calli from the cultivar Koshihikari that were transformed, and stained deep blue with X-gluc. However, thus far, we have not been successful in regenerating transformed plants from this cultivar. Hiei et al (1994) also noted that, of the cultivars examined, Koshihikari was relatively recalcitrant to regeneration.

Although both *Agrobacterium* strains used were competent for transformation of Taipei 309, we were not successful in transforming Gulfmont with EHA101(pIG12lhm) (Table 1). From these findings, it is evident that strain and cultivar compatibilities exist. However, it seems likely that modifications in culture conditions for both the plant and bacterial strains could yield successful results.

Agrobacterium-mediated transformation of many agronomically important dicot species has provided a sound approach for the development of commercial crops having new traits. The proven ability to use a similar approach for transformation of rice, one of the most important monocot crops in the world, promises to be revolutionary in the application of biotechnological advances to agriculture.

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Biolistic transformation of rice: now efficient and routine for japonica and indica rices

C.M. Fauquet, S. Zhang, L. Chen, P. Marmey, A. de Kochko, and R.N. Beachy

Rice transformation using microbombardment has been used for 4 yr, but it is only since 1994 that we can consider the process routine for both japonica and indica rices. For japonica transformation, the rate of efficiency of transformation now averages 25%. Our protocol does not generate escapes for hygromycin resistance and the duration of our protocol has been shortened to 8 wk from shooting time to obtaining plantlets. Transformation is accomplished by mixing plasmids containing the hygromycin resistance gene and the gene(s) of interest (GOI). Cointegration of two genes averages 70% and greatly depends on the DNA ratio in the plasmid mixture. Several types of explant tissues such as immature embryos, embryogenic calli, and embryogenic suspensions have been tested and transgenic plants were produced. Embryogenic calli are preferred but, in some cases, embryogenic suspensions can be advantageous. Indica transformation using embryogenic suspensions and the biolistic method is performed regularly with varieties that are difficult to regenerate such as IR72, IR64, and BG90-2 but with a lower efficiency ranging from 1 to 5%. Transgenic plants have been carried through to seven generations, which proves stability in the inheritance of the integrated genes and shows that normal segregation is maintained over generations. Many different GOIs have now been inserted, including the *Xa21* bacterial blight resistance gene, demonstrating that large pieces of DNA coding for large proteins can be successfully integrated and expressed in rice and can produce the expected phenotype.

For some time now, rice has been transformed using protoplast transformation mediated by polyethylene glycol (Toriyama et al 1988; Zhang et al 1988; Zhang and Wu 1988; Shimamoto et al 1989; Datta et al 1990, 1992; Hayashimoto et al 1990; Peng et al 1990, 1992; Terada and Shimamoto 1990; Hayakawa et al 1992; Fujimoto et al 1993;

Rathore et al 1993). Most of the genotypes have been japonica and the fertility of the transgenic plants has always been a problem. Rice transformation has also been successfully achieved with microbombardment of immature embryos or embryogenic calli (Christou et al 1991, Cao et al 1992, Li et al 1993, Sivamani et al 1996), but it is only recently that biolistic transformation of indica rice has been shown to be effective and capable of producing fertile group I indica plants (Zhang et al 1996). More recently, other methods such as *Agrobacterium*-mediated transformation (Hiei et al 1994) and electroporation of intact seed embryo cells (Xu and Li 1994) have been published.

While fertile japonica transgenic plants can be obtained using other methods, the biolistic system has become the method of choice because it alleviates the need of preparing protoplasts, reduces the time needed to regenerate transgenic plants, and results in transgenic plants with higher fertility. *Agrobacterium*-mediated transformation of rice may provide another alternative technique to biolistic transformation if it is proven to be genotype-independent and routinely efficient.

At the International Laboratory for Tropical Agricultural Biotechnology (ILTAB), successful japonica rice transformations have been carried out since 1991, but it was only in 1994 that we really had an efficient system in place where the production of transgenic independent lines per experiment averaged 25%. It is only now that we can really do experiments properly designed to check a number of variables that are important when transforming rice. In this paper, we discuss results of a number of tissue culture experiments aimed at making the protocol more precise, shorter, and simpler. We also provide information on DNA variables tested so far in our experiments.

Indica rice transformations have always been much more difficult to achieve. We first used the immature embryo system (Rancé et al 1994, Tian et al 1994) and then switched to embryogenic suspensions (Zhang 1996. Zhang et al 1996). Using the latter method, we have had variable success.

Finally, through collaboration with the University of California, Davis, we managed to transfer the bacterial blight resistance gene *Xa21* first to japonica rice (Song et al 1995, Ronald et al 1996) and recently to indica rice (S. Zhang et al, unpubl. data). It is now possible to transfer and express a phenotype resulting from a single gene to any rice genotype. DNA mapping, gene cloning, and genetic engineering are indeed complementary tools that should enable breeders to develop some interesting transgenic lines.

Biolistic transformation of japonica rice

ILTAB's transformation protocol was established in 199 1 and modified and improved in 1994 for our model japonica variety, TP309. The protocol is a model for optimizing transformation methods for other japonica varieties and, possibly, for a few indica varieties. Using embryogenic suspension and/or embryogenic calli as the target tissue (Sivamani et al 1996), the new protocol is faster and more efficient in selection. One major improvement is the use of osmotic pressure to ensure that wounded cells have a better chance of recovery. Another major improvement is the early visual identification of the transgenic calli, which has tremendously boosted transformation efficiency.

Nature of the explants used for bombardment

Early biolistic transformations used immature embryos, but it is very difficult to obtain immature embryos suitable for transformation on a regular basis. We investigated using other tissues such as embryogenic calli and embryogenic suspensions. Figure 1 shows a diagrammatic representation of the protocol used at ILTAB to produce such embryogenic material for T309 and other japonica genotypes.

Nature of the targeted tissue

We compared the results of transformation efficiency between immature embryos, embryogenic calli, and embryogenic suspensions as target tissues, using T309 in all experiments (Figure 2). Embryogenic calli are much more efficient in producing transgenic plants compared with immature embryos (25 vs 3%). Another set of experiments demonstrated that embryogenic suspensions and embryogenic calli are equally effective in producing transgenic calli (41±3 vs 45±9%).

Tissue size and age

When using embryogenic suspensions for transformation, the size and age of the explants used for bombardment are critical. The suspension should be replicated at least 7 d before—but no more than 14 d prior to—bombardment and the size of the explants should not be smaller than 2 mm for optimal transformation.

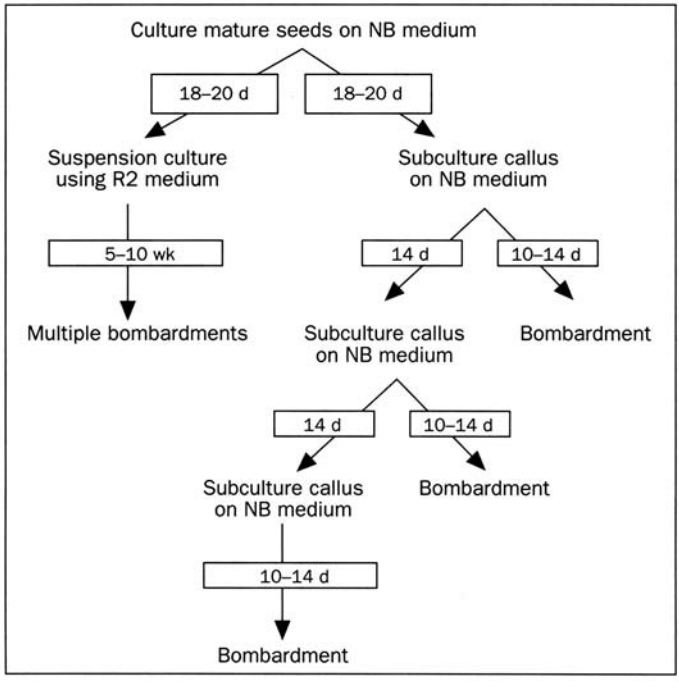

Fig. 1. Preparation of the subcultured embryogenic explants for bombardment.

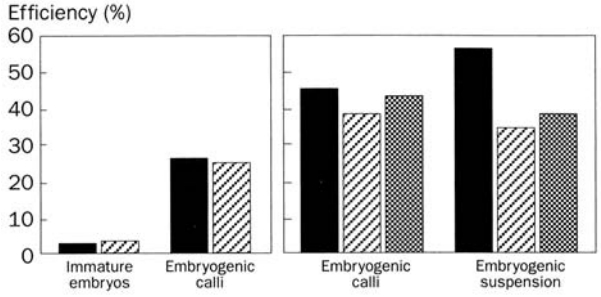

Fig. 2. Comparison of target immature embryos, embryogenic calli, and embryogenic suspension as target tissues used to produce transgenic plants. The patterns designate different experiments (three replicates for each experiment).

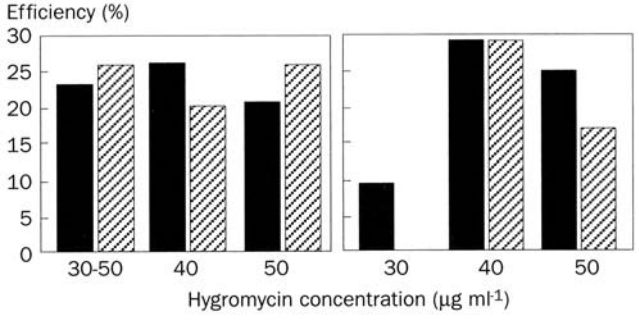

Fig. 3. Comparison of different selection treatments used to produce transgenic rice plants. Each experiment is an average of four or five replicates.

Osmotic treatment

Gold particle bombardment is detrimental to cells. However, it has been demonstrated that wounded cells can be protected by keeping them under high osmotic pressure before and after bombardment. Vain et al (1993) demonstrated this with maize and we confirmed it with rice. It is possible to obtain eight times more transgenic plants (average of three experiments) when using osmotic pressure treatment. We have also found that an optimum osmotic pressure can be reached to optimize rice transformation (data not presented).

Selection with one hygromycin treatment is preferred

Since false-positive plants in transformation work have always been a problem, we investigated various protocols that differed in the amount of selectable marker (30, 40, and 50 mg hygromycin B L^{-1}) and in the number of selection times. Figure 3 shows that a moderate selection pressure is preferable (40 mg hygromycin B L^{-1}) and that one selection is effective.

Need for experienced technicians

An important change at ILTAB since 1992 is the exclusive use of well-trained, experienced technicians to select visually the transgenic calli as early as 21 d after bombardment. Our technicians are trained to observe color, glossiness, and callus structure—all important factors when selecting transgenic calli. Figure 4 provides a diagrammatic representation of the ILTAB protocol for japonica rice transformation, which includes the improvements discussed above.

Transformation efficiency

Transformation efficiency is determined by calculating the number of transgenic lines obtained per explant bombarded. If several plants are regenerated from one explant, they are considered to be siblings and counted as one. In a few experiments, we reached a 50% transformation efficiency but the average is closer to 25%.

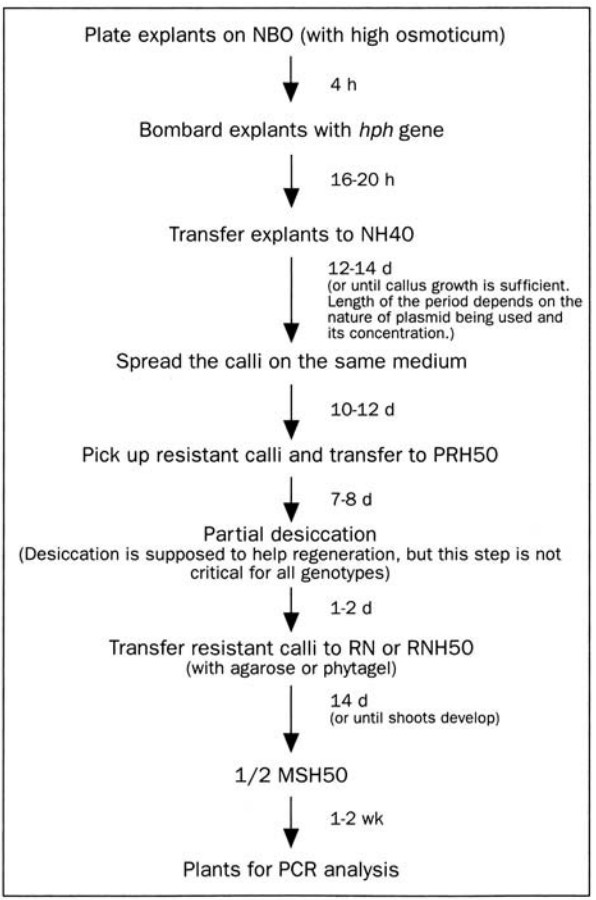

Fig. 4. ILTAB protocol for japonica rice transformation.

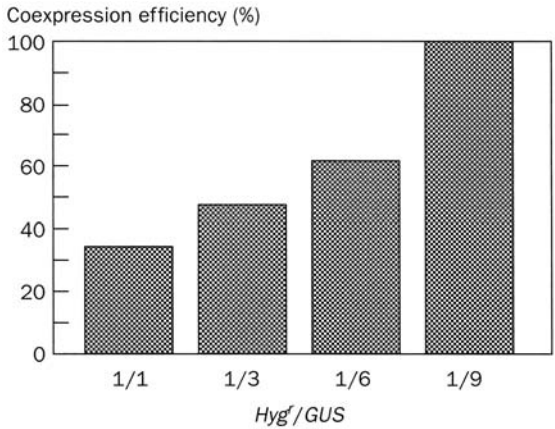

Fig. 5. Percentage of co-expression efficiency for hygromycin resistance and *GUS* expression for various ratios of the DNAs coding for *Hyg*^{r} and *GUS*.

Cointegration efficiency

Because we select with one gene while working to insert another gene, the efficiency of the integration of both genes (cointegration) is a major concern in rice transformation when using the biolistic method—specially since we use mixtures of plasmids rather than plasmids carrying both genes. The percentage of cointegration varies between 5 and 100%. Our experience shows that DNA quality and the nature of the gene are also very important in achieving positive results.

To improve our results, we have studied the molar ratio of the DNA of the gene of interest (GOI) and the DNA of the selectable marker, relative to the efficiency of cointegration and, in some cases, of coexpression. Figure 5 provides preliminary results showing that this ratio is important and that an increase in the molar ratio of the plasmid coding for the GOI increases the coexpression ratio. In this experiment, the two genes were *hph* and *iudA* and 100% coexpression was obtained when using a ratio of 1/9 for *Hyg*^{r}*/GUS* genes.

Fertility and germination

Recently, more than 80% of the transgenic rice plants developed at ILTAB were at least partially fertile (ranging from highly fertile to highly sterile, based on IRRI criteria) and only 18% were completely sterile. In cases where R_0 transgenic plants were partially fertile, fertility was generally restored in the next generation, indicating that partial sterility, in most cases, is not an inherited trait.

Germination rates of seeds from most transgenic rice lines were usually higher than 90%, comparable with the seeds from nontransgenic plants. Lower germination rates were also observed in a few cases, ranging approximately from 10 to 60%.

It is our opinion that transformation itself does not generally induce sterility, but it is the passage through tissue culture and, above all, the conditions under which the transgenic plants are growing that are critical in obtaining high fertility.

Inheritance mode, copy number, and stability of the transgenes

For a majority of the transgenic rice plants assayed so far, the transgenes were inherited as dominant genes in a Mendelian way. However, non-Mendelian inheritance and 1:1 ratio (at some time over several generations) are observed.

Generally, the copy number of the transgenes integrated into the rice genome ranges from one to several when the plants are transformed biolistically. In one study, we used Southern analysis to investigate 41 transformation events and found that 6 (14.5%) had one transgene copy, 31 (80.5%) had from 2 to 10, and only 2 (5%) had more than 10 copies. Using biological assays (*hyg*^{r}), we have detected expression of the *hph* gene in transgenic plants with various copy numbers. The expression of the GOI in some transgenic plants with single- or multiple-copy numbers of the relevant genes was also detected using Northern or Western hybridization. Usually, only a random portion of the vector DNA is integrated into the rice genome, and this portion is different from one transgenic plant to another as revealed in many Southern hybridization analyses. In many cases, when the Southern pattern shows multiple copies, all copies have been inherited together, indicating they were inserted at the same genetic locus.

So far, the *hph* gene has been stably inherited up to the R_7 generation, and the GUS gene up to the R_5 generation, except for one offspring line in which *GUS* expression was mostly suppressed. Studies involving other genes are under way.

Japonica transformation at ILTAB

Over the last 18 mo, one experienced technician and two assistants produced 2,135 independent transgenic lines with 187 different constructs. These numbers affirm that japonica transformation is now routine at ILTAB. We can now address scientific and technical questions that need to be answered before transferring any GOI to a genome of interest, including indica rice.

Recent improvements in the biolistic method used for japonicas will have an impact on all rice transformation research. It is now possible to look for the expression of an inserted gene and to study a large number of genes and promoters in rice. These developments will permit rice biotechnology to progress rapidly over the next few years.

Major advantages in the ILTAB protocol are summarized below.

- Preparation of the target tissue is independent of the environment. Instead of immature embryos, we now use subcultured embryogenic calli (Sivamani et al 1996) obtained from mature seeds, as well as embryogenic suspensions (Zhang et al 1996).
- The transformation protocol is rapid. Generally, it does not take more than 8-9 wk from bombardment to appearance of transgenic shoots/plantlets.

- There are no escapes in terms of hygromycin resistance.
- Transformation efficiency is consistent and uniform. An experienced technician can achieve efficiency ranging from 8 to 54% with an average of 25%, i.e., 25% of the bombarded explants will produce an independent transgenic line.
- The cointegration efficiency has improved to an average of 76%. In some cases, 100% cointegration has been obtained.
- Most of the transgenic plants are fertile or at least partially fertile. Fertility problems are associated more with greenhouse conditions than with the transformation protocol itself.

Much can still be done to improve the transformation protocol. For example, there is too much variability in transformation efficiency and we need to investigate DNA concentration and quality. And the nature of the sequences to be inserted is an important issue since we see a lot of variation from among the plasmids.

Biolistic transformation of indica rice

Four laboratories (Peng et al 1990, 1992; Christou et al 1991, 1992; Datta et al 1992; Xu and Li 1994) have reported accomplishing transformations using group 1 (Glaszmann 1987) indica rice, but with limited success in obtaining fertile transgenic plants. Fertile lines were regenerated only through bombarding immature embryos (Christou et al 1991, 1992) or electroporating embryogenic cells (Xu and Li 1994). Unfortunately, these techniques are genotype-dependent and cannot be extrapolated to a wide range of indica varieties. In addition, both the immature embryo and electroporation techniques are time-consuming and labor-intensive. Since immature embryos are environment-dependent, it has been difficult to maintain a continuous supply of suitable explants at ILTAB.

ILTAB protocol for indica transformation

The ILTAB protocol calls for using regenerable embryogenic suspensions as the target tissue for bombardment. Over the last 2 yr, we have obtained fertile transgenic plants from elite varieties IR24, IR64, and IR72 and advanced breeding line IR57311-95-2-3 and from indica varieties popular in Vietnam (Nang Huong Cho Dao), India (Basmati, Co 45, Malaysia (MR80, MR81), Thailand (Khao Dawk Mali), and West Africa (BG90-2).

Although indica transformation efficiency is quite low compared with that of japonica transformation, the steps described in the following sections are simple and repeatable and can be used to introduce GOIs into indica germplasm. This protocol has produced 163 green plants, representing 49 independent transgenic lines, expressing marker genes and GOIs of IR24, IR64, IR72, IR57311-95-2-3. Most of the plants produced seeds.

Callus induction and cell suspension. Small, compact, and globular embryogenic calli can be induced in equal quantities using either mature seeds or immature embryos (Zhang 1995). ILTAB prefers using mature seeds to avoid the extra work of maintaining a constant supply of immature embryos. The most difficult step—and a limiting

factor—in establishing embryogenic suspensions is cell browning and death in the liquid media at the suspension initiation stage. To avoid cell death, small, compact, and loosely attached globular embryogenic calli are carefully selected and cultured in R2 medium with 2 mg 2,4-D L^{-1} and 20 g sucrose L^{-1}. If the callus begins to turn brown, the R2 medium is changed to 2 mg 2,4-D L^{-1} and 20 g maltose L^{-1} and the cultures are subcultured at short intervals (1-5 d) for 2-3 wk. Following this protocol, group 1 indica rice suspensions can be established in 6-8 wk. These suspensions can be used as target tissue for transformation for up to 3.5 mo; after this time, plants can be regenerated, but with a low fertility. Depending on experimental needs, large quantities of this embryogenic suspension can be easily produced.

Effect of osmotic pressure on transformation efficiency. As in japonica transformation, it is crucial to maintain cell turgor after wounding with the gold particles. An osmotic treatment (30 g mannitol L^{-1}, 30 g sorbitol L^{-1}) of cell suspension cultures for 4 h before and 16-20 h after bombardment (Vain et al 1993) enhances transformation efficiency as much as fourfold.

Transformation and selection of embryogenic rice cell suspension. We use hygromycin B (hyg B) as the selection agent in our studies. The effect of hyg B on the growth of embryogenic suspensions of each variety is examined prior to initiation of an experiment. Cell growth is significantly reduced on solid medium containing 30 mg hyg B L^{-1}, and growth is greatly inhibited on medium containing 50 mg hyg B L^{-1}. However, if cells are plated from suspension cultures on solid growth medium without hyg B for a few days, the selection on hyg B becomes more difficult, presumably because the rapidly growing cell clusters do not allow for efficient selection on hyg B. Therefore, bombarded cell suspension cultures, kept on high osmotic pressure medium for 16-20 h after bombardment, are then transferred directly to selection medium with 30 mg hyg B L^{-1}.

Most explants gradually turn brown 2-3 wk later and it is easy to identify any white, growing cell clusters. At this stage, however, not all growing clusters are transgenic, so they are carefully removed from dying explant tissue and transferred to fresh selection medium containing 50 mg hyg B L^{-1} for further selection. To increase plant regeneration, after 2 wk of selection on 50 mg hyg B L^{-1}, the hyg^r calli are transferred to preregeneration medium containing 50 mg hyg B L^{-1} before transferring to the regeneration medium.

Regeneration of hygromycin-resistant plants. When the hyg^r calli reach approximately 2-3 mm in diameter, they are transferred to regeneration medium containing 50 mg hyg B L^{-1}. In preliminary experiments, we had observed that the absence of hyg B leads to regeneration of nontransgenic plants, although the calli were previously grown on medium with hyg B for more than 1 mo. Plantlets are then transferred to a rooting medium without hyg B; rooting in the absence of hyg B appears to be important in retaining fertility of the R_0 plants.

Fertility of transgenic rice plants and germination of transgenic seeds

Transgenic group 1 indica plants developed from protoplast transformation protocols have reportedly been sterile (Peng et al 1990, 1992; Datta et al 1992). This may be

Table 1. Fertility of transgenic IR72 lines and germination rates of transgenic seeds.

Transgenic rice line	Fertility[a] (%)	Seeds germinated[b]/ seeds planted
IR72-1	NT	47/50
lR72-2	NT	139/150
lR72-5	45.1 ± 14	47/49
lR72-7	40.1 ± 17	55/56
IR72-10	49.5 ± 18	47/47
IR72 (control)	65.3 ± 7	63/63

[a] Mean of fully set seeds per hundred seeds collected from six random panicles. NT: not tested. [b] Seeds were germinated in magenta boxes containing half-strength MS salts, 30 g sucrose L^{-1} , 0.1 mg NAA L^{-1} and with 0.25% (w/v) phytagel medium.

Table 2. Inheritance of the hyg^r trait and *uidA* gene expression in transgenic IR72.

Transgenic line	Seeds planted (no.)	R_1 germinated seedlings (no.)	hyg^r seedlings (%)	GUS-positive seedlings (no.)	c^2
IR73-1	50	47	35 (74.5)	35	0.007
IR72-2	100	91	76 (83.5)	76	5.280
lR72-5	49	47	34 (72.3)	34	0.177
lR72-7	56	55	39 (70.9)	39	0.491
IR72-10	47	47	35 (74.5)	35	0.007
IR72 (control)	63	63		0	

[a] Transgenic rice seeds (R_1) were planted on 1/2 MS salts medium for 6 d, then transferred to medium containing hyg 8 (50 mg L^{-1}). c^2 tests indicate good agreement with segregation ratios of 3:1 except in line IR72-2.

due, in part, to the time involved in establishing the cell suspension cultures. In our experiments, cell suspensions are used for biolistic transformation 6-8 wk after initiation. All of the regenerated transgenic plants tested were fertile. Table 1 shows the fertility level of three independent R_0 transgenic IR72 lines. Their fertility is lower than that of nontransgenic plants, probably due to the effects of the tissue culture protocol. However, normal fertility can be restored in the R_1 plants (data not shown). The seed germination rate in R_0 transgenic rice lines is comparable with that of the nontransgenic plants (Table 1).

Inheritance of *hph* and *uidA* genes

Integration of the *hph* gene into the genome of transgenic rice plants was confirmed by Southern blot hybridization using the hph sequence as probe. All lines tested contained the intact cassette (promoter, *hph* gene, and Nos terminus), as expected. Some lines contained more than one copy. The segregation of the hyg^r trait and *uidA* gene expression among offspring of the transgenic plants were demonstrated by germinating R_1 seeds on medium containing 50 mg hyg B L^{-1} and by GUS assays,

respectively (Table 2). Results showed that the *hph* and *uidA* genes segregated together in tested offspring and most of the lines exhibited a 3: 1 segregation ratio among the offspring, indicating Mendelian inheritance from a single genetic locus of functional *hph* and *uidA* genes. Inheritance of *hph* and *uidA* by Southern blot and GUS assay of R_3 transgenic plants was also demonstrated (data not shown).

Production of bacterial blight-resistant rice plants

We collaborated with the University of California, Davis (UCD), to transfer the bacterial blight resistance gene *Xa21* from *Oryza longistaminata* to *O. sativa.* The gene, which had previously been transferred through crossing, has been mapped on chromosome 11 and cosmid-bacterial artificial chromosome (BAC) libraries containing it have been made from line IRBB21. Through cloning and hybridization, UCD researchers managed to isolate eight clones corresponding to the *Xa21* locus. These constructs were integrated, as such, into 306 independent T309 transgenic lines, produced in 4 mo. Most of the R_0 plants were then challenged with *Xanthomonas oryzae.* Eighty percent of the 15 lines corresponding to construct B were found extremely resistant to *X. oryzae* under laboratory conditions. After sequencing the clones, it was found that only the construct possessing the entire *Xa21-B* gene induced the resistance phenotype (Song et al 1995, Ronald et al 1996). For six resistant lines, it was possible to obtain seeds and check the inheritance of the trait, and indeed the resistance phenotype followed a 3:l ratio as expected for a transgene present at a unique locus.

Since then, we have transformed the indica rice varieties IR64 and IR72, with the same *Xa21-B* construct, and we have managed to produce several independent transgenic lines for both varieties that have been challenged with *X. oryzae* and subsequently shown to be extremely resistant. These plants are now producing seeds, and we should be able to challenge the second generation very soon. After that, seeds will be mass-propagated and bacterial blight resistance tests will be performed on a large scale by bacteriologists. Breeders will then evaluate these transgenics for other traits to determine their characteristics.

These results demonstrate that an attempt to transform indica rice can be made with less than complete molecular information. Even in the case of a very large protein (the Xa21 protein is about 113 kDa), it is possible to express it correctly and above all to obtain the expected phenotype. It will be possible to use BAC subclones directly in transformation experiments and to immediately begin the search for the phenotype—if a very efficient transformation system like that currently used for japonicas becomes available. When the effective clone has been precisely identified, we have demonstrated that it is possible to transfer a useful gene to improve indica varieties.

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Notes

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Transformation of rice for producing agronomically useful transgenic plants and/or analyzing gene expression, including the gibberellin-dependent signaling pathway

R. Wu, X. Chen, B. Wang, M. Chang, D. Xu, X. Duan, and P. Chen

Transformation of rice cells followed by regeneration of fertile plants constitutes a new method for producing agronomically useful transgenic rice plants. We have transformed rice with a plasmid harboring a potato protease inhibitor gene (*PINII*) joined to a wound-inducible pin2 promoter. The transgenes in plants of the R_0, R_1, and R_2 generations have been analyzed, and the results showed that the transgene is stably inherited. Transgenic plants have also proven to be useful in analyzing the expression of a gene or a modified gene. Transfer of genes into rice cells, followed by determining the amount of the gene products several hours later, constitutes a convenient way to analyze transient gene expression. We have used this transient assay to look for gibberellin (GA)-responsive sequences in GA-induced genes such as the rice a-amylase gene, *Osamy-c.* In this assay, different lengths of the promoter region were fused to a reporter gene such as the luciferase cDNA. Several plasmids were constructed and transferred into rice aleurone cells by the biolistic method, and the luciferase activity was measured. We have also used the transient assay system to look for intermediates in the GA-dependent signal-transduction pathway by transactivation experiments. These involve transferring two genes on two separate plasmids together into rice cells and observing how the gene product of the first gene affects the expression of the second. By using this approach, we have shown that a ubiquitin-conjugating enzyme gene and a calcium-dependent ATPase gene are putative intermediates in the GA-dependent signal-transduction pathway because either gene can transactivate the expression of a target gene, *Osamy-c.* Identification of other putative intermediates in the GA-signaling pathway is also presented.

The success of our ability to introduce foreign genes or modified genes into rice opens up new opportunities for producing agronomically useful transgenic rice plants and for allowing detailed analyses of gene expression. regulation, and biological function. This success has been made possible by the following developments: first, improvements in cell culture methodologies and regeneration procedures to produce fertile rice plants from either protoplasts or suspension cells; second, development of efficient methods for gene transfer; third, construction of useful plasmids that include strong or regulatable promoters, suitable reporters and selectable markers; and fourth, identification of agronomically useful genes.

A useful plasmid requires a strong or regulatable promoter. An example of a strong constitutive promoter is the rice actin 1 gene (Actl) promoter (McElroy et al 1991). An example of a regulatable promoter is a wound-inducible promoter from the potato protease inhibitor gene; when used in conjunction with the Actl intron, it gives high-level, wound-inducible gene expression in rice (Xu et al 1993).

There are a number of reporter genes and selectable marker genes that have been used for plant gene transfer experiments. The most useful assayable marker genes include the bacterial b-glucuronidase gene (*Gus*) and the firefly luciferase gene (*Luc*). Three selectable markers have been widely used in rice transformation: the phosphinothricin acetyl transferase gene (*bar*), the hygromycin phosphotransferase gene (*hpt*), and the neomycin phosphotransferase gene (*npt* or *neo*).

Since genes from any source can be introduced into rice using the established gene transfer technology, there are several genes that are potentially useful for rice genetic engineering (for a review, see Toenniessen 1991). However, to date, only a few genes have been transferred into rice and proven to be beneficial, at least in greenhouse tests. Thus, more genes need to be transferred into rice and tested for their usefulness for rice genetic engineering.

Production of agronomically useful transgenic rice plants

Production of insect-resistant transgenic rice plants

The loss of rice yield due to direct insect damage is estimated to cost at least several billion dollars a year (Heinrichs et al 1985). By using conventional plant breeding, several useful disease-resistance genes from wild rice have been introduced into cultivated rice. However, the availability of useful rice germplasm is limited, and the introduction of beneficial genes into elite cultivars is time consuming. Moreover, the control of insect pests in rice cultivation by chemically synthesized insecticides often results in environmental pollution and affects food safety. Genetic engineering offers a safe and effective way to deal with these problems. Currently, there are two major types of useful genes that can be transferred into rice for genetic engineering of insect resistance: the insecticidal crystal protein genes from *Bacillus thuringiensis* (*Bt*) and the plant-derived insecticidal proteinase inhibitor genes.

Bt crystal protein genes. Several *Bt* genes encoding different crystal proteins have been isolated from bacterial strains, and two of these genes have been introduced into monocot plants such as maize and rice. For example, Fujimoto et al (1993)

introduced a *cryI(A)b* gene into rice protoplasts and regenerated fertile transgenic plants.

Proteinase inhibitor genes. Proteinase inhibitors bind and inhibit serine-type proteinases, including trypsin and chymotrypsin. A cowpea trypsin inhibitor gene (*CpTi*) was cloned and introduced into tobacco plants. The transgenic plants became resistant to tobacco insect pests (Hilder et al 1987). Similarly, a potato protease inhibitor gene (*PINII*) was cloned and introduced into tobacco plants (Johnson et al 1989). The transgenic tobacco plants that produced relatively high levels (1% of soluble leaf proteins) of *CpTi* or *PINII* showed resistance to typical tobacco insect pests.

We have constructed a plasmid including the wound-inducible pin2 promoter, the first intron of rice Act1 (to increase the level of expression in rice), the pin2 coding sequence, and the *bar* gene (for selection) driven by the 35S promoter. This plasmid was introduced into rice suspension cells (two japonica varieties: Tainung 67 and Pi 4) by the biolistic method. Of 37 Southern blot-positive lines, 27 were fertile. Southern blot analysis of the second (R_1) and third (R_2) generations plants from a number of transgenic lines showed that almost all of the R_2 and R_1 plants from a given R_0, plant had the same hybridization pattern, indicating that the *pin2*-containing transgene is stable in transgenic rice plants (Duan et al 1996).

At the same time, the level of PINII protein in R_0, R_1, and R_2 plants was determined by a quantitative spectrophotometric assay. The results showed that the PINII level in 15 R_0 transgenic plants varies between 0.4 and 2.6% of soluble leaf proteins. Ten R_1 plants from each of two R_0 lines were analyzed in detail. For example, in line No. 12, the PINII level in the R_0 plant was 0.6%, but it was 1.2% in 4 of the 14 R_1 plants. These R_1 plants are likely to be homozygous, and this has been confirmed by analyzing 14 R_2 plants from each R_1 line. In all cases, the PINII level in the R_2 plants was close to 1.2% (Duan et al 1996). Our conclusion that these R_2 plants are homozygous is further supported by our herbicide resistance test (using Basta) with R_3 plants. All 300 R_3 plants from 19 R_2 plants derived from plants No. 12-3 and No. 6-8 were resistant to 0.5% Basta (data not shown). As mentioned in an earlier section, different copies of the transgene segregate as a single-copy gene in each line of the transgenic plant. Thus, if these R_2 lines are not homozygous, we would expect 75 of the 300 R_3 plants (25%) to be sensitive to Basta. However, our results showed that none of the 300 R_3 plants were herbicide-sensitive.

Small-scale greenhouse test of transgenic rice plants for insect resistance. The fifth generation (R_4) plants of two primary transgenic lines, No. 6 and No. 12, were used for the preliminary insect resistance test. The progeny of the same generation from transformation procedure-derived nontransformed plants were used as controls. Individual plants were grown in enclosed pots kept in the greenhouse. Second-instar larvae of rice pink stem borer were artificially applied to the rice plants at the early heading stage. In our bioassay, a significant difference was observed between transgenic plants and nontransformed control plants in their response to the infestation by rice pink stem borer and development of the whitehead symptom. The number of shoots (tillers) developing the whitehead symptom in transgenic plants was significantly lower than that of nontransformed plants. More than 70% of the tillers in nontransformed

Tainung 67 control plants showed the whitehead symptom (hollow stem and dead panicles), whereas only 16% of the tillers in transgenic plants (no. 6-7-4-1) showed the whitehead symptom. In addition, there was a significant difference between the larvae applied to the transgenic plants and those applied to the control plants in their growth and development during the 5-wk experimental period. Larvae in the transgenic plants showed little weight increase and stayed at the second- or third-instar stage, while larvae in the control plants showed a 3- to 4-fold weight increase and developed to the fourth- or fifth-instar stage. Similarly, 100% of the tillers in the nontransformed Pi 4 control plants and only 17.5% of the tillers in transgenic plants (no. 12-8-4-2) showed whiteheads.

Production of transgenic rice tolerant of drought, salt, and temperature

Environmental stresses, such as drought, increased soil salinity, and extreme temperature, are major factors that limit plant growth and productivity. Recent progress in plant genetic transformation and the availability of potentially useful genes characterized from different sources make it possible to generate stress-tolerant crops using transgenic approaches (Tarczynski et al 1993, Pilon-Smits et al 1995, Xu et al 1996).

There are two general types of molecules that are potentially useful in conferring stress tolerance to plants. The first type includes low-molecular weight compounds such as mannitol and proline. The introduction of the mannitol 1-phosphate dehydrogenase (*MtDH*) gene into tobacco plants led to an increase in the level of this enzyme and accumulation of mannitol in these plants (Tarczynski et al 1993). Transfer of the delta^1-pyrroline-5-carboxylase synthase (*P5CS*) gene into tobacco led to an increase in this enzyme level and proline accumulation (Kishor et al 1995). In both cases, the transgenic tobacco plants showed increased tolerance to salt stress compared with nontransformed plants.

The second type includes high-molecular weight compounds such as late embryogenesis abundant (LEA) proteins, which highly accumulate in the embryos at the late stage of seed development (Dure III 1993). The expression of LEA genes in vegetative tissues is induced by various environmental stresses, such as drought, salt, and extreme temperature (Shriver and Mundy 1990). However, clear experimental evidence supporting the exact functions of LEA proteins is still lacking.

A group 3 LEA protein, HVA1, was characterized from barley. Expression of the *HVA1* gene is rapidly induced in seedlings by dehydration, salt, and extreme temperature (Hong et al 1992). In our current study, we have taken a transgenic approach to investigating the probable function of the HVA1 protein in stress protection. A plasmid, pBY520, was constructed, which includes the cDNA of the barley *HVA1* located downstream of the rice Act1 promoter. On the same plasmid, the bar coding region is located downstream of the 35S promoter. This plasmid was introduced into rice suspension cells by the biolistic method. Sixty-three independent lines of transgenic plants were regenerated, and 85% of them were fertile. DNA blot hybridization, using the HVA1 cDNA as a probe, showed that 80% of the lines gave the predicted band pattern (Xu et al 1996). Constitutive, high-level expression of the barley *HVA1* gene

in transgenic rice plants was detected by quantitative immunoblot analysis. We found that accumulation of the barley HVA1 protein in the vegetative tissues of transgenic rice plants conferred increased tolerance to drought and salt stresses; further, the extent of stress tolerance was correlated with the level of HVA1 protein accumulation. Our results not only demonstrate a role of the barley LEA protein in stress protection, they also suggest the potential usefulness of *LEA* genes for genetic engineering of stress tolerance (Xu et al 1996).

Analysis of gene expression using transient assays

In this section, examples of transient gene expression are presented. In addition, the transient assays used to identify intermediate steps in the gibberellin (GA)-dependent signaling pathway are described.

Functional dissection of a rice high-pI α-amylase gene promoter

During germination in cereals, GA is known to induce the transcription of genes that encode hydrolytic enzymes such as α-amylase, glycosidase, protease, and nuclease. These enzymes mobilize resources that promote the growth of seedlings. We have established a quantitative transient assay based on expression of a luciferase reporter gene in rice aleurone cells, and we have characterized the Osamy-c promoter for GA-dependent regulatory sequences. Using this method, we have shown that the DNA sequence between -158 and -46 is sufficient to confer GA-responsive activation in Osamy-c (see Fig. 1, experiment C). In addition, this sequence is capable of directing GA-dependent expression from a heterologous minimal promoter (data not shown; Tanida et al 1994).

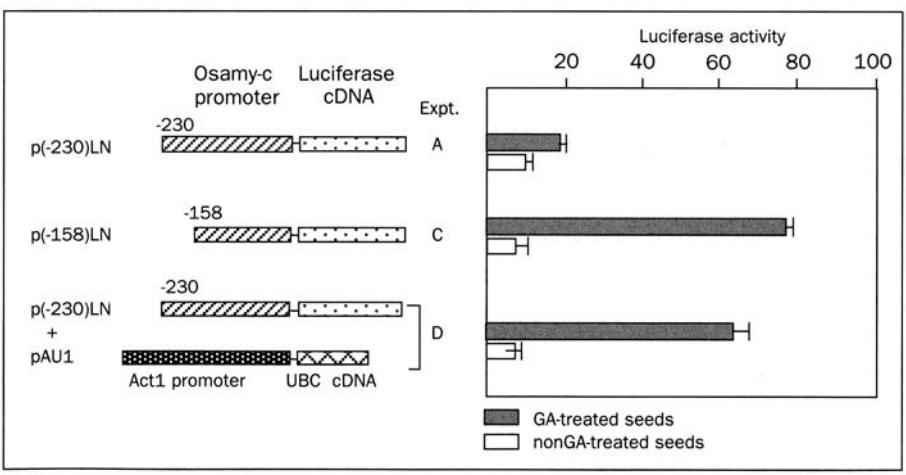

Fig. 1. Transient assay for testing the function of the rice UBC cDNA in regulating α-amylase gene expression.

Transient assays to identify intermediate steps in the GA-dependent signaling pathway

Until now, studies in different laboratories on GA-induced genes in aleurone cells of various cereals have focused on target genes, such as α-amylase genes (for a review, see Hooley 1994). However, the identity of the intermediate steps of the GA-dependent signaling pathway in aleurone cells remains unknown. In this section, examples will be presented to show how transient assays contributed to the discovery of two intermediate steps in the GA-dependent signaling pathway.

A ubiquitin-conjugating enzyme gene as an intermediate in the GA-dependent signaling pathway. A ubiquitin-conjugating enzyme (*UBC*) gene, induced by GA within 1 hr, was identified in rice (*Oryza sativa*) seeds by the mRNA differential display technique (Liang et al 1993). A full-length UBC cDNA clone and a genomic clone have been isolated and sequenced. The deduced amino acid sequence shares significant identity with several known UBC sequences, which are probably involved in the pathway responsible for degrading short-lived regulatory proteins. In vivo transient assays using the UBC gene promoter, joined to the luciferase cDNA as the reporter gene, showed that the sequence located between positions 231 and 159 upstream of the transcription start site of this promoter is crucial for GA-dependent activation of luciferase cDNA. Finally, transactivation experiments indicated that this UBC gene is involved in GA-stimulated α-amylase gene expression (Chen et al 1995).

Presence of a repressorlike protein that represses the activity of Osamy-c promoter. For testing GA response, the plasmid p(-230)LN was used. This plasmid contains a 230-bp sequence from the 5' noncoding region of Osamy-c fused to the coding region of the firefly luciferase gene, followed by the Nos 3' terminator sequence (Tanida et al 1994). When plasmid p(-230)LN was introduced into aleurone cells of deembryoed rice half seeds, the luciferase activity was low, and there was only a twofold GA stimulation (Fig. 1, experiment A). The interpretation of this result is that a repressor-like protein binds to the region between positions -230 and -158 of the Osamy-c promoter (Kim et al 1992) and strongly represses the promoter activity. This conclusion is consistent with the finding that, in experiment C, deleting the sequence between -230 and -158 resulted in a large increase in the luciferase activity.

UBC's role in regulating the expression of Osamy-c. Transactivation experiments were carried out by introducing two plasmids into rice aleurone cells (Fig. 1, experiment D), followed by the same transient assay described above. One of the plasmids, pAU1 , contains the actin 1 gene (Act1) promoter and the rice UBC cDNA; the other one, p(-230)LN, includes the promoter region (between positions -230 and +29) of the *Osamy-c* gene and the coding region of the luciferase gene. By comparing the results shown for the GA-treated half seeds in experiments A and D (Fig. l), the luciferase activity in experiment D is three times higher than that in experiment A. As a result, there is a sevenfold GA effect in experiment D, instead of the twofold GA effect in experiment A. This result can be interpreted to indicate that the protein product of the rice UBC gene (*pAU1*) directly or indirectly degraded the repressorlike protein that binds to the Osamy-c promoter region.

A calcium-dependent ATPase gene as an intermediate in the GA-dependent signaling pathway. By using a differential display approach, a GA-responsive Ca^{2+}-ATPase gene was identified. A genomic clone and a cDNA were isolated and sequenced. To investigate the possible role of this early GA-responsive endoplasmic reticulum (ER) Ca^{2+}-ATPase gene in the rice aleurone cell during GA induction, the coding sequence of this gene was fused transcriptionally with the rice Act1 promoter. The resulting plasmid was introduced into rice aleurone cells from nonGA-treated deembryoed half seeds by the biolistic method. mRNAs were isolated 16 or 24 h later and converted to cDNA, which was amplified by polymerase chain reaction (PCR). cDNAs corresponding to Osamy-c mRNA were detected in the lanes (designated 16 and 24) corresponding to mRNAs isolated at 16 and 24 h after introducing the Ca^{2+}-ATPase cDNA fusion plasmid into the nonGA-treated samples (Fig. 2). However. no Osamy-c cDNA was found in the control (lane 1, a plasmid consists of Act 1 promoter/bar cDNA, was delivered into aleurone cells; 24 h later, mRNAs were isolated, converted to cDNA, and amplified by PCR). The results suggest that rice ER Ca^{2+}-ATPase was able to trigger the synthesis of Osamy-c mRNA from the endogenous gene. It appears that the overproduced ER Ca^{2+}-ATPase bypassed the GA requirement and induced the transcription of Osamy-c. Thus, the ER Ca^{2+}-ATPase gene plays an important role in the GA-dependent signal-transduction pathway in rice aleurone cells.

The relation of Ca^{2+} release from ER and the increase of the inositol 1,4,5-triphosphate (IP_3) level in the aleurone cell. We have provided preliminary evidence to show that Ca^{2+} release from ER is critical for the transcription of Osamy-c; moreover, ER Ca^{2+}-ATPase is induced by GA and is involved in the GA-dependent signal-transduction pathway. Therefore, it suggests that the regulation of Ca^{2+} transport into

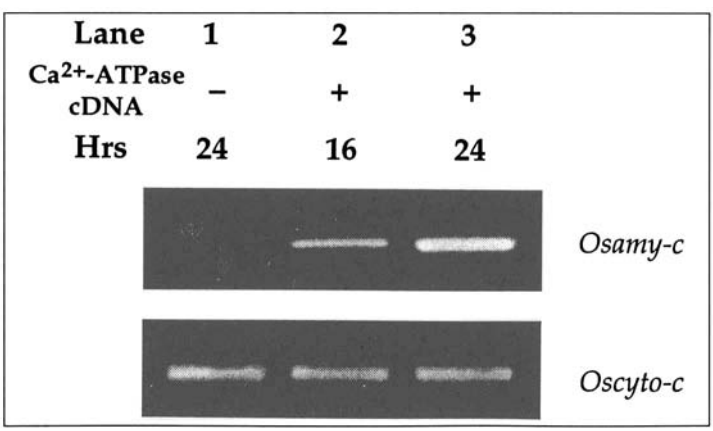

Fig. 2. A transient assay showed that overexpression of rice ER Ca^{2+}-ATPase cDNA in rice aleurone cells can bypass the GA requirement in the synthesis of Osamy-c mRNA. *Oscyto-c,* the expression of the rice cytochrome c gene (a nonGA-responsive gene), as monitored by reverse transcriptase-based PCR, was used as an internal standard for comparing the band density.

the ER is directly linked to the downstream steps of this pathway, which lead to the activation of target genes, such as *Osamy-c*. According to information on other systems, Ca^{2+} release from the ER membrane is initiated by inositol 1,4,5-triphosphate (IP_3). After it is produced, IP_3 can bind to receptors on the ER membrane in just minutes to release Ca^{2+} into the cytoplasm (Miyazaki 1995). We examined the possible role of IP_3 in the rice aleurone cells by measuring the amount of IP_3 after GA treatment of rice half seeds in a receptor-binding assay. Our results showed that the level of IP_3 increased more than four times in the seeds after 20-25 min of GA treatment compared with that of nonGA-treated samples. After 40 min, the IP_3 level dropped back down to near the original level. Therefore, it appears that the increase in IP_3 was an immediate response to the GA signal.

Examining the role of the PLC enzyme in the GA-dependent signal-transduction pathway. The experiment described above showed that the accumulation of IP_3 was transiently induced by GA in 25 min. Since IP_3 is produced by the action of the phospholipase C (PLC) enzyme, which is located on the plasma membrane, the possible involvement of the PLC enzyme in the GA-dependent signal-transduction pathway also needs to be examined. A PLC antagonist, U73122 (Powis et al 1992), was used to block the activity of PLC, and the expression level of *Osamy-c* was measured by

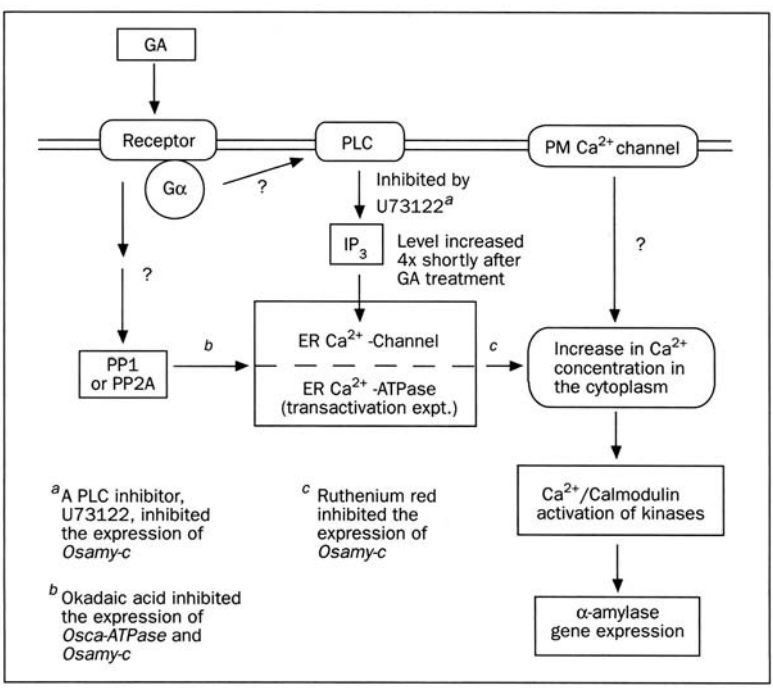

Fig. 3. A proposed model of the GA-dependent signal-transduction pathway in rice aleurone cells. G_a = alpha subunit of G protein; PLC = phospholipase C; IP_3 = inositol 1,4,5-triphosphate; PM = plasma membrane; ER = endoplasmic reticulum.

reverse transcriptase-PCR. Our results showed that 4 μM U73122 completely abolished the synthesis of Osamy-c mRNA. However, an inactive derivative of U73122, U73344, did not affect the transcription of *Osamy-c.*

Probable role of protein phosphatase 1 and 2A (PPI and PP2A) in GA-dependent expression of two GA-induced genes. Okadaic acid (OKA) is a known inhibitor of PP1 and PP2A in many systems. We found that OKA inhibited GA-dependent germination of rice seeds. In addition, the GA-dependent expression of *Osamy-c* and *Osca-ATPase* in embryo-less half seeds was inhibited by 1 μM OKA. A control experiment showed that OKA did not give a general inhibition of mRNA synthesis in rice seeds. Thus, we conclude that PP1 and/or PP2A are likely to be involved in the GA-dependent signal-transduction pathway in rice aleurone cells (B. Wang, M. Chang, X. Chen, and R. Wu, manuscript in preparation).

The preliminary results described in the above sections and other data (not shown), have led us to propose a plausible model of a GA-dependent signal-transduction pathway in rice aleurone cells based on analogy to animal systems. In our proposed model (Fig. 3), the putative GA-activated receptor (Hooley 1994) triggers the activation of the PLC enzyme on the plasma to produce IP_3 (Verhey and Lomax 1993). IP, then diffuses to the ER membrane and increases the local concentration of Ca^{2+} in the cytoplasm (the Ca^{2+} level is coregulated by the ER Ca^{2+}-ATPase). Next, Ca^{2+}-binding proteins (calmodulin or calmodulinlike proteins) bind Ca^{2+} and activate one or several protein kinases (based on information from other well-known systems). Then, presumably a protein kinase phosphorylates a transcription factor which leads to the activation of a -amylase gene expression.

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Transgenic indica rice for the benefit of less developed countries: toward fungal, insect, and viral resistance and accumulation of ß-carotene in the endosperm

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Indica rice provides the staple food for more than 2 billion poor in developing countries. In humid and semihumid Asia where rice is the basic food, the population is expected to increase by 58% over the next 35 yr. Increased yields to meet the demand for more food may come from reducing the present 40% harvest losses attributed to biotic and abiotic stresses. We focus on developing disease and insect resistances and improved nutrition quality through the use of genetic engineering. Toward this goal, we have a) developed a gene transfer technology for indica rice, b) introduced numerous genes that code for proteins with antifungal activity with an initial success in increasing resistance to sheath blight, c) produced *crylA(b)*-transgenic IR58 with good resistance to yellow stem borer and other lepidopteran insect pests, d) regenerated numerous transgenic offspring populations that harbor a series of RTBV-DNA sequences representing several anti-tungro disease strategies, and e) activated the terpenoid pathway with an endosperm-specific, transgenic phytoene synthase such that the endosperm synthesizes good quantities of phytoene for further (transgenic) conversion to b-carotene. A series of additional experiments include approaches toward nitrogen fixation, enhanced phosphate uptake, improved iron availability, and apomictic stabilization of hybrid vigor.

Indica rice feeds more than two billion poor, predominantly in less developed countries (LDCs). In humid and semihumid Asia where rice is the basic food, the population is expected to increase by 58% over the next 35 yr. In 30 yr, the world will need 70% more rice than it requires today. And these additional 780 million t of rice will have to be grown with considerably fewer inputs of agrochemicals and under sustainable conditions (IRRI 1993). This immense task will require that traditional rice breeding and production be supported by every possible contribution from science, especially genetic engineering. For maximum benefit to LDCs, the application of genetic

engineering should focus on problems for which solutions are, so far, not available from traditional approaches. Such problems have been identified in a joint study of IRRI and the Rockefeller Foundation (Khush and Toenniessen 1991). We have focused our research on a number of the priority problems identified by IRRI/RF, namely finding resistance to blast (caused by *M. grisea*), sheath blight (caused by *Rhizoctonia solani*), yellow stem borer (*Scirpophaga incertulas*), and rice tungro bacilliform virus (RTBV). We are also working to reduce health problems caused by malnutrition such as vitamin A deficiency, which affects 134 million children. For all of these problems, there are, to date, no conventional solutions.

Fungal resistance

The main fungal pathogens of rice are *M. grisea* and *R. solani,* which cause blast and sheath blight, respectively. In most parts of a plant, the natural defense mechanism consists of a number of genes that are induced to action upon infection. In some parts of a plant, some of these genes are expressed constitutively. A variety of such genes has been identified. In most cases, their precise mode of action is not understood, but the most obvious functions of the encoded proteins allow some deductions to be made. Defense genes encode 1) hydrolases (chitinases and glucanases) that are thought to act by digesting fungal cell walls and/or by producing elicitors from fungal or plant cell walls that induce the plant's defense reaction; 2) ribosome-inactivating proteins (Rips), which may inactivate fungal ribosomes; 3) proteins that alter the plant's cell wall; 4) proteins that may detoxify compounds present in the infected cell; 5) proteins that may interfere with fungal or cellular membrane stability (thaumatin-like proteins, thionins, etc.); 6) proteins that are involved in the synthesis of antifungal compounds, 7) inhibitors of fungal proteases, and 8) a number of proteins with even less well-known functions (Linthorst 1991).

Antifungal activities of some of these proteins have been proven using in vitro assays in microinjected plant cells and recently in transgenic plants. In a variety of transgenic dicot plants, constitutive expression of a single defense gene strengthened the plant's defense against fungal infection (Alexander et al 1991, Broglie et al 1991, Hain et al 1993, Jach et al 1992, Logeman et al 1992). So far, only a few defense genes have been transformed in plants, particularly monocot plants. Only in one case has the effect of more than one gene in a plant been analyzed (Zhu et al 1994). In this case, a synergistic effect between a chitinase and a glucanase in transgenic tobacco was observed, similar to that found in in vitro experiments (Leah et al 1991).

We have started to transform rice with a number of putative defense genes. These genes include different chitinases from bean (Broglie et al 1989), rice (Huang et al 1991), and barley (Leah et al 1991); b-1,3-glucanases from tobacco (Ohme-Takagi and Shinshi 1990) and barley (Leah et al 1991); a ribosome-inactivating protein from barley (Leah et al 1991); and an osmotin-like protein (AP24) from tobacco (Singh et al 1989). Several genes that are induced in a defense reaction against powdery mildew in wheat have also been cloned into monocot gene expression constructs: a peroxidase, a glutathione-S-transferase, a thaumatin-like protein, and a putative cell wall-associated

protein (Dudler et al 1991, Rebman et al 1991). Similarly, thionins from barley (Bohlmann 1994) and an antimicrobial peptide from onion (Terras et al 1995) were cloned. In in vitro assays, the antimicrobial activity of the onion protein against *M. grisea, R. solani,* and the bacterial rice pathogen *Xanthomonas oryzae* was superior to 13 other antimicrobial proteins. In the constructed genes, expression is under the control of the CaMV35S promoter, enhanced by enhancer duplication or by intron insertion. For some coding regions, expression cassettes with different promoters have been produced. This allows for different expression characteristics and also for a later combination of different transgenes by crossing without complications of gene silencing due to sequence duplications. We have also produced some multigene expression cassettes in which a chitinase gene is combined either with a glucanase, a Rip, or a thaumatin-like protein gene.

These constructs are transformed into rice by particle bombardment to either calli derived from the scutella of immature embryos or to fresh cell suspensions. We use mainly japonica rice lines such as Taipei 309 or Kinuhikari, but also use other lines including Chinsurah Boro II and several Chinese varieties. Transgenic plants have been obtained expressing a rice chitinase that showed some resistance against *R. solani* (Lin et al 1995). Other plants containing barley chitinase, glucanase, and Rip under control of a dicot promoter have been obtained but not yet analyzed for gene expression levels and antifungal effects. Similarly, plants containing genes coding for a wheat peroxidase and a barley Rip under control of an intron-enhanced CaMV35S promoter have been generated and are currently being analyzed. Generation of transgenic plants with the other constructs described above is in progress.

Insect resistance

Among the wide range of insect pests affecting rice, the stem borers are generally considered to be the most destructive ones. In Asia, the yellow stem borer (*Scirpophaga incertulas*) and the striped stem borer (*Chilo suppressalis*) are responsible for a steady annual damage to the rice crop of 5-10%, with occasional outbreaks of up to 60% (Pathak and Khan 1994). Before penetrating the stem, stem borer larvae remain only for a short time on the outer surface of the rice plant. This makes chemical control very difficult and ineffective.

Despite the fact that more than 30,000 rice varieties have been screened for stem borer resistance, genes imparting sufficient levels of resistance have not been found and none of the rice varieties developed so far have more than a moderate level of resistance (Khan et al 1991).

An attractive alternative is producing proteins with insecticidal activity in the rice plant itself. The soil bacterium *Bacillus thuringiensis* (*Bt*) offers a promising range of genes that encode for specific endotoxins with insecticidal activity. We transformed indica rice breeding line IR58 with a synthetic version of the crylA(b) gene from *Bt* via particle bombardment. Southern analysis from R_0, R_1, and R_2 generation plants demonstrated the stable integration of this gene into the genome of IR58. Inheritance of the transgene from the R_0 to the R_1 was determined by DNA dot

blot analysis of 50 selfed R_1 plants. A 3:1 segregation ratio was found, indicating that the transgene was stably inherited and inserted in a single locus on one chromosome.

The activity of the transgene was determined by enzyme-linked immuno-sorbent assay, where expression levels of up to 84 ng *Bt* protein mg^{-1} of total soluble protein were found in the leaves of 12 to 14-wk-old plants. Western analysis showed the presence of a protein with the expected size of 60 kDa.

The insecticidal activity of plants expressing *cryIA(b)* was demonstrated by insect bioassays. Neonate larvae of both yellow stem borer and striped stem borer showed a mortality rate of up to 100% when feeding for 2-4 d on *Bt*-expressing leaves. Damage to these leaves was consistently limited, indicating early termination of feeding by the larvae. Larvae feeding on control leaves developed normally.

R_2 plants are currently being analyzed at IRRI where the insecticidal potential of whole plants or even small plant populations can be examined under more natural conditions. Furthermore, this material will be used for insect resistance studies and for evaluation of the role of *Bt*-expressing plants in integrated pest management.

To minimize the risk of developing resistant insect populations, not only one *Bt* gene should be expressed in a widely grown crop. The combination of different *Bt* genes with different binding characteristics or the combination with other insecticidal genes should be achieved. In addition to that, tissue- or stage-specific promoters should be used to reduce the selection pressure in the field.

We are therefore transforming different combinations of such promoters with different, independent insecticidal genes to rice variety Taipei 309 to identify the most suitable construct combinations. The best candidates for sustained insect control will then be used for transformation of other IRRI breeding lines.

Tungro virus resistance

Tungro is probably the most important viral disease of rice. No natural, durable resistance gene appears to exist in the rice gene pool. Genetically engineered resistance against a large number of plant RNA viruses has been achieved in recent years. In most cases, resistance was obtained by simply expressing a normal or mutated, single viral gene product in transgenic plants (Beachy 1993). A similar approach should also be feasible for RTBV, although the differences in the viral life cycle between the RNA viruses and the pararetrovirus RTBV—particularly the presence of RTBV as a DNA copy in the nucleus in contrast to the more labile cytoplasmic RNA copies of the RNA viruses—may demand different strategies and make protection more difficult. It is noteworthy that, so far, efficiently engineered resistance against a plant pararetrovirus has not been described.

We have produced a number of fertile, transgenic rice plants (Taipei 309 or Kinuhikari) containing genes that code for complete or mutated RTBV proteins. Southern blot analysis confirmed that these plants contain the RTBV open reading frames (ORFs) I, III, and IV and subfragments of ORF III comprising the different regions of the coat protein, the reverse transcription, and mutated versions of these proteins. The respective genes are under the control of a CaMV35S promoter or the

corresponding RTBV promoter. The activities of the promoters was enhanced by the presence of the first exon and parts of the intron of the RTBV ORF IV mRNA (Fütterer et a1 1994). Promoter activity was verified by the analysis of transgenic plants containing a b-glucuronidase gene under the control of the respective promoters. Gene expression analysis and analysis of antiviral effects of the RTBV transgenes are in progress. (For details see Klöti et al, these proceedings.)

Genetic engineering of provitamin A biosynthesis in rice endosperm

According to UNICEF statistics, more than 134 million children worldwide suffer from vitamin A deficiency (Humphrey et al 1992). Improved vitamin A nutrition would most likely prevent approximately 1-2 million deaths annually among children in the 1-4 yr age group. An additional 0.25-0.5 million deaths could be avoided if improved vitamin A nutrition could be achieved during later childhood. Improved vitamin A nutrition alone could, therefore, prevent 1.3-2.5 million out of nearly 8 million late infancy and preschool child deaths that occur annually in highest risk countries (West et al 1989).

Rice in its milled form—as it is consumed by most people in Southeast Asia—is characterized by the complete absence of provitamin A. The milled rice kernel consists exclusively of the endosperm; the embryo and the aleurone layer have been removed during processing of the rice grain.

The aim of this project is to initiate carotenoid biosynthesis in the rice endosperm tissue to increase the daily provitamin A uptake of people who rely predominantly on rice as a food source.

To provide the minimum requirements of relevant carotenoids to infants, and assuming rice as the sole dietary source, 1-2 mg b-carotene g^{-1} of uncooked rice would be needed in the endosperm (The Rockefeller Foundation 1993). This is roughly 1/4-1/2 of the amount produced in maize endosperm.

Our strategy is to produce transgenic rice varieties that contain either single carotenoid biosynthesis genes or several genes in combination. For this approach, we have chosen sequences encoding phytoene synthase and phytoene desaturase from daffodil (*Narcissus pseudonarcissus*) as a monocot plant. Phytoene synthase and phytoene desaturase cDNAs have been characterized recently (P. Beyer, University of Freiburg, Germany, 1995, unpubl. data).

These cDNAs have been combined with a constitutively expressed CaMV35S promoter, as well as with the endosperm-specific rice Gt1 glutelin promoter (Okita et al 1989). The gene products are targeting amyloplasts, using geranyl geranyl diphosphate (GGPP) that is available in this cellular compartment. As a selectable marker, a hygromycin phosphotransferase gene under the control of a CaMV35S promoter has been linked to these constructs. Subsequently, precultured immature embryos of the japonica rice variety Taipei 309 have been transformed. Two hundred and three transgenic plants have been recovered from these experiments of which 59 contained a DNA fragment integrated in the genome of the right size for cDNAs representing either the phytoene synthase, the phytoene desaturase, or both. Among

these transgenic plants, several lines have been identified that accumulate high levels of phytoene in the endosperm of the mature seed. Phytoene levels were as high as 0.74 mg phytoene g^{-1} dry weight. Correct integration and expression of the transgene were further confirmed by Western analysis and reverse transcriptase-polymerase chain reaction.

Discussion

Getting these novel traits into the hands of farmers

The Rockefeller Rice Biotechnology Program has entered a phase where transgenic rice varieties are becoming available. These varieties carry novel characters with potential for ensuring future food security in LDCs. Our laboratory has, so far, contributed increased tolerance for sheath blight and resistance to yellow stem borer and related insect pests. We expect to follow soon with resistance to tungro and, hopefully, incorporation of provitamin A in the endosperm.

For now, a transgenic rice plant with a novel trait is far from being in the hands of farmers. From the experience with transgenic varieties produced by companies for commercial purposes, it is evident that numerous additional problems begin after success in the laboratory. Carrying a genetic engineering success through to the marketplace requires substantial and persistent inputs in funding and manpower.

Since we consider IRRI the ideal partner for such necessary followup work, we have initiated joint Swiss Federal Institute of Technology (ETH)-IRRI-RF postdoctoral fellowships. J. Wünn, responsible for the insect resistance project, and A. Klöti, responsible for the tungro resistance project, have conducted their first joint release experiments in IRRI's containment greenhouse; others will follow.

Funding is scarce and we do not expect IRRI to carry the burden alone. We hope that the Rice Biotechnology Program will find solutions. It would not be wise to develop novel transgenic traits and then allow them to disappear somewhere or to bypass the present obstacles to working with transgenic plants by performing follow-up experiments in a country that does not comply with regulations.

Below, we present what we believe to be necessary follow-up work to support getting a novel trait into the hands of farmers. We use yellow stem borer-resistant IR58 as an example.

- Preparatory work to obtain permission.
- Glasshouse tests—effectiveness, stability, gene transfer, level of expression; effects on beneficial insects, etc.
- Field tests—preparatory work for obtaining permission, gene stability and expression, effects on insect pests and beneficial insects, effectiveness in integrated pest management schemes, pollen flow, spread of the transgene, horizontal gene transfer, yield, seed quality.
- Food tests—alterations in food characters, toxic compounds, allergenicity, feeding studies, receptor binding studies with gut membranes from potential "eaters" and "feeders".

- Prevention of resistance building—addition of *cryIA(c), cryIH,* and *cryII;* regulation by leaf sheath-specific signals; regulation by stage- and tissue-specific signals; addition of protease inhibitor genes.
- Improvement of stability of expression—transformation with *Agrobacterium* for simple integration pattern, deletion of methylation signals from constructs.
- Transfer of optimized genes to most advanced IRRI lines, e.g., IR72, IR64, "new plant type".
- Antibiotic-independent selection—development of selection schemes independent of antibiotic resistance genes.
- Biocontainment—transfer of the genes into the chloroplast genome to prevent spread of transgenes via pollen.
- Pyramiding of transgenic characters—combination with *cryIIIA* for defense against storage pests, with RTBV resistance, with pro-vitamin A endosperm, with sheath blight resistance.
- Additional field tests and food tests with the new varieties, etc.
- Biological and socioeconomic risk assessment.
- Involvement of the NGO opposition.
- Public awareness for consumer acceptance.

Comparable followup work like that outlined above will be necessary for every novel transgenic trait. It is obvious that this effort cannot be taken for any given transgenic rice, and that, therefore, some authority will have to take the responsibility to decide which case should be followed and, consequently, should be further funded.

Additional experiments in rice biotechnology at ETH

A series of additional approaches, which we consider important for food security and sustained rice production in LDCs, has been initiated in our laboratory. These include enhanced iron availability, improved phosphate uptake, apomictic stabilization of hybrid vigor, selectable gene-independent transformation, signal transduction analysis for rice root nodulation, N_2 fixation following the heterocyst concept, and N_2 fixation via operon transformation into plastids. For the first two to three projects, we expect applicable results within 3 yr. Successes with the remaining projects are too difficult to predict.

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Genetic engineering of rice for resistance to homopteran insect pests

J.A. Gatehouse, K. Powell, and H. Edmonds

The rice brown planthopper (BPH; *Nilaparvata lugens*) is a serious pest of rice crops throughout Asia, damaging plants both through its feeding behavior and by acting as a virus vector. Like many homopteran pests of crops, it is primarily a phloem feeder, abstracting sap via specially adapted mouthparts. An artificial diet bioassay system for this pest was developed to allow the effects of potentially insecticidal proteins to be assayed. Several lectins and oxidative enzymes were found to be toxic to BPH. Snowdrop (*Galanthus nivalis*) lectin (GNA) was selected for further study as it is nontoxic to higher animals. A cDNA encoding GNA was assembled into constructs for expression in transgenic plants, with the aim of producing transgenic rice plants that would express the foreign protein in their phloem sap and be resistant to BPH. Constitutive expression of GNA in model plant systems was shown to have deleterious effects on the development of lepidopteran and homopteran pest insects. Phloem-specific promoters for expressing GNA in transgenic rice were isolated and characterized with the aim of increasing the effectiveness and specificity of the protection against BPH. A construct containing the GNA coding sequence driven by the promoter from the rice sucrose synthase *RSs1* gene was tested in tobacco and transformed into rice. Transgenic rice plants containing this construct are currently being evaluated.

Sucking insects of the order Homoptera can cause serious damage to rice, both directly and by acting as vectors for plant pathogens. The major pests in this order are the rice brown planthopper (BPH, *Nilaparvata lugens*) and the rice green leafhopper (GLH, *Nephotettix* spp.). Both BPH and GLH are economically serious pests of rice and can be the major cause of crop losses. Control by chemical insecticides is incomplete and, in any case, too expensive for poor farmers. It also poses health and environmental risks. Biological control and especially breeding for resistance are attrac-

tive alternatives to chemicals, but both methods could be augmented by genetic engineering. Insects can rapidly adapt to become resistant to control measures, so it is essential to use a pluralistic approach. It is now widely recognized that genetic engineering of "exotic" resistance genes is a significant new approach that offers possible solutions within several years.

Most work on resistance of plants to sucking pests has concentrated on the role of semiochemicals and plant secondary metabolites as feeding deterrents. The feasibility of engineering transgenic plants to confer the ability to produce secondary metabolites has yet to be demonstrated, and the ability to do this on a routine basis for given secondary compounds is in the future due to the complexity and species-specificity of the biochemical pathways involved—although this approach is now being addressed (Hallahan et al 1992). For some insect pests, the expression of *Bacillus thuringiensis* (*Bt*) endotoxin genes in transgenic plants has been shown to be an effective means of control, although the long-term use of *Bt* may depend on devising suitable management strategies to delay the buildup of *Bt*-resistant insect populations. However, sucking insects are not amenable to control by *Bt* bacteria, or toxins, at present, since no reported strain of *Bt* is effective against homopterans. To tackle the problem of producing transgenic plants with resistance to sucking pests, it was necessary to go back to insect bioassays. Products of genes that could be obtained reasonably easily, and which could be expressed in transgenic plants using existing technology, were assayed for their effect in artificial diet bioassay.

We have shown that certain plant lectins and enzymes have insecticidal properties toward BPH and other homopteran insect pests, and have assembled the necessary elements of a technology for producing transgenic rice plants with engineered insect resistance. The main emphasis in the program has been the production of rice with resistance to BPH, as this was identified as the pest most difficult to control using insecticides, to which it readily developed resistance. Although the use of biological control measures and the breeding of new resistant rice varieties have prevented BPH from becoming an uncontrollable pest, it remains a serious problem to rice growers throughout Asia.

Identification of insecticidal proteins

Insect bioassays

BPH feeds exclusively on the phloem and xylem saps of rice plants, with the phloem sap only providing a source of nutrients. An artificial diet system for this insect must thus mimic its natural foodstuff. A liquid diet formulation, containing sucrose, amino acids, and vitamins is used; portions of this diet are enclosed in parafilm sachets (which can be put under pressure, to simulate the normal phloem pressure in the plant), and the insects feed by probing the parafilm and sucking the diet in the same way in which they normally probe plant tissues and suck phloem sap. The diet allows the insects to develop through several nymphal stages to adults quite successfully with survivals of more than 50%, but is not suitable for rearing successive generations of insects (Powell et al 1993).

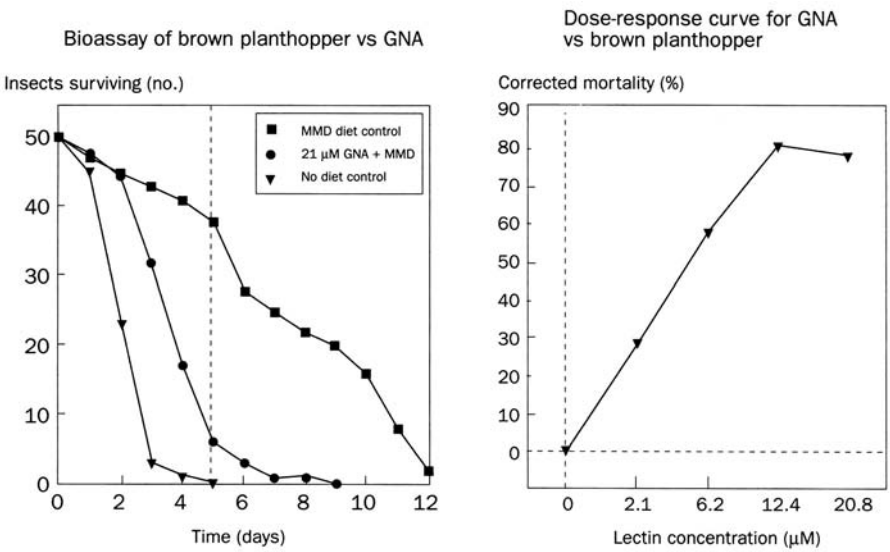

Fig. 1. Bioassay of BPH on artificial diets containing snowdrop lectin (GNA).

Bioassays of potentially insecticidal proteins are done by incorporation into the liquid diet, and the survival of insects is compared on control diet, diet + treatment, and a "no diet" control where the insects are given moisture but no nutrients. The "no diet" control allows the corrected mortality for the treatment to be calculated (see Fig. 1).

At the time when mortality on "no diet" = 100%, corrected mortality %/100 = 1 - {([survival on control] - [survival on treatment])/ survival on control}.

Standard statistical methods can be used to evaluate the significance of corrected mortality figures; however, these techniques are relatively insensitive and analysis of the survival vs time curves for treatments and controls can be used to identify more subtle effects on insect development. Some typical results for the insect bioassay are shown in Figure 1, which demonstrates that an effective insecticidal protein can be expected to give corrected mortality figures in excess of 50%.

Proteins with insecticidal properties toward BPH

Assay of a number of plant and other proteins against BPH in the bioassay system described above showed that the presence of an inert protein, such as ovalbumin, had no deleterious effects on survival, but some biologically active proteins were toxic (Powell et al 1993). Inhibitors of digestive enzymes, such as cowpea trypsin inhibitor and wheat a-amylase inhibitor, had no effect, as would have been expected on the basis that sap-sucking homopteran insects do not rely on protein or starch digestion for nutrients. On the other hand, two types of protein did show deleterious effects:

lectins and oxidative enzymes such as lipoxidase, and, to a lesser effect, polyphenol oxidase. The toxicity of lectins varied considerably from those that had very little effect on the corrected mortality at the concentration used (0.1% w/v in the liquid diet), e.g., the lectin from garden pea, to those that gave corrected mortality values of nearly 90%, e.g., the lectins from wheatgerm and from snowdrop.

The results of many similar bioassays have suggested that BPH is generally sensitive toward insecticidal proteins, so that results obtained with this species must be extended with caution to other insect pests. Nevertheless, assays with GLH showed that the lectins from snowdrop and wheatgerm were both strongly toxic toward this species also, although it was not sensitive to lipoxidase.

Toxicity of lectins

The bases for the toxicity of lectins toward animals, in general, are still the subject of research. In higher animals, binding of lectins to gut epithelial cells is well demonstrated, and effects on the growth of gut tissues, particularly in terms of effects on the normal structures of villi, are well documented (Pusztai 1991). Certain lectins also show systemic effects by crossing the gut wall intact and passing into the circulatory system. An additional factor is the effects of lectins on the attachment of gut microflora to the gut epithelium, which can lead to breakdown of the gut wall and bacterial invasion of gut tissues. All these effects are thought to be mediated through the carbohydrate-binding properties of lectins, which lead to interactions with cell surface glycoproteins, both on gut epithelial cells and on bacteria.

The situation in insects is less clear. Binding of lectins to gut surfaces in insects has been observed by several researchers, but the results of this binding are not characterized. The toxicity of wheatgerm lectin toward a range of insects and its specificity of binding toward chitin have led to suggestions that the peritrophic membrane, a thin porous chitin layer that covers the gut epithelium in many insects, is the target of its action. However, other chitin-binding lectins are not toxic and lectins with other carbohydrate-binding specificities are toxic (Powell et al 1993, 1995b).

The toxicity of many lectins toward higher animals limits their usefulness in the protection of crop plants that are intended for consumption. In particular, wheatgerm lectin, which is strongly insecticidal, is also significantly toxic to mammals and other higher animals. However, certain lectins, in particular those from the plant family Amaryllidaceae, show low or no toxicity toward higher animals, but are toxic to insects. This type of lectin is exemplified by snowdrop (*Galanthus nivalis*) lectin (GNA), which had been identified as toxic to BPH. GNA was thus selected as the "best candidate" gene for engineering of BPH-resistant rice.

Snowdrop (*Galanthus nivalis*) lectin (GNA)

Snowdrop lectin (like other Amaryllidaceae lectins) binds specifically to mannose residues in a -l,3 or a -l,6 glycosidic linkages. The protein is a tetramer of polypeptides of M_r approx. 11,600, and is accumulated in snowdrop bulbs, and to a lesser extent in other tissues. It is encoded by a multigene family and many isomeric forms are present in snowdrop tissues. The polypeptides are synthesized as preproproteins and are sub-

ject to both cotranslational N-terminal processing and post-translational C-terminal processing. GNA protein and its encoding genes have been extensively characterized in the laboratory of W. Peumans and E. van Damme (Leuven, Belgium), from where a cDNA clone containing the complete coding sequence of a GNA isoform was obtained (van Damme et al 1991).

To generate a subclone containing only the GNA coding sequence, with convenient restriction sites for construct assembly, polymerase chain reaction (PCR) amplification of the desired sequence, with appropriate primers containing added restriction sites, was carried out. PCR products were cloned into pUC vectors, and selected clones were checked for PCR errors by DNA sequencing. The clone selected was identical in sequence to the reported GNA coding sequence, apart from one silent base change.

Effects of GNA on BPH

Further assays carried out with BPH were used to estimate the lower limit of effectiveness of GNA in an artificial diet. At 0.05% (w/v), the protein is as effective as at 0.1 %, but below this level effectiveness declines with an LC_{50} of approximately 0.02%; this is equivalent to a protein concentration of approximately 6 mM (Fig. 1). Elevated levels of GNA do not increase insect mortality beyond approximately 90% in this system, although this reflects the way in which mortality is measured, rather than an ability of the insect to survive the treatment, since all insects on the GNA diets (at GNA concentrations of $\geq^3$ 0.05%) die within 7-10 d. Effects on insect survival are significant at the lowest levels tested (0.01% w/v; approx. 3 mM).

GNA has antifeedant properties toward BPH. This has been shown in two ways: first, indirectly, by measuring the production of liquid excreta, or honeydew, as an indication of food ingested (Powell et a1 1995a); and secondly, directly, by examining the feeding behavior of the insect by the electrical penetration graph method. Honeydew production suggests that insects exposed to GNA at 0.1% (w/v) ingest virtually no diet over an initial 24-h period, and a reduced amount compared with control diet for the next 12 h. After this, ingestion approaches control levels. Similarly, the feeding behavior data also show a failure to ingest a diet containing GNA during a 4-h exposure period; whereas control insects spend approximately 25% of their time ingesting diet, of which approximately 70% is spent in actively sucking in the liquid diet, GNA-fed insects spend only 3%, and no active ingestion was observed. The decreased palatability of diet containing GNA seems unlikely to account for the high levels of mortality observed with this lectin; a more likely explanation is that once the insect is forced to feed on GNA-containing diet, a toxic effect then manifests itself. The shape of the survival curve with GNA-containing diet also suggests that an initial lag phase (when the insect is deterred from feeding) in the toxic effect of GNA is present.

An antifeedant effect might be viewed as disadvantageous in transgenic plants, since it will increase movement of hoppers and probing actions, and could thus increase the possibility of virus transmission. However, virus transmission by homopterans is not instantaneous; the time for transfer varies with virus, vector, and

host, but many viruses need prolonged feeding for efficient transfer to take place, which would be prevented by an antifeedant effect.

Expression of insecticidal proteins in transgenic plants

Phloem-specific promoters

Although the constitutive CaMV35S gene promoter, used in many constructs for expression in transgenic plants, is expressed efficiently in phloem tissue, it was felt desirable to identify promoters that would show phloem-specific expression for use in producing rice with BPH resistance. Use of such promoters could give higher levels of expression in the phloem and would minimize exposure of nontarget insects and other consumers of the plant material to GNA. Use of an endogenous phloem-specific promoter was decided on. Protein concentrations in phloem of different plant species have been estimated at 0.03-0.2% (w/v) in most species or as much as 10% in cucurbits, and thus the lower limits of effectiveness of GNA lie within achievable expression levels.

Sucrose synthase is known to be specific to phloem tissue and studies on the gene that encodes the enzyme from maize had suggested that the promoter was active and phloem-specific. A gene, designated *RSs1,* corresponding to the maize *Sh1* locus was isolated from rice, and fully characterized and sequenced (Wang et al 1992). The promoter sequence from this gene has been fused to the glucuronidase (*gus*) gene coding sequence in a promoter-reporter gene construct, and transformed into tobacco plants by standard techniques. Histochemical staining of the transformed plants with X-glc has shown that the *RSs1* promoter fragment used (approx. 1.2 kb of 5' flanking sequence, the transcription start, the first intron and the translation start) is sufficient to direct phloem-specific expression of *gus* in transgenic tobacco plants. Expression is observed in phloem sieve tubes and companion cells in roots, stems, petioles, and leaves and is not seen in mesophyll cells or other vascular tissues. The phloem-specific expression directed by this promoter is thus confirmed (Shi et al 1994). Expression levels observed in tobacco were low due to the presence of the first intron of the *RSs1* gene in the 5' untranslated sequence between the transcription start and the translation start.

An alternative strategy to isolate a phloem-specific promoter was also followed by attempting to isolate the promoter from a gene encoding one of the phloem-specific P-proteins. An advantage of these genes is that their products are not selectively accumulated in developing seeds, unlike sucrose synthase. A gene encoding a P-protein was isolated by a protein to cDNA to gene route. Relatively large amounts of phloem exudate from *Cucurbita maxima* (pumpkin) plants were collected and used as a source for purification of the chitin-binding phloem lectin protein designated PP2, a major protein in phloem sap. The partially purified protein was run on SDS-polyacrylamide gel electrophoresis and the most abundant polypeptide was blotted onto PVDF membrane and subjected to protein sequencing. This polypeptide was found to have a blocked N-terminus so, to obtain useful sequence information, the separated polypeptide was cleaved in the gel slice by CNBr and the resulting frag-

ments were purified by reverse phase high-performance liquid chromatography and sequenced. Two fragments were identified. Amino acid sequence data from these polypeptides were used to generate oligonucleotide sequences of lowest redundancy. These were used as probes on a Northern blot of RNA isolated from different organs of developing pumpkin seedlings. Hybridization was observed to an mRNA species of approx. 0.9 kb in RNA from hypocotyls and this tissue was used as a source for cDNA library construction. The library, in the 1 phage vector ZAPII (Stratagene), was screened with the labeled oligonucleotide and positive plaques were purified. Three clones were fully sequenced. These proved to contain identical PP2 lectin-encoding sequences (Wang et al 1994). The sequence predicted by these clones was in complete agreement with the 78 residues of amino acid sequence determined for the PP2 protein, confirming their identity.

The PP2 cDNA was used as a probe to screen a cucurbit genomic library in the vector 1 EMBL3 to obtain a gene encoding the PP2 protein. The gene was fully characterized and sequenced. The sequence of the coding region is given in Figure 2. The predicted amino acid sequence in this gene was not identical to that predicted by the cDNA, but encoded a PP2-like protein. PCR of the *C. maxima* seedling cDNA library using primers specific for the gene sequence amplified a fragment of the expected size (data not presented). This result suggests that a cDNA corresponding to the gene is present in the library, and that the gene is highly likely to be expressed. However, when the promoter region was fused to a *gus* reporter gene and the construct was transformed into tobacco plants, no expression of the reporter gene was observed. The reasons for this failure to observe expression are under investigation.

The *RSs1* gene had provided a viable phloem-specific promoter, which was used in subsequent constructs.

Gene constructs

The GNA coding sequence was assembled into two constructs for expression in transgenic plants. A standard transcriptional fusion with the CaMV35S promoter was made for expression in model systems in experiments to "prove" the technology, and a translational fusion between the *RSs1* promoter and the GNA sequence, which introduced the translational start of sucrose synthase and some ''linker" amino acids onto the N-terminus of the GNA precursor was made for expression in both model systems and rice.

Testing constructs in a model plant system

Expression in phloem. The *RSs1 -GNA* construct was introduced into tobacco via standard *Agrobacterium tumefaciens* transformation procedures. The phloem-specific expression pattern observed with the reporter gene *gus* driven by the *RSs1* promoter was also evident with GNA in the transformed plants. GNA accumulation was determined by immunohistochemical staining (Shi et al 1994) and the presence of GNA in the phloem vessels and companion cells was demonstrated. However, expression levels were indicated to be low, as was found when *gus* was expressed from the *RSs1* promoter in tobacco (see above). The presence of GNA in the phloem sap of these plants

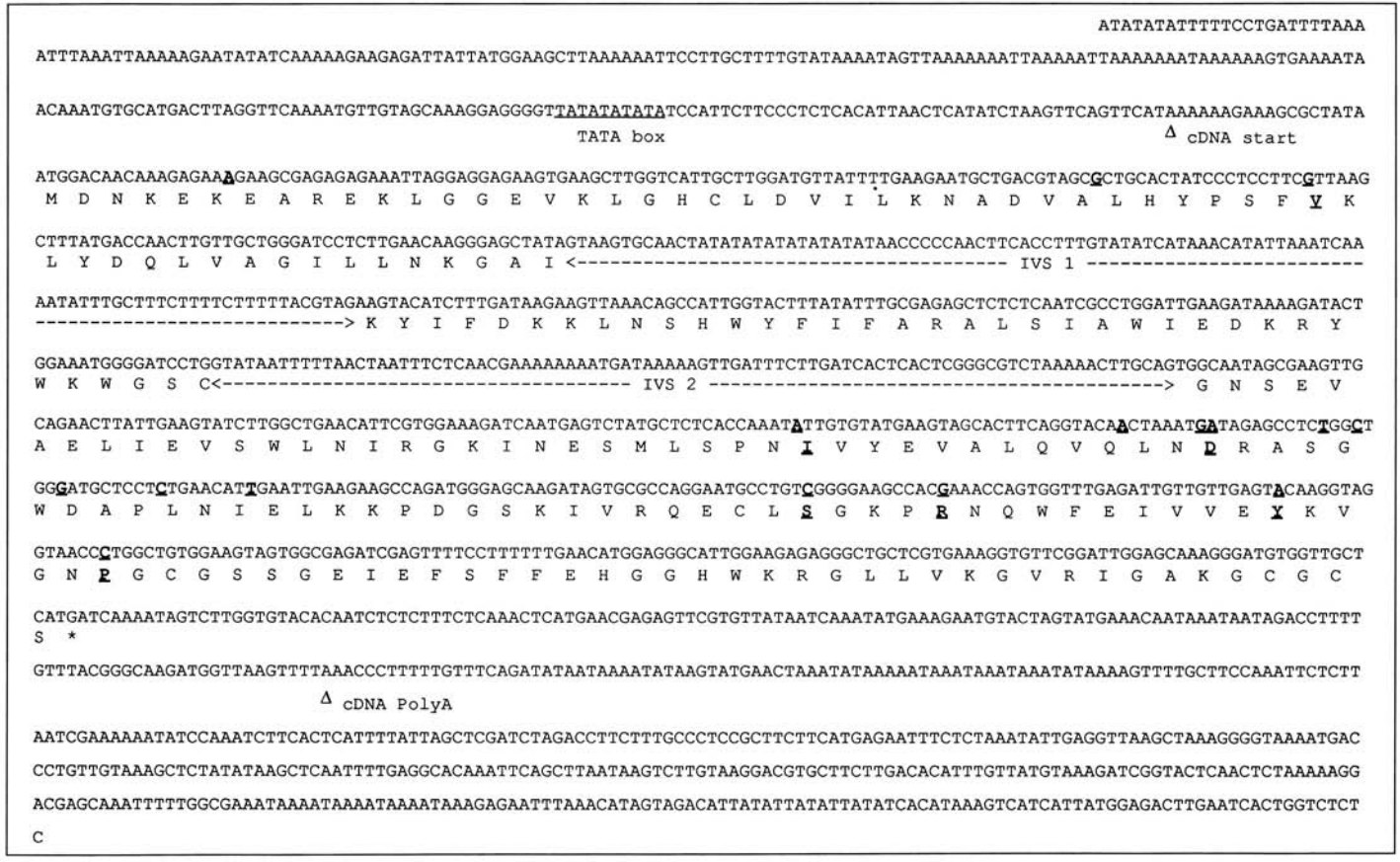

Fig. 2. Sequence of the gene encoding a PP2-like protein from *Cucurbita maxima*. Sequence differences in coding sequence from the cDNA reported by Wang et al (1994) are underlined and in boldface.

was also shown by immunoassay. Peach-potato aphids (*Myzus persicae*) were fed on transgenic and control tobacco plants and the honeydew produced by the aphids was collected on filters. The filters were then processed as immunoblots. This showed the presence of GNA in the honeydew of the aphids feeding on the transgenic plants, but not the control plants. The experiment showed that it was possible to deliver the insecticidal gene product to a sucking insect pest by phloem-specific expression of its encoding gene and proved that the lectin had been transported into the phloem sap from its site of synthesis. The targeting information for this was assumed to have come from the intact GNA leader sequence in the translational fusion construct.

Insecticidal properties. The *CaMV-GNA* construct was introduced into potato using *A. tumefaciens*-based vector systems. Expression of GNA in transformants was measured by dot-blot immunoassay, and was estimated at 0.2-1.0% of total protein, depending on the transgenic line and tissue selected for assay. All work with potato was done on primary transformants, which were vegetatively propagated via shoot cuttings and tubers. Tissue blots showed the presence of GNA in all parts of the plant, but the protein was observed to be selectively accumulated in vascular tissue, and thus would be available to phloem-feeding insects. Potato lines expressing GNA from the CaMV promoter have been subjected to bioassay against lepidopteran and homopteran insect pests, both in the growth room and in the glasshouse. These assays have confirmed that GNA has insecticidal effects and have shown that these effects extend to insect species other than rice pests, although the high level of mortality observed with BPH is not duplicated with more polyphagous pests. Potato plants expressing GNA are protected against attack by larvae of *Lacanobia oleracea*, the tomato moth, with plant damage, larval survival, and larval biomass per plant all significantly reduced. These effects are seen both in growth room and glasshouse bioassays. More relevantly, GNA-expressing potato plants also show resistance to attack by a homopteran pest, the potato aphid *Aulacorthum solani.* In this case, no mortality of insects is observed, but the parthenogenetic production of nymphal offspring is affected, so that the normal population increase of the pest is slowed. Once again, these effects have been observed both in the growth room and in glasshouse trials. Preliminary results showing deleterious effects of GNA expression in transgenic tobacco plants or the peach-potato aphid have been reported (Hilder et al 1994).

These results confirm that GNA expression in transgenic plants is sufficient to confer protection against insect pests. although the degree of protection against polyphagous pests observed in potato is lower than would be expected (or desirable) for transgenic rice exposed to BPH.

Production of transgenic rice

Constructs as described above have been supplied to collaborators in the Rockefeller Foundation Rice Biotechnology Program, who have used the best existing technologies to produce transgenic rice. Both electroporation of protoplasts and the biolistic method, where immature embryos are bombarded, have been used successfully to produce transgenic rice. Details of these technologies are given elsewhere (Hall et al 1993).

Assay of transgenic rice

Putatively transgenic rice plantlets, at the stage where the plantlets have formed root systems, but are still under tissue culture conditions, can be tested for the presence of transgenes by polymerase chain reaction (PCR) on tissue samples. Leaf samples of 0.1 cm^2 can be tested successfully by this method. The technique has been used in Durham to test plantlets for the presence of the GNA gene. using appropriate primers (Fig. 3). To avoid false positive results, control samples must be processed with the

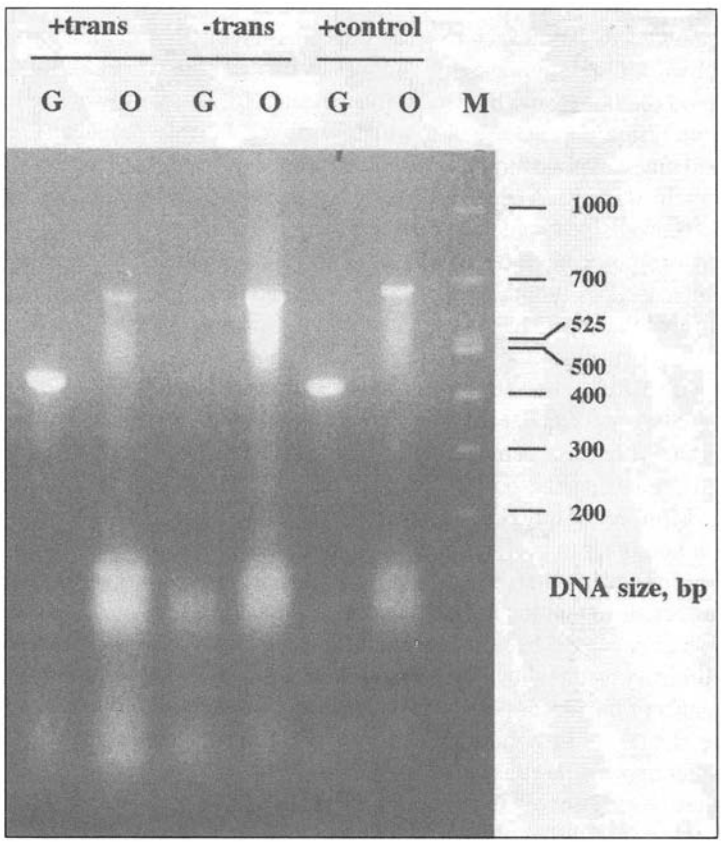

Fig. 3. Assay for presence of GNA coding sequence in putative transgenic rice plants by PCR. G = amplification with GNA primers (expected size of fragment 415 bp); O = amplification with oryzacystatin primers (internal control; expected size of fragment 680 bp); M = size marker calibration. "+ trans" is a transgenic plant containing the GNA coding sequence; "-trans" is a negative transgenic plant; "+control" is a positive control containing approx. 0.1 pg of GNA plasmid DNA. Putative transgenic rice plantlets supplied by M. Davey, University of Nottingham, UK.

putative transgenics; to avoid false negative results, both a control sample spiked with amounts of GNA plasmid (0.1 pg), and amplification of an internal control in the putative transgenics is necessary. The internal control should be a single-copy endogenous rice gene. Amplification of this sequence from the DNA samples extracted from putative transgenic plantlets demonstrates that amplification would work successfully on the transgene as well. Transgenic rice plantlets that contain the GNA transgene have been obtained.

Assay for protein expression using dot-blot, or better, Western blotting techniques using antibodies raised against purified GNA, can be done on rooted plantlets. False positive results have been obtained in dot-blots of rice extracts when reacted with anti-GNA antibodies, and thus Western blotting is necessary to confirm GNA expression. Rice plants transformed with the *RSs1-GNA* construct described above, produced by Prof. Hodges' group at Purdue University, have been assayed for expression of GNA by Western blotting both at Purdue and Durham. Expression levels in the progeny of plants assayed at Durham, raised from seed supplied by Prof. Hodges, have been very low, but some primary transformant plants at Purdue show better expression of levels (T. Hodges, pers. commun.). These plants are being allowed to set seed to provide material for insect bioassays with BPH.

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Construction of transgenic rice plants resistant to rice yellow stunt virus, a plant rhabdovirus

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W.-S. Qin, L. Zhang, S.-Y. Cao, W.-Z. Tian, and L.-C. Li

Rice yellow stunt is an important disease found in China and some other Asian countries. The pathogen of the disease was identified as a rhabdovirus, which contains a single-stranded, negative-sense RNA molecule as its genome. We have cloned and completely determined the nucleotide sequence of the gene coding for the viral nucleocapsid protein, a structural protein associated with the viral genome. To develop a novel strategy against the infection of plant rhabdoviruses, a cDNA copy of the rice yellow stunt virus (RYSV) nucleocapsid protein gene, or a reading frame-shift mutant of the gene has been inserted into a rice expression vector under the control of the rice Act1 promoter. The constructs have been introduced into rice genomes of two RYSV-susceptible cultivars, Xiushui 11 and Bing 88, both japonicas, by an improved biolistic method. Numerous rice plants have been regenerated. Virus resistance assays were performed on 23 plants from seven independent primary transgenic plant lines by using viruliferous rice leafhoppers. The preliminary results showed that plants transformed with both constructs displayed resistance to the RYSV infection, indicating that this resistance might be mediated by the RNA transcripts of the nucleocapsid protein gene.

Rice yellow stunt disease was first observed in China in 1957 and became epidemic during the 1960s and 1970s in eastern and southern China. The disease pathogen was identified as a bullet-shaped virus (rice yellow stunt virus, RYSV) and was taxonomically included in the genus *Nucleorhabdovirus.* Conventional breeding of major rice cultivars resistant to RYSV has not been successful.

Genetically engineered resistance to plant virus infection has been obtained by transformation of plants with viral genes or gene fragments, including those coding for structural proteins, replicases, and movement proteins (Beachy et al 1990, Wilson 1993). More than 30 viruses from at least 10 different taxonomic groups have been

targeted. Most of the viruses involved contain positive-sense single-stranded RNA genome. Only two examples were reported from viruses with ambisense coding strategies, tomato spotted wilt virus (Gielen et al 1991) and rice stripe virus (Hayakawa et al 1992). For DNA viruses, a coat protein-mediated resistance strategy has been described to combat tomato yellow leaf curl virus (Kunik et al 1994). Genetically engineered resistance to plant rhabdoviruses has not been reported yet. A plant rhabdovirus has a nonsegmented, single-stranded RNA genome of negative polarity, which contains, usually, five open reading frames (ORFs) in the order 3' N-P-M-G-L 5', where N encodes the nucleoprotein, P the polymerase-associated protein, M the matrix protein, G the glycoprotein, and L the polymerase. In some rhabdoviruses, a sixth ORF that encodes a nonstructural protein of unknown function has been observed. Besides, there are leader sequences located at the 3' and 5' end of the genomic RNA. Fang et al (1992) reported that the RYSV genome contains three major structural proteins: G (84 kDa), N (60 kDa), and M (31 kDa), and two minor proteins: L (170 kDa) NS (corresponding to P protein, 42 kDa). Genes coding for the RYSV N protein, the 3' leader, NS protein, and L protein have been cloned and sequenced (Fang et a1 1994, and unpubl. data). The availability of the RYSV cDNA clones allows us to test the ability of selected RYSV genes in conferring virus resistance in rice plants using a genetic engineering approach. We report the production of transgenic rice plants expressing the RYSV N gene and the preliminary results of virus resistance assays on the T_0 generation of the transgenic plant lines.

Results and discussion

Construction of the full-length N gene and its reading frame-shift mutant

A cDNA clone, named pN2, was obtained by screening RYSV genomic cDNA library; pN2 was completely sequenced. It covers the entire region of the N gene except for 60 bp at the 5' end (messenger sense) and its sequence extends for 418 bp into the downstream region containing the NS gene (Fang et al 1994). The remainder of the N gene and the 3' leader sequence were cloned by 3' RACE on in vitro polyadenylated RYSV genomic RNA, resulting in clone pG3. The complete N gene (*pN*) was constructed by combination of pN2 and pG3 through an overlapping restriction site, *Sph*I at the 5' region of the N gene. To clarify whether resistance in the plant would be based on the presence of the N protein or just mediated by the N gene transcript, a reading frame-shift mutation was introduced first in the pG3 by removing 4 bp at the *Pst*I site (the 59th to 62nd bp downstream of the initiation codon ATG), resulting in pG3'. The frame-shift mutant of the N gene (*pN'*) was then constructed by combining pN2 and pG3' in a way similar to generating pN. In the *pN'* mutant, an ORF starting from the authentic initiation codon only extends to the 28th codon followed by a termination codon TAG. The next in-frame ATG occurs at the 75th codon of the wild type N gene and would allow the synthesis of a polypeptide of 447 amino acid residues. It is very unlikely that the putative polypeptide would be produced.

The coding capacity of the constructed N and N' genes was verified by the bacterial expression system. A 1.58-kb *Ase*I-*Nsi*I fragment, spanning the entire N or N'

gene region with 5 bp of the 5' untranslated sequence and 15 bp of the 3' untranslated region, was inserted into an *E. coli* thermoinducible expression vector pBV220 under the control of P_RP_L promoter (Zhang et al 1990). Western blot analyses of soluble bacterial proteins probed with the RYSV N protein specific-antisera revealed that about a 58-kDa band, corresponding to the size of the N protein, was obtained from the clone containing the N gene and no band of the expected size of the N protein was found in the extract of the clone containing the N' gene. A 29-kDa band was observed in extracts from both clones that contain N and N' genes, indicating that there might be translational events occurring in *E. coli* from the downstream in-frame ATG. We have no evidence if this downstream initiation of the N polypeptide synthesis happens in plants.

Transformation of rice with N or N' genes

A rice expression vector pDA12 was constructed in our laboratory. It contains the pBR322-derived ampicillin resistance gene and an origin of replication, a 35s-HPT-NOS 3' selection marker for plant transformation, and the rice Actl promoter/Act1 intron1-polylinker-NOS 3' cassette derived from pActl.cas. The N gene or N' gene was inserted into the polylinker region of pDA12 downstream of the Act1 promoter/ Actl intron1, resulting in pDA12N or pDA12N', respectively.

After introducing pDA12N or pDA12N' into the rice cells, the N gene is expected to be transcribed and translated, while the N' gene would only be transcribed and no intact N protein would be synthesized in transformed rice plants.

Two RYSV-susceptible japonica rice cultivars, Xiushui 11 and Bing 88, were transformed with pDA12N or pDA12N' by bombarding the immature embryos or mature embryogenic calli with BioRad's Biolistic PDS-1000/He System (Li et al 1993). Transformants were selected in a medium containing hygromycin (50 mg L^{-1}) and plants were regenerated on the regeneration medium containing hygromycin (50 mg L^{-1}). Regenerated plants were then planted in soil in plastic pots.

Virus resistance assay

Twenty-three rice plants from seven independent transgenic lines were selected for a virus resistance assay conducted in an insect-free greenhouse using viruliferous rice leafhoppers as inoculators. Among the 23 plants, seven plants were derived from three transgenic lines transformed with pDA12N and 16 plants from four transgenic lines transformed with pDA12N'. Integration of the N gene or N' gene in the rice genome was analyzed by polymerase chain reaction (PCR) assay on rice DNAs using a pair of primers specific for the RYSV N gene. The PCR results show that 16 plants from six transgenic lines (three pDA12N and three pDA12N') were positive and seven plants from one pDA12N'-derived plant line were negative. Further analysis of transgene expression in these plants has not been done yet.

In the virus challenge experiments, 14 of 16 PCR-positive plants showed no symptoms while two became infected (12.5% infection rate). More specifically, one of seven pDA12N-derived plants was infected and one of nine pDA12N'-derived plants developed symptoms, showing approximately the same protection rate for either

pDA12N- or pDAl2N'-derived plants. On the other hand. five of seven PCR-negative plants developed symptoms (71% infection rate), close to that of the untransformed rice plants, which had an 80-90% infection rate.

The virus-resistant characteristics of the tested rice plants were correlated with the data of DAS-ELISA, which implicates the multiplication of RYSV in these plants (data not shown).

Conclusion

For biosafety reasons, RNA-mediated protection to virus infection is safer and more preferable. The preliminary virus resistance assay has shown that the RYSV N gene sequence could provide rice plants with resistance to RYSV infection. The protection rate for the transgenic plants was at the same level, regardless of whether they were transformed with the translationally functional N gene or the translationally defective N' gene. We thus postulate that the engineered resistance to RYSV in rice plants might be mediated by RYSV N gene transcripts. Viral RNA-mediated resistance has been reported for several other plant viruses, including coat protein genes of potato leaf roll luteovims (Kawchuk et al 1991), coat protein genes of tobacco etch virus (Lindbo and Dougherty 1992), and potato virus Y (van der Vlugt et al 1992)—both potyviruses—and nucleocapsid protein genes of tomato spotted wilt tospovirus (de Haan 1993, Pang et al 1993).

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FLP/*FRT*-mediated manipulation of transgenes in the plant genome

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Site-specific recombinases catalyze recombination reactions between two nucleotide sequences referred to as recombination sites. If such recombination sites are integrated into genomic DNA, depending on the orientation of these sites relative to each other, i.e., direct or inverted, the intervening genomic DNA sequence can be either inverted or excised by certain site-specific recombinases. If the recombination sites are on two different chromosomes, some of the recombinases can catalyze the exchange of chromosomal fragments. Thus, site-specific recombination reactions have the potential to have substantial practical applications in genetic engineering. We have investigated one of these site-specific recombinases, the FLP/*FRT* system from the 2-µm plasmid of *Saccharomyces cerevisiae,* with respect to its function in cells of monocots, especially maize and rice. We have shown that the FLP recombinase can recognize and recombine *FRT* sites located in a plasmid molecule in maize or rice protoplasts, and if the *FRT* sites are on two different plasmids, they can be recombined by FLP. A recombinase test target vector containing *FRT* sites (pUFNeoFmG), the complete sequence being ubiquitin promoter/*FRT* site/neo gene/a mutated *FRT* site/*gusA* gene, was introduced into protoplasts. Calli were selected on kanamycin and suspension cultures were made. Protoplasts of these suspension cultures were treated with a FLP expression vector (pUbiFLP) containing the maize ubiquitin promoter driving FLP. b-glucuronidase (GUS) activity resulting from the recombination-mediated excision of *neo* (resulting in activation of *gusA)* was observed in 3-4% of all treated protoplasts. Southern blot analyses of putatively retransformed calli (GUS^+) indicated that all of the calli contained the product of the site-specific recombination reaction. Protoplasts from the same suspension culture were also treated with pUbiFLP and pHyg (containing the hygromycin resistance gene), and calli were selected on hygromycin. A recombined *FRT* site in the genome of

one of the selected GUS$^+$ callus lines was sequenced, which confirmed that the recombination reaction indeed produced the chimeric *FRT/FRT*m site. To eliminate the second retransformation step in the genomic DNA excision procedure, the target vector, pUFNeoFmG, was cointroduced into maize protoplasts together with the *FLP* gene driven by a soybean heat-shock promoter. *FLP* expression was activated (based on Western blots) in transgenic calli by a heat-shock treatment. Some of these heat-shocked transgenic calli had also lost NPTII activity and gained GUS activity. Southern blot analyses indicated that the expected recombination product had been produced; however, not all the *neo* genes had been excised from the genome. This was due to the initial integration of multiple copies of the target vector (intact or fragmented) into the genome.

Manipulations of transgenes after incorporation into the genome of a host have both practical and fundamental applications in genetic engineering. In plants, it would be desirable, for example, to remove antibiotic resistance genes or herbicide resistance genes after they have been used for their primary purpose, i.e., following selection of transformed cells or after providing resistance to specific herbicides, respectively. Other applications utilizing transgene excisions or gene inversions could be useful for controlling developmental processes. Such manipulations or modifications of transgenes in the plant genome will soon be possible using certain DNA site-specific recombinase systems (Odell and Russell 1994, Ow and Medberry 1995).

Site-specific recombinases are enzymes that recognize short DNA sequences, and in the presence of two such recombination sites, they catalyze the recombination of DNA strands. With some of these recombinases, the orientation of the recognition sites relative to each other (i.e., direct or inverted) affects whether the recombination results in the intervening strand of DNA being either excised or inverted. Some site-specific recombinase systems do not require other factors for their function; thus, they represent relatively simple systems that are capable of functioning accurately in heterologous systems.

Examples of site-specific recombination systems that have been shown to be functional in plants include 1) *Crellox* from *Escherichia coli* phage P1, where the Cre (control of recombination) recombinase recognizes *lox* (locus of x-over) sites (Sauer 1987, Sauer and Henderson 1989, Hoess and Abremski 1990); 2) FLP/*FRT* from the 2-μm plasmid of *Saccharomyces cerevisiae*, where the FLP recombinase acts on the FLP recombination target, *FRT* sites (Cox 1989, Huang et al 1991); 3) R-*RS* from *Zygosaccharomyces rouxii*, where R and *RS* are the recombinase and recombinase site, respectively (Araki et al 1985, Matsuzaki et al 1990); and 4) a mutant Gin protein (G inversion) and the *gix* site from enteric bacteriophage Mu (Kahmann et al 1985). Dale and Ow (1990) and Odell et al (1990) first reported the Cre-*lox* function in transgenic tobacco subsequently followed by Dale and Ow (1991), Bayley et al (1992), Russell et al (1992), Odell et al (1994), and Qin et al (1994). FLP-*FRT* was first reported to function in rice and maize cells by Lyznik et al (1993) and then in tobacco

(Lloyd and Davis 1994, Kilby et al 1995) and in *Arabidopsis* (Kilby et al 1995, Sonti et al 1995). The mutant Gin-*gix* (Maeser and Kahmann 1991) and the R-*RS* (Onouchi et al 1991, 1995) systems have been found to function in tobacco and/or *Arabidopsis thaliana.*

In this presentation, we will summarize our progress on analyzing the performance of the FLP/*FRT* system in maize and rice cells.

FLP/*FRT* site-specific recombination in plant cells

General description of the FLP/*FRT* system

The FLP enzyme is a 48-kDa protein that covalently binds as four monomeric units to two *FRT* sites and catalyzes the cleavage and ligation of these sites (Cox 1989). FLP-mediated DNA excision or inversion occurs depending on the orientation of the *FRT* sites and when they are in *cis* (Fig. 1). Also shown in Figure 1 is the FLP-mediated

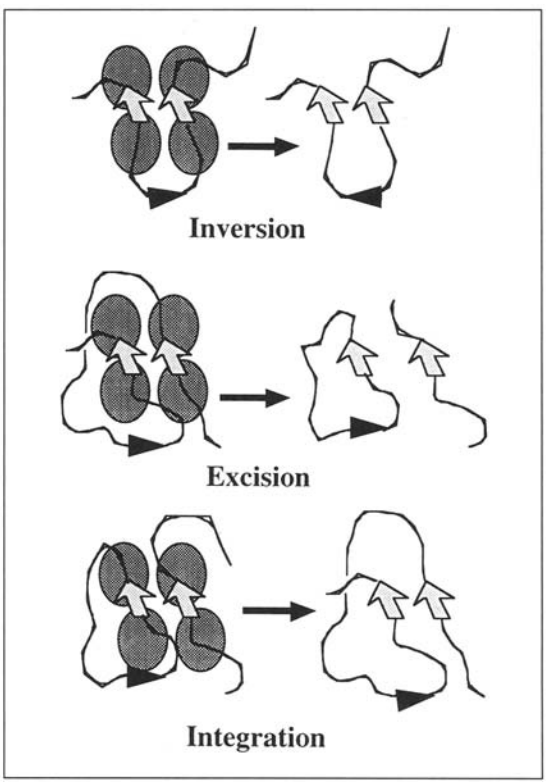

Fig. 1. Diagram depicting reactions catalyzed by FLP upon interaction with *FRT* sites. Depending on the orientation and location of the *FRT* sites, either excision, inversion, or integration of DNA fragment occurs.

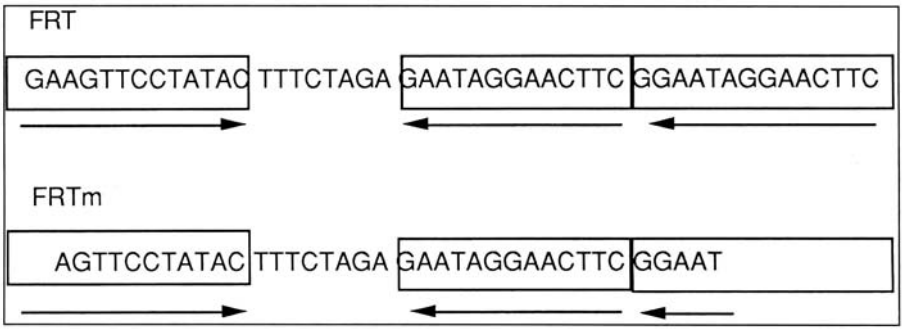

Fig. 2. Nucleotide sequence of a full-length I and a truncated *FRT*m site. The truncated *FRT* m site is referred to in the text as either FRTm or Fm.

integration (combining) of two DNA molecules when the *FRT* sites are in *trans.* The *FRT* site consists of three repeated DNA sequences of 13 bp each; two repeats in a direct orientation and one repeat inverted relative to the other two (Fig. 2; Futcher 1988). In addition, there is an 8-bp spacer region between the repeats that determines the overall orientation of the *FRT* recombination site. The mechanism of DNA exchange and the requirements of the FLP-catalyzed reaction have been studied extensively (Qian et al 1992, Serre and Jayaram 1992, Luetke and Sadowski 1995). The first step in the recombination reaction involves FLP binding to the recognition target sites followed by cleavage of the phosphodiester bond at the border of the spacer region by the tyrosine hydroxyl group of the FLP active site (Evans et al 1990, Pan et al 1993). The subsequent strand exchange reaction generated a transient Holliday intermediate (Dixon and Sadowski 1993). The exchange of the second pair of DNA strands completes the recombination reaction.

Intramolecular recombination of plasmids in protoplasts

To test whether the FLP/*FRT* system would function in plant cells, FLP-mediated recombinations were investigated in maize and rice protoplasts. A target vector was constructed that contained two *FRT* sites flanking a 1.2-kb DNA fragment inserted between the maize ubiquitin promoter and *gusA* (Lyznik et al 1991). One *FRT* site consisted of 48 nucleotides (denoted as FRT or F), and the other *FRT* site was truncated to consist of 39 nucleotides (denoted as FRTm or Fm) (Fig. 2). This vector was cointroduced into maize (hybrid A 188 × BMS) or rice (IR54) protoplasts with an FLP expression vector (pUbiFLP) containing the maize ubiquitin promoter driving FLP. The activity of the FLP recombinase, which would lead to excision of the 1.2-kb DNA fragment, thus removing the block in *gusA* expression, was monitored by determining GUS activity 24 h after the pUbiFLP treatment (Table 1). The results demonstrated that the FLP recombinase could function in plant cells to catalyze the excision of a segment of DNA from the target vector. In addition, FLP catalyzed the reversible or cointegration event (the treatment involving all three plasmids—pUbiFRT + pFRTGUS + pUbiFLP). Interestingly, the combination of a full-length and a truncated version of the *FRT* sites (pU2FRTmG) was more effective than two full-length *FRT*s

Table 1. GUS activities in maize protoplasts treated with vectors containing *FRT* sites and the *FLP* gene.[a]

Plasmid DNA	GUS activity (nmole MU min^{-1} mg $protein^{-1}$)
-DNA	0.11±0.05
pUbiGUS	70±8
pUFRTmG	136±32
pUFRTG	40±3
pU2FRTmG	1.5±0.1
pU2FRTmG rev.	1.9±0.1
pU2FRTG	0.3±0.05
pU2FRTG rev.	0.14±0.04
pUbiFRT	0.1±0.05
pFRTGUS	6±1
Cotransformed with pUbiFLP:	
pU2FRTmG	116±26
pU2FRTmG rev.	1.6±0.1
pU2FRTG	19±2
pu2FRTG rev.	0.4±0.1
pUbiFRT + pFRTGUS	26±1

[a]Maize protoplasts were treated with 20 µg of test plasmid DNA and 25 µg of pUbiFLP DNA where applicable. Values represent average GUS activity of four independent measurements (total of 12 time points) ± standard error. Taken from Lyznik et al (1993).

(p2FRTG). Also, if the *FRT* sites had been altered (indicated as rev in Table 1), FLP would not have been able to recombine these altered *FRT* sites. Thus, the results shown in Table 1 demonstrate that the FLP/*FRT* system can function in plant cells. Furthermore, the FLP/*FRT* system in plant cells appeared to be as efficient as in animal cells (O'Gorman et al 1991), indicating that in both of these heterologous systems, no additional transacting factors are necessary. Based upon these results, we then carried out experiments to determine whether FLP could find and catalyze *FRT* sites after they had integrated into the plant genome.

Intramolecular recombination via retransformation

Experimental system. A target vector was constructed (pUFNeoFmG, Fig. 3), which contained the maize ubiquitin promoter driving a fully functional *neo* gene whose coding region was flanked by *FRT* sites followed by a promoterless *gusA* gene. This vector was introduced, via polyethylene glycol (PEG), into maize protoplasts derived from a suspension culture (identified as PCE). Calli were selected in the presence of kanamycin, and suspension cultures of these callus lines were established and confirmed to contain the intact 5.5-kb *Xho*I-*Sac*I fragment containing the *neo* and *gusA* genes (Fig. 4).

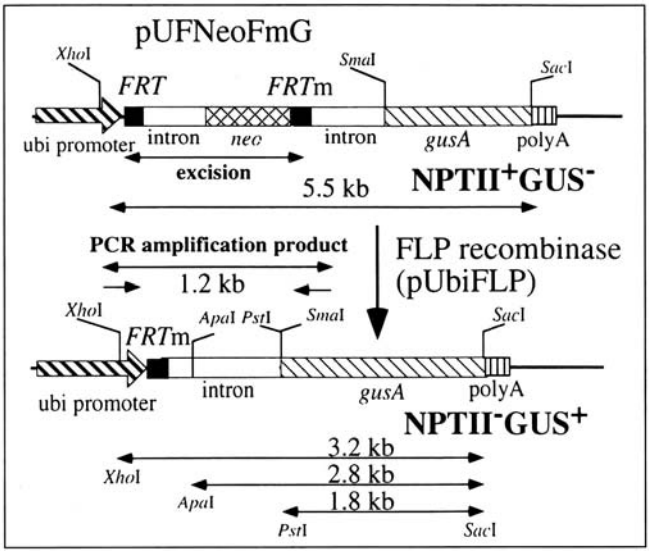

Fig. 3. Diagram of the recombination test vector, pUFNeoFmG, and the potential recombination product having a phenotype that is NPRII$^+$GUS$^-$. Only restriction sites pertinent to this study are indicated. *Xho*I and *Sac*I restriction enzymes would yield a 5.5-kb fragment before FLP-mediated recombination and a 3.2-kb fragment after recombination. From Lyznik and Hodges (1996).

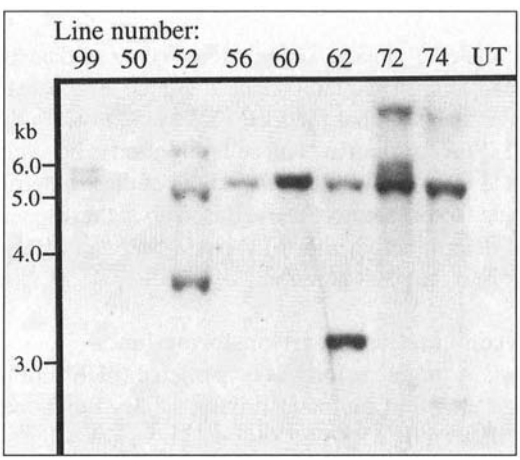

Fig. 4. Southern blot of maize callus DNA to test for the presence of the target vector (pUFneoFmG—see Fig. 3) sequence. The DNA was restricted with *Xba*I and *Sac*I restriction enzymes and probed with the *gusA* coding sequence. The expected 5.5 kb fragment was seen in all lines, plus some modified fragments in certain lines. From Lyznik and Hodges (1996).

Excision of chromosomal neo from protoplasts by pUbiFLP. Three suspension cultures (56, 60, and 302), which contained the functional *neo* gene and were negative for GUS (i.e., the $NPT^+ GUS^-$ phenotype), were chosen for further study. Protoplasts isolated from these three suspension cultures were then incubated with pUbiFLP in the presence of PEG and assayed for GUS within 48 h. Protoplasts from two of these lines, 56 and 60, exhibited an activation of GUS, indicating that FLP recombinase had catalyzed excision of the *neo* gene. The parental suspension culture line (PCE) as well as one of the Kan^r lines (302) did not exhibit GUS activity after treatment with pUbiFLP. These results indicate that transient expression of *FLP*, as well as the levels and activity of the FLP recombinase, is sufficient to recombine the genomic *FRT* sites resulting in excision of the *neo* gene, and simultaneously positioning the ubiquitin promoter proximal to *gusA* resulting in expression of *gusA* and GUS activity.

In similar experiments, protoplasts of line 56 ($NPT^+ GUS^-$) were treated with pUbiFLP and then cultured for 4 wk with no selection on kanamycin. Individual microcalli were then assayed for GUS activity using MUG as the substrate (Jefferson et al 1987), and the frequency of GUS expressing calli was in the order of 3-4% (Fig. 5). As expected, GU^+ lines G8, E11, and E2 (all derived from line 56, which had the *neo* gene, expressed NPTII), did not exhibit NPTII activity while some of the others still retained NPTII activity (Fig. 6b). The strong GUS activity of one of the lines (G8) is illustrated in Figure 6a. A polymerase chain reaction (PCR) analysis of DNA using primers that would amplify only the recombination product (i.e., ubiquitin promoter to *gusA*—the 1.2-kb fragment shown in Figure 3) confirmed that lines E11, G8, and E2 (lanes 3,4, and 5, respectively) had the expected fragment, indicating that excision of the *neo* gene had occurred (Fig. 7a). A Southern blot of genomic DNA

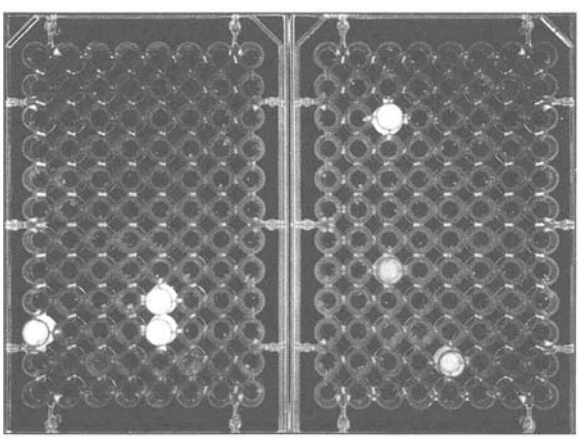

Fig. 5. A screening procedure for determining GUS activity from several callus lines derived from treating protoplasts of line 56 with pUbiFLP. Young calli were chosen randomly and grown for 4 wk without selection and stained with X-gluc according to Jefferson et al 1987.

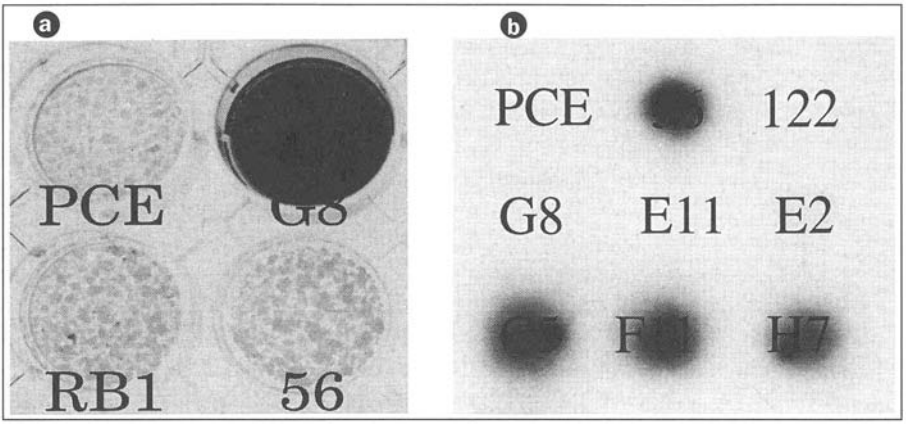

Fig. 6. a) GUS and b) NPTII activities in calli obtained from protoplasts of callus line 56 (NPTII$^+$GUS$^-$) transformed with pUbiFLP. PCE is the original parent callus of line 56. a) Callus stained with X-gluc according to Jefferson et al (1987) for demonstrating GUS activity. b) NPTII activity in various cell lines assayed according to Peng et al (1993).

from these lines that had been restricted with *Xho*I-*Sac*I and hybridized with *gusA* contained a 3.25-kb band—the expected size following *neo* excision (Fig. 7b). Expected size fragments were also obtained for line G8 when other restriction enzyme treatments were used (Fig. 7c). All GUS$^+$ lines derived from line 56 possessed the 3.25-kb band (Fig. 7b); however, some of the lines also still contained a 5.5-kb band. The latter results indicate that not all of the target sites had been recombined to remove the *neo* gene or that these callus lines may have been chimeric, or possibly that a reintegration might have occurred after the excision. Nevertheless, these experiments illustrate that the frequency of transient FLP-catalyzed excisions are quite high and easy to detect. However, since it is possible that these calli could have been chimeric, i.e., some of the cells might have had the *neo* gene excised and thus expressed GUS$^+$ while other adjacent protoplasts/calli may not have had the *neo* gene excised, and we wished to do a molecular analysis to confirm that the recombination had indeed occurred, these experiments were repeated by treating the protoplasts not only with pUbiFLP, but also with pHyg, and then selected the calli on hygromycin.

Protoplasts from line 56 (NPT$^+$GUS$^-$), which had been cotreated with pUbiFLP and pHyg, were cultured and allowed to grow on medium containing 100 mg hygromycin L^{-1}. These lines were screened initially for GUS activity to provide an indication that FLP had excised the *neo* gene, and then they were tested for the loss of NPTII activity. To obtain additional evidence that the *neo* gene had been excised, the genomic DNA from the initial cell line used in this study (PCE), the daughter line (56) containing the target vector (pUFneoFmG), and a granddaughter line (122) retransformed with pUFLP were restricted with *Xho*I and *Sac*I and hybridized to either the *neo* gene or the *gusA* gene (Fig. 8). In line 56, the *neo* and *gusA* genes banded at

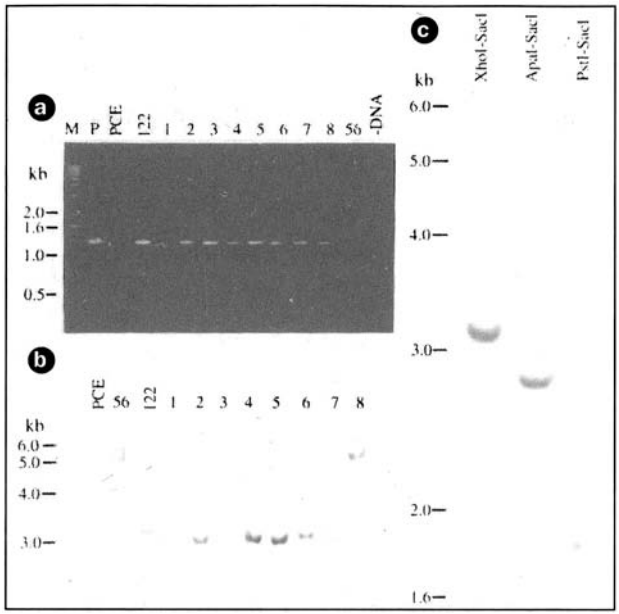

Fig. 7. a) PCR and b,c) Southern blot analysis of genomic DNA isolated from callus of the original parent line never transformed (PCE), line 56 transformed with pUFNeoFmG, line 122 contains the product of the recombination reaction (positive control), and a sample of GUS$^+$ callus lines selected after transient retransformation with pUbiFLP is shown in lanes 1-8. a) The P lane represents the amplification product of the pUFRTG vector and M lane indicates molecular markers. DNA was isolated from the callus 4 wk after treatment with pUbiFLP. The PCR amplification product (1.2 kb) is shown in Figure 3. DNA was isolated from the callus 4 wk after treatment with pUbiFLP. b) DNA was digested with *Xho*I-*Sac*I restriction enzymes. The length of the restriction fragment hybridizing to the *gusA* probe in line 56 (5.5 kb) should be reduced to a fragment size of 3.2 kb after a successful recombination event (compare lanes 56 and 122). c) DNA from transgenic maize suspension present in lane 4 (part B of this figure) was additionally digested with *Xho*I-*Sac*I, *Apa*I-*Sac*I, and *Pst*I-*Sac*I to verify the recombination event. The size of the fragments hybridizing to the gusA probe (3.2,2.8, and 1.8 kb, respectively) was as expected from the product of the site-specific recombination event, see Figure 3. From Lyznik and Hodges (1996).

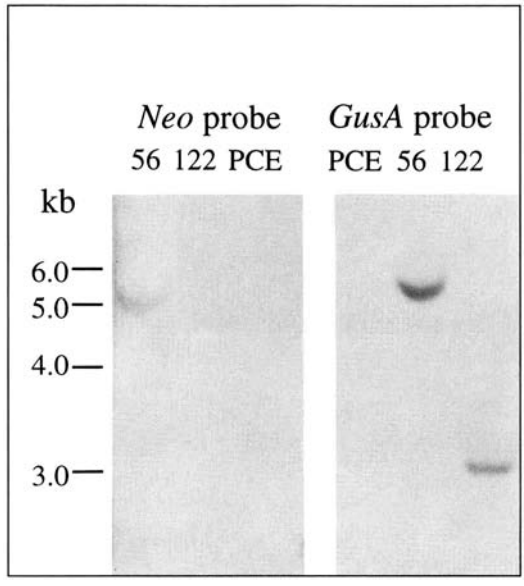

Fig. 8. Southern blot analysis of genomic DNA from the grandmother callus line PCE, the daughter line 56 (transformed with pUFNeoFmG), and granddaughter line 122 (line 56 retransformed with pUbiFLP) and probed with either the *neo* (left panel) or the *gusA* (right panel) genes. The DNA was digested with XhoI-SacI restriction enzymes and 10 µg lane^{-1} was added. A 5.5kb band hybridized to both probes as predicted; a band of 3.2 kb, which is the product of a site-specific recombination reaction mediated by the FLP protein, hybridized only with the *gusA* probe. From Lyznik and Hodges (1996).

5.5 kb. In the derivative line 112, the *neo* gene was missing and the *gusA* gene was present as the expected 3.25-kb band.

To prove that FLP mediated the recombination. as the above results indicated, the sequence of the recombination product was determined (Fig. 9). This was achieved by amplifying a DNA fragment consisting of the 5' untranslated region of the *gusA* gene from line 122 via PCR. This fragment was subcloned into the pGEM7(z) vector and its 5' end, containing the putatively recombined *FRT,* was sequenced. The results confirmed that the recombined *FRT* site was chimeric between the original full-length *FRT* and the original mutated *FRT*m (Fig. 9).

Based on Southern analyses (Fig. 8) and finally on sequencing of the recombined DNA (Fig. 9), we have shown that chromosomal DNA containing the selectable marker gene *neo* flanked by *FRT* sites can be cleansed of the *neo* gene by the expression of the *FLP* site-specific recombinase. Thus, this procedure provides an effective way of

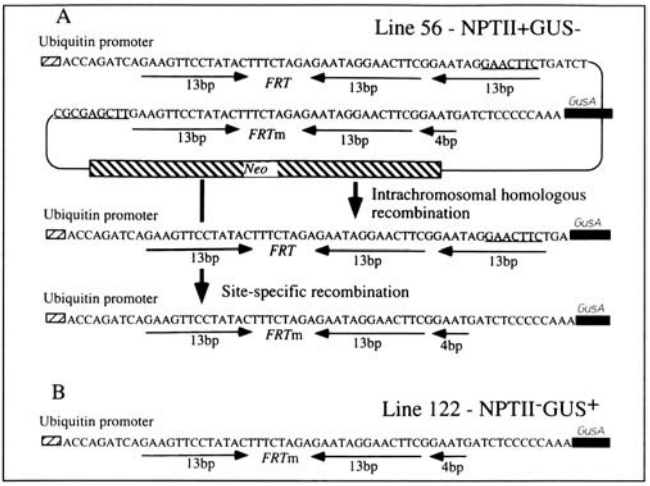

Fig. 9. Sequence of the *FRT* sites integrated into the maize genome before (a) and after (b) site-specific recombination. The two *FRT* sites in line 56 share a region of 39-bp identity. The sequences are different outside of this region (underlined nucleotides). Any conservative DNA recombination within the 39-bp identical regions of the *FRT* sites, including the site-specific recombination, should exchange flanking DNA segments producing a chimeric *FRT* site shown at the bottom of (a). The actual sequence of the *FRT* site in line 122 is shown in (b). This sequence corresponded exactly to the predicted product of the site-specific recombination reaction. From Lyznik and Hodges (1996).

removing a previously integrated gene (*neo* in this case) and simultaneously activating another gene (*gusA* in this case).

Excision of chromosomal neo via inducible expression of chromosomal FLP. In addition to being able to excise DNA fragments from the genome by using retransformation treatments, it would be desirable to achieve this result by a more efficient method. An alternative approach that has worked for the Cre/*lox* site-specific recombination, but one that is rather slow, is to put the two components (Cre and the *lox* sites) in separate plants and then cross breed the plants (Dale and Ow 1991, Russell et al 1992). Although this procedure is useful, it is time-consuming and somewhat difficult. Another approach we have pursued is to integrate the recombinase gene (*FLP*) into the genome under the control of an inducible promoter. With the entire site-specific recombinase system (FLP/*FRT*) integrated into the genome, but with the recombinase gene itself controlled by an inducible-promoter, one should be able to utilize the target gene, such as a selectable marker gene, and then activate expression of FLP by an external signal to excise the target gene. To achieve this, we employed a heat-shock promoter to control expression of *FLP* (Lyznik et al 1995), which had proven to be effective in *Drosophila* (Golic and Lundquist 1989, Golic 1991, Konsolaki

et al 1992). Our results in maize have recently been substantiated by Kilby et al (1995) in *Arabidopsis.*

We used a soybean heat-shock promoter (Czarnecka et al 1985, Ainley and Key 1990) to drive expression of *FLP* and this gene (in pHsFLP) was integrated into the genome in association with the target vector (pUFneoFmG) (Lyznik et al 1995). Following selection of calli on kanamycin, a heat-shock treatment of Kan^r-calli activated expression of FLP as determined by Western blotting, which then recombined the DNA to apparently excise some of the integrated *neo* genes and activate *gusA* expression (+/-heat shock). Southern blot and PCR analyses (Lyznik et al 1995) indeed illustrated that some of the *neo* genes (but not all) had been excised and the expected recombined product was produced. In these experiments. the results were complicated by the integration of more than one vector molecule (intact or fragmented). Clearly, the FLP enzyme was capable of locating and recombining some of the *FRT* sites, but not all of them. This illustrates the need to screen callus initially for single-copy or simple-gene integrations for the *FLP* to be most effective. which should now be easily attained in rice using *Agrobacterium tumefaciens* (Hiei et al 1994, Aldemita and Hodges 1996). These results illustrate that it is possible to have the entire FLP/*FRT* recombination system integrated into the genome and functional, i.e., capable of excising a transgene and simultaneously activating a second one.

Summary

The FLP/*FRT* site-specific recombinase system was shown to catalyze DNA recombinations between two plasmid molecules when introduced into maize or rice protoplasts. It was also shown that *FRT* sites integrated into the maize genome could be located and recombined by FLP following transient expression of the *FLP* gene, and that this occurred in nonselected, randomly chosen calli at a frequency of 3-4%. Molecular evaluations involving both Southern analyses and nucleotide sequencing of the recombined chimeric *FRT* site proved that the expected DNA recombinations had occurred. Finally, it was demonstrated that a heat-shock promoter driving expression of genomic *FLP* was effective in controlling the expression and activity of FLP such that it could locate and recombine *FRT* sites within the genome.

In addition to our studies of the FLP/*FRT* system in maize and rice (Lyznik et al 1993, 1995; Lyznik and Hodges 1996). Lloyd and Davis (1994), Kilby et al (1995), and Sonti et al (1995) have shown this site-specific recombinase to operate in tobacco and *Arabidopsis,* respectively. In tobacco, Lloyd and Davis (1994) used a cross breeding experiment to introduce *FLP* from one plant into a receptor plant containing previously inserted *FRT* sites into the genome and FLP-catalyzed excisions occurred at a frequency of about 70%. Kilby et al (1995) used a heat-shock promoter to drive expression of FLP, and they demonstrated that cell fate could be determined in *Arabidopsis* using this system.

Use of the FLP/*FRT* and the other site-specific recombinase systems for the excision of unwanted transgenes, the inversion of either active or quiescent transgenes, the removal of blocking genes to activate other genes, the development of plant hybrids

by excision or inversion of sterility or fertility genes, the control of the recombinase genes by environment-sensitive (temperature, chemical) or tissue-specific (endosperm, floral) promoters, etc. provides the opportunity to manipulate the plant genome for the development of transgenic plants in a very sophisticated fashion.

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Notes

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Introduction of multiple genes into elite rice varieties—evaluation of transgene stability, gene expression, and field performance of herbicide-resistant transgenic plants

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Transgenic rice plants from indica, japonica, and javanica varieties that express several foreign genes were generated via particle gun bombardment of immature embryos. Molecular and genetic characterization of large numbers of these plants (more than 500 independent transgenic plants) provided information on structure, expression, and stability of integrated DNA through multiple generations. Such evaluations were carried out in the greenhouse (five generations) and in the field (three generations). Stability of foreign DNA was found to be dependent on the nature of the promoter and the transgene, and in specific cases, on gene copy number. A general conclusion of the results presented here is that direct DNA transfer utilizing electric discharge particle bombardment for the delivery of foreign DNA into rice tissue results in the recovery of large numbers of independently derived transgenic plants in a variety-independent fashion.

Rice is one of the world's most important food crops. Accordingly, great effort has been directed toward biotechnology to complement conventional breeding methods. Previously, we reported the recovery of transgenic rice plants using direct gene transfer of exogenous DNA into immature zygotic embryos by electric discharge particle acceleration (Christou et al 1991, 1992). The method bypasses traditional variety-dependent, tissue-culture procedures involving protoplast and embryogenic suspension cultures (Zhang and Wu 1988, Shimamoto et al 1989) thus enabling the rapid recovery of desirable transgenic phenotypes in agronomically important cultivars.

A number of advantages make microprojectile bombardment the method of choice for engineering major agronomic crops:

- Stable transformation of a wide range of cell types in organized tissue is possible. The ability to engineer organized and easily regenerable tissue permits introduction of foreign genes into elite germplasm directly. Consequently,

backcrossing is not required to restore the original line as compared with other transformation methods limited by genotype and host specificity. Recovery of transformed R_1 seed is considerably shortened and this saving in time is of paramount importance in commercial programs where timing is vital in bringing products to market.

- Transient gene expression has been demonstrated in almost all tissues for many crops, and this provides an easy method for rapid gene expression studies.
- Stable transformation of recalcitrant species: most of the important agronomic crops, including previously recalcitrant legumes and cereals can be engineered effectively only through bombardment-based technologies. In the case of soybean, *Agrobacterium* host specificity restricts utilization of this technology to specific varieties that are of no commercial importance (Hinchee et al 1988); for cotton, tissue culture limitations only allow engineering of a specific variety (Umbeck et al 1987, Finer and McMullen 1990). Wheat transformation using electroporation of protoplasts resulted in the recovery of infertile plants (He et al 1994). Bombardment-based methodology allowed effective engineering of important species such as soybean (Christou et al 1990), cotton (McCabe and Martinell 1993), maize (Gordon-Kamm et al 1990, Fromm et al 1990), wheat (Vasil et al 1993, Weeks et al 1993), barley (Wan and Lemaux 1994), and others (Vain et al 1996). Breakthroughs have been the consequence of improved transgene engineering (Callis et al 1987, Christensen et al 1992), efficient selection (Gordon-Kamm et al 1990, Fromm et al 1990), screenable markers (Jefferson et al 1987), and reliable tissue culture techniques (Christou et al 1991, Weeks et al 1993).
- Study of basic plant developmental processes: by utilizing chromogenic markers, it is possible to study developmental processes and also better clarify the origin of the germline in regenerated plants (Christou and McCabe 1992).

A number of factors were found to influence successful particle and DNA delivery into regenerable tissues of rice. Parameters examined included condition of the explant prior to bombardment, environmental factors including temperature and humidity, and influence of such parameters on transient activity of the *uidA* marker gene; and depth of particle penetration and degree of tissue damage as a function of accelerating voltage and effect of selective agents. Experiments were carried out to develop a selection system that would eliminate the need to screen all regenerated plants derived from transformation experiments. These studies have been reported elsewhere (Christou et al 1995a,b,c). Selection procedures were optimized to eliminate potential escapes resulting from cross-protection of wild-type cells. This was achieved effectively by incorporating a secondary selection phase (P. Vain et al, pers. commun.).

Until recently, the key barrier in achieving effective transformation of agronomically important species was the DNA delivery method. Particle bombardment has had a tremendous impact on this limitation. The challenge now is shifting to the biology of the explant used in bombardment experiments and also to the integration and subsequent expression of transgenes. It is apparent that conversion frequency of transient to stable transformation events is low. This makes recovery of large numbers

of independently derived transformation events labor-intensive and rather expensive. More attention needs to be paid to the biology of explants prior to and following bombardment. We need to identify how more cells can be induced to become competent for stable DNA uptake and regeneration. Optimization of biological interactions between physical parameters and target tissue needs to be better studied and understood. Not much is known about the fate of DNA from the time particles are introduced into plant cells. Recipient tissue variation and variability due to bombardment conditions complicate the picture even further. Additional issues such as irregular particle size and uniformity as well as improvements in hardware design need also to be addressed. However, recent results from a number of different laboratories are beginning to address some of these problems. Further progress in plant transformation techniques would also depend on developing techniques that allow controlled integration and expression of foreign DNA into the plant genome (Finnegan and McElroy 1994).

Once an efficient transformation system has been developed, it is necessary to assess the fate and function of the exogenous genes in the host organism and its progeny. Of particular interest are the structure and expression of unselected genes, which often encode the desired transgenic phenotype. Goto et al (1993) demonstrated cointegration and coinheritance of selected and unselected exogenous genes transformed on separate plasmids. We also reported cointegration and coinheritance as well as stable coexpression of multiple genes transferred on a single plasmid into rice by electric discharge particle acceleration (Cooley et al 1995).

The transformation method

Ten to fifteen-day-old rice immature seeds from various cultivars (Table 1) were harvested from expanded panicles and sterilized with 2% sodium hypochlorite for 5 min. They were subsequently rinsed repeatedly with sterile distilled water and the glumes were removed under a dissecting microscope. In some cases, immature seeds were resterilized for 10 min as described above. The embryos were then aseptically removed and plated on MS or CC media supplemented with 0.5 or 2.0 mg 2,4-D L^{-1}, respectively, with the adaxial side in contact with the medium. Particle bombardment was carried out as described elsewhere (Christou et al 1991). Explants were bombarded at an accelerating voltage of 14-16 kV. Following bombardment, embryos were plated on fresh medium, and embryogenic calli and plantlets were recovered as described (Hartke and Lorz 1989, Datta et al 1990). Transformed calli and plants were recovered under selective conditions. Explants were plated on hygromycin-containing media (usually 50 mg L^{-1}) at 2 d following bombardment. Once tissue was subjected to selection pressure, this pressure was maintained throughout the proliferation and regeneration phase.

Differential responses of the explants were observed depending on the input plasmid and timing of selection. As expected, nontreated embryos and embryos bombarded with plasmids not conferring resistance to hygromycin or only gold particles (no DNA) died within 48 h of plating on hygromycin-containing media. Explants bombarded with the hygromycin-resistance plasmid proliferated when plated on media

Table 1. Transformation frequencies, genotypes, and fertilities of transgenic plants generated through electric discharge particle bombardment.

Genotype	Explants (no.)	Independently derived transformed plants (no.)	Transformation frequency	Fertility		
				75-100%	50%	Sterile
Gulfmont	650	108	17	94	14	0
Koshihikari	600	93	16	52	41	0
Sasanishiki	300	48	16	29	15	4
CM101	250	42	17	40	2	0
S201	200	36	18	8	28	0
IR26	100	9	9	2	7	0
IR36	350	28	8	22	5	1
IR54	300	67	22	52	15	0
IR72	900	131	15	82	35	14
Cypress	200	23	12	23	0	0
Bengal	200	8	4	8	0	0
Lido	250	15	6	2	8	5
Carnaroli	50	1	2	1	0	0
Thaibonnet	50	3	6	1	0	2
ITA212[a]	766	20	3	nd	nd	nd
WAB56-104[a]	627	3	0.5	nd	nd	nd
Lac26[a]	493	5	1	nd	nd	nd
IDSA6[a]	408	8	2	nd	nd	nd

[a]These results include optimization experiments.

supplemented with the antibiotic. In parallel experiments in which a plasmid conferring resistance to the herbicide Bialaphos (or glufosinate) was introduced into rice immature embryos, similar results were obtained, i.e., when the bombarded explants where plated on media supplemented with hygromycin, they died, but proliferated when cultured in the presence of Bialaphos.

To monitor and confirm transformation events conveniently, a *uidA* gene (Jefferson et al 1987) was linked to the hygromycin (*hpaIV*) gene. The plasmid containing both genes as well as a third gene (*bar*) was introduced into rice immature embryos and transformation events were monitored using a GUS histochemical assay. The timing of selection was shown to influence the phenotype of transgenic callus lines, and subsequent recovery of transgenic plants. In experiments in which immature embryos were bombarded and plated on hygromycin at various times following bombardment, we observed that a small number of transformed cells on each explant allowed proliferation of wild-type cells by effectively detoxifying the antibiotic. As a result calli from these explants were shown to be chimeric. When selection was delayed for up to 10 d following bombardment, we obtained chimeric calli comprising of transformed and nontransformed cells. Subsequently, when plants regenerated from these cultures, only a small fraction of them was shown to be transformed. In experiments in which selection was applied early, i.e., 48 h following bombardment, we recovered transformed embryogenic calli, as shown by GUS activity. All transgenic plants recovered under the conditions of our experiments were clonal in nature, segregating in a Mendelian fashion in the R_1 generation. The selection procedure was

optimized further by incorporating a secondary selection step to allow complete elimination of wild-type cells, which were cross-protected by transformed cells overexpressing the gene(s) for detoxification of the selectable marker(s). More details on the selection procedure will soon be found in the primary literature (P. Vain et al, pers. commun.).

Table 1 summarizes transformation frequencies, genotypes, and fertilities of transgenic plants generated through these procedures.

Recently, transgenic plants from four West African varieties were recovered. These plants were engineered with the Oryzacystatin-1-delta-86 gene (Urwin et al 1995), which confers resistance to parasitic nematodes. Western blots confirmed expression of detectable levels of the gene in root tissue. Biochemical analysis of these plants will be reported elsewhere (P. Vain et al, pers. commun.).

Transgene integration, levels of expression, and stability

Transgenic plants transformed with a single plasmid containing the selectable gene hygromycin phosphotransferase (*hpaIV*) and one or two unselected genes (*uidA* coding for b-glucuronidase or *bar* coding for phosphinothricin acetyltransferase) were analyzed by Southern blot analyses to determine copy number and cointegration frequencies. Coexpression frequencies for selected and unselected genes were also determined. The physical linkage of transgenes was confirmed by Southern blot patterns and cosegregation of transgenic phenotypes in the R_1 and R_2 generations. In one group of experiments (Cooley et al 1995), Southern blot analysis of primary transformants was used to identify 56 independent transformation events. The analysis was designed to characterize independent events by comparing patterns of bands, which include both the chimeric gene insert and flanking rice genomic DNA. Cointegration of selected and unselected marker genes was determined by probing blots for all chimeric genes (*uidA, bar,* and *hpaIV*; Cooley et al 1995). The transforming DNA in each of these transgenic individuals consisted of one of six different plasmid constructs. Southern blot analysis using gene-specific probes indicated that all genes on the transforming DNA were integrated at least once in all independent events with a 100% cointegration frequency of selected and unselected genes on the same plasmid. For most transformants, the complexity of the Southern band patterns was comparable from one gene-specific probe to another, indicating similar copy numbers of the various transferred genes. This suggests that the transforming plasmids generally integrate as a complete unit. Battraw and Hall (1992) also reported a 100% cointegration frequency when using linked genes to produce transgenic rice plants from protoplasts. This cointegration frequency is about 25% higher than other linked-gene transfer methods described for transgenic soybean callus (Christou and Swain 1990) and *Phaseolus vulgaris* (Russell et al 1993).

We examined the inheritance patterns of unselected transgenes in the R_1 progeny of 19 *uidA*- and/or *bar*-expressing transformants. Production of transformants with multiple genetic loci occurred at a very low frequency. Expression of *uidA* and *bar* in transformed plants demonstrated a 3:1 segregation ratio consistent with Mendelian

Table 2. Segregation of transgenes in progeny of primary transformants.

	Transformant	GUS+	BAR+	Total assayed	Expected ratio	c^2	P
R_1	495-1	41	51	53	3:1 GUS	0.039	0.80-0.95
					15:1 BAR	0.035	0.80-0.95
	496-1	0	74	101	3:1 BAR	0.040	0.80-0.95
	496-2	31	31	47	3:1 GUS	0.512	0.20-0.50
					3:1 BAR	0.512	0.20-0.50
	496-3	0	41	45	3:1 BAR	1.557	0.20-0.50
	496-4	57	57	86	3:1 GUS	0.870	0.20-0.50
					3:1 BAR	0.870	0.20-0.50
	496-5	0	31	46	3:1 BAR	0.355	0.20-0.50
	517-5	17	17	30	1:1 GUS	0.133	0.80-0.95
					1:1 BAR	0.133	0.80-0.95
R_2	495-1-5	56	56	78	3:1 GUS	0.107	0.50-0.80
					3:1 BAR	0.107	0.50-0.80
	495-1-12	27	44	46	3:1 GUS	1.630	0.20-0.50
					15:1 BAR	0.018	0.50-0.80
	495-1-13	0	33	46	3:1 BAR	0.065	0.50-0.80
	496-3-1	0	72	90	3:1 BAR	0.300	0.50-0.80
	496-4-1	48	48	59	3:1 GUS	0.318	0.50-0.80
					3:1 BAR	0.318	0.50-0.80

inheritance of a single dominant locus in all but two plants (Table 2). One plant, 517-5, demonstrated a 1:1 segregation ratio for BAR expression. This aberrant segregation can be explained by the passage of the transgene exclusively through one gamete. A second plant, 495-1, produced a 15:1 segregation ratio for BAR expression consistent with the presence of two unlinked genetic loci. However, analysis of GUS expression resulted in a 3:1 ratio in the same group, indicating the presence of only one locus with a functional *uidA* gene. Southern blot analyses using both *bar* and *uidA* probes on multiple R_1 seedlings from 495-1 indicated that *bar* and *uidA* DNA were inherited in all progeny resulting in three distinct band patterns. Two of the patterns had no common bands between them, while the third pattern exhibited a combination of the other two. These patterns represented the progeny with either one or two loci, respectively. Subsequent Southern blot analysis demonstrated that at each locus, there was one full size and one partial plasmid insert with nonplasmid DNA between the two inserts at both loci. At one locus, the partial plasmid copy lacks the *bar* gene, while at the other locus, the partial copy lacks the *uidA* gene. The single *uidA* gene at the latter locus is nonfunctional as determined by lack of GUS staining of progeny possessing only this locus. All three types of progeny were resistant to Basta® with no obvious difference in resistance between R_1 plants that had either or both loci. Southern blot analysis of other independent families demonstrated complete concordance between the presence of enzyme activity and the corresponding genes. Southern blots on progeny resulted in the same banding pattern as the primary regenerant. Segregation analysis from this and other systems indicates that these multiple integration events are genetically linked. In this study, multiple inserts were not generally tandem concatemeric arrays, but had multiple genomic DNA borders

indicated by sequencing. This suggests that the linked multiple inserts are significantly fragmented and/or separated by nonplasmid DNA.

We observed a typical range of 1-10 copies of plasmid DNA per haploid genome. This is consistent with other reported methods of direct gene transfer (Schocher et al 1986, Linn et al 1990, Battraw and Hall 1992, Peng et al 1992, Goto et al 1993). Our experiments have shown that in the vast majority of transformants, Southern blot patterns are conserved in R_0, R_1, and R_2 generations and reflect Mendelian inheritance of a single locus. The tightly linked nature of gene fragments associated with multicopy integration events has also been reported elsewhere with both T-DNA integration (De Block and Debrouwer 1991) and direct transfer of linked (Christou et al 1989, Battraw and Hall 1992) and unlinked genes (Kartzke et al 1990, Saul and Potrykus 1990, Rathore et al 1993, Goto et al 1993).

To determine if copy number plays a role in coexpression of transgenes, the number of functional copies of transgenic inserts per individual transformant was estimated (Cooley et al 1995). When the number of *uidA* transgenes exceeded 10, the frequency of GUS expression in hygromycin-resistant individuals decreased sharply from 44 to 13%. In the individuals with *uidA* driven exclusively by 35S, however, all low-copy (1-2 copies) transformants expressed GUS, but none of the individuals with greater than 10 integrated copies were GUS-positive. Conversely, 35S-*bar/hpaIV* plants demonstrated the opposite relationship, with coexpression rate stable or increasing with copy number from 80% for one or two copies to 100% for greater than 10 copies. In parallel experiments in which the *uidA* and *bar* genes were evaluated separately, we found that, in general, levels of expression were reduced with increasing copy number for *uidA*, but not for the *bar* gene. These results suggest that correlation between gene-silencing and multicopy integration events may be gene- or construct-specific.

Our experiments suggest that the key variables affecting gene silencing are inherent to the unselected gene and/or its promoter. All 35S-*uidA* and 35s-*bar* constructs exhibited 66 and 90% coexpression, respectively. Plants transformed with pWRG2426 contained both 35S-*uidA* and 35S-*bar* as unselected transgenes on the same plasmid. However, while more than 90% of these plants expressed BAR, only half of them expressed GUS. These results indicate that GUS expression may be more susceptible to inactivation than BAR expression. The larger *uidA* gene is twice as likely than *bar* to be truncated during integration based on gene size alone. Variation in the levels of expression of transgenes is typical of genetically engineered plants. These variations have been observed utilizing either T-DNA or direct DNA transfer methods and are largely attributed to a number of phenomena including integrative fragmentation or rearrangement (Jongsma et al 1987, Kartzke et al 1990, Battraw and Hall 1992, Peng et al 1992, Rathore et al 1993), position effects (Shirstat et al 1989, AI-Shawl 1990, Allen et al 1993, Assaad et al 1993), and gene silencing (Jorgensen 1991, Hart et al 1992, Allen et al 1993, Assaad et al 1993, Rathore et al 1993). In the present study, these variations are indicated by lack of GUS activity or susceptibility to Basta® in R_0 hygromycin-resistant plants.

Our data indicate a relationship between copy number and gene silencing in plants transformed with *uidA* constructs. Allen et al (1993) also reported an inverse

relationship between copy number and expression of 35S-*uidA* transgenes in transformed tobacco NT1 cell lines. Linn et al (1990) reported reduced gene expression with increased copy number in petunia plants engineered with an unselected 35S-dihydrofoliate reductase (*dhfr*) gene. Shirstat et al (1989) and van der Krol et al (1990) reported no correlation between expression and transgenic copy number of transgenic leguminin and *dhfr* genes, respectively, and Stockhaus et al (1987) reported an increase in gene expression with increased copy number. These apparent discrepancies support our hypothesis that factors affecting gene silencing may be gene- or construct-specific. The sensitivity of the assays used to measure and compare various unselected gene product activities and the level of gene expression required to produce detectable enzyme activity are additional factors that may affect apparent gene silencing. It may be that significantly less protein is required to produce the Basta® -resistant phenotype than is necessary to produce the GUS phenotype. We have shown that choice of promoter is important in attaining detectable levels of GUS expression. Of the tissue-specific promoters used, only the *Arabidopsis ssu* promoter produced GUS expression albeit at a lower frequency (33%; 5 of 15 plants examined). However, it is possible that these weak promoters would be sufficient to produce a positive BAR phenotype.

In experiments in which multiple genes were introduced into rice plants utilizing one construct, we determined that coexpression frequencies of all genes were dependent on promoter nature and orientation and also on the number of genes present on the plasmid. In general, promoter orientation does not seem to be important when two genes are present on the plasmid. However, as the number of genes increases, it becomes crucial (Table 3).

Table 3. The nature and orientation of promoters that influence coexpression frequencies of multiple genes introduced into rice plants utilizing one plasmid.[a]

Transgenic plants analyzed (no.)	hmr	gus	bar	x	Plants expressing all genes (no.)
30	+	+			30
30	+	-			30
74	+	+	+		48
45	+	+	-		45
25	+	-	-		15
38	+	+	+	+	8
42	+	+	+	-	20
50	+	+	-	-	30
28	+	-	+	-	16
28	+	-	*Arab. ssu*	Rice ssu	26

[a]Plus and minus signs indicate multiple 35S promoters driving multiple genes in a clockwise or counterclockwise orientation.

Molecular characteristics of field-derived plants

Fifteen lines representing 11 independent transformation events were analyzed. Southern blot analysis of the transgenic lines was used to characterize patterns of integration of the unselected genes (*uidA* and *bar*) carried on the transforming WRG 2426 plasmid DNA (Cooley et al 1995). Analysis was performed on one plant only of a segregating population for each line. Consequently, only general statements regarding the fate of the transgenes can be made as we are not dealing exclusively with homozygous populations for the transgene(s). Northern blot analysis was used to determine the transcriptional state of the *uidA* and *bar* transgenes. Southern blot analysis with gene-specific probes showed that in nine of the plants representing the 15 lines, both the *uidA* and *bar* transgenes were present (Fig. 1). The complexities of band patterns and intensities were similar when the banding patterns within the same line were compared for the *uidA* and *bar* transgenes, indicating that the transforming plasmid was inherited as a complete unit in the nine lines. Comparison of the complexities of banding patterns for the *uidA* and the *bar* transgenes within independent lines showed six different patterns of integration of the *uidA* transgene, whereas at least eight different integration patterns were revealed for the *bar* gene suggesting recombination events. DNA from two lines, GFMT 517-5-R1 and GFMT 517-2-R1, showed hybridization to the *bar* gene probe but not to the *uidA* gene probe. In the remaining four lines (GFMT 526-1 GFMT 517-1-R1, GFMT 517-7-Rl, and KOSH 496-3-R2), neither the *bar* nor the *uidA* transgene was detected. Since these plant lineages exhibited resistance in the field following herbicide application and since KOSH 496-3-R1 contains both *bar* and *uidA,* this shows that we are dealing with a mix of transformed and nontransformed plants derived from segregating populations. We are currently developing homozygous lines for the transgenes, which will be used for a more in-depth molecular analysis of transgene structure and function.

A comparison of the Southern and Northern data provide evidence for mechanisms of gene silencing operating at both the transcriptional and posttranscriptional levels. Nucleic acid extracted from the GFMT 517-3-Rl line showed hybridization to the *uidA* gene probe at the DNA level, but no transcript was detected in these plants for the *uidA* transgene at the RNA level. Of the 11 lines in which the *bar* transgene was detected at the DNA level, four lines (KOSH 496-2-R1, KOSH 496-3-R1, KOSH 496-4-Rl, and KOSH 496-4-R2) showed no hybridization to the *bar* gene probe at the RNA level. That the silencing effect is seen in plants having anywhere between 1 and 5 hybridizing bands in Southern gel-blot analyses suggests that the transcriptional silencing effect may be independent of the number of integration sites of either the *uidA* or *bar* transgenes. Our data also provide evidence for gene silencing occurring at the posttranscriptional level in some of the plants we analyzed. The expected size of the transcript from the *uidA* gene in plasmid WRG 2426 is 2 kb; such a transcript was observed in RNA only from lines KOSH 495-1-Rl, KOSH-495-1-R2, and KOSH-496-2-R1 (Fig. 2). These lines exhibited the highest frequency of GUS expression (three of the nine plants tested, data not shown). GUS activity was rarely detected in any other line. Transcripts of more than 12 kb, detected in RNA from lines KOSH

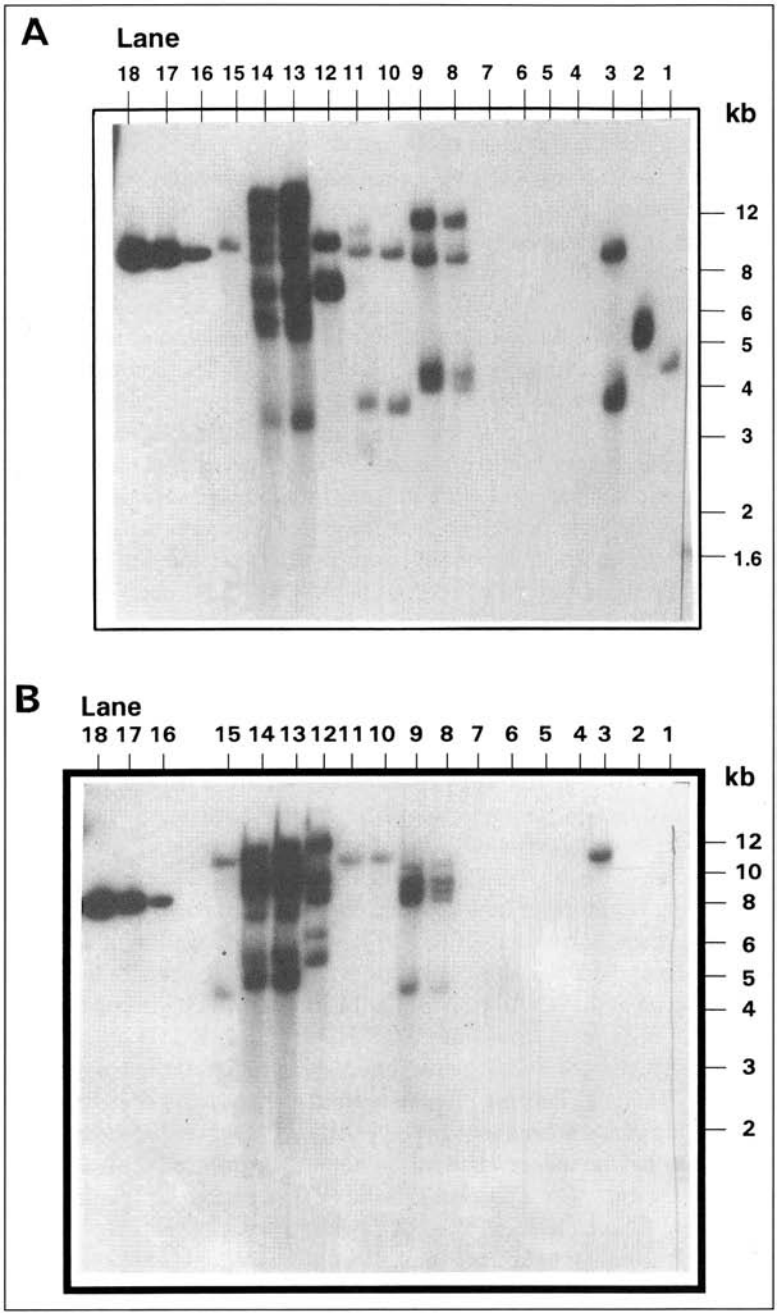
A
Lane
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Fig. 1. Southern blots of transgenic lines tested in the field. Genomic DNA was digested with *Sac*I (blot A) or *Xba*I (blot B), transferred to nylon membranes and probed with ^{32}P-labeled *uidA* gene probe DNA (blot A) or similarly labeled *bar* gene DNA (blot B). Blots A and B have identical lane loadings of genomic DNA isolated from the following lines: GFMT 517-2-R1 (lane 1), GFMT 517-5-R1(2), GFMT 517-3-R1(3), GFMT 517-1-R1(4), G 526-1 (5), KOSH 496-3-R2 (6), GFMT 517-7-R1(7), KOSH 496-4-R1 (8), KOSH 496-4-R2 (9), KOSH 495-1-R2 (10), KOSH 4951-R1 (11), KOSH 496-3-R1 (12), KOSH 496-1-R2 (13), KOSH 496-1-R1 (14), and KOSH 496-2-R1 (15). Lanes 16,17, and 18 contain pWRG2426 at 1, 5, and 10 copies, respectively, per genome, digested with either *Sac*I (blot A) or *Xba*I (blot B).

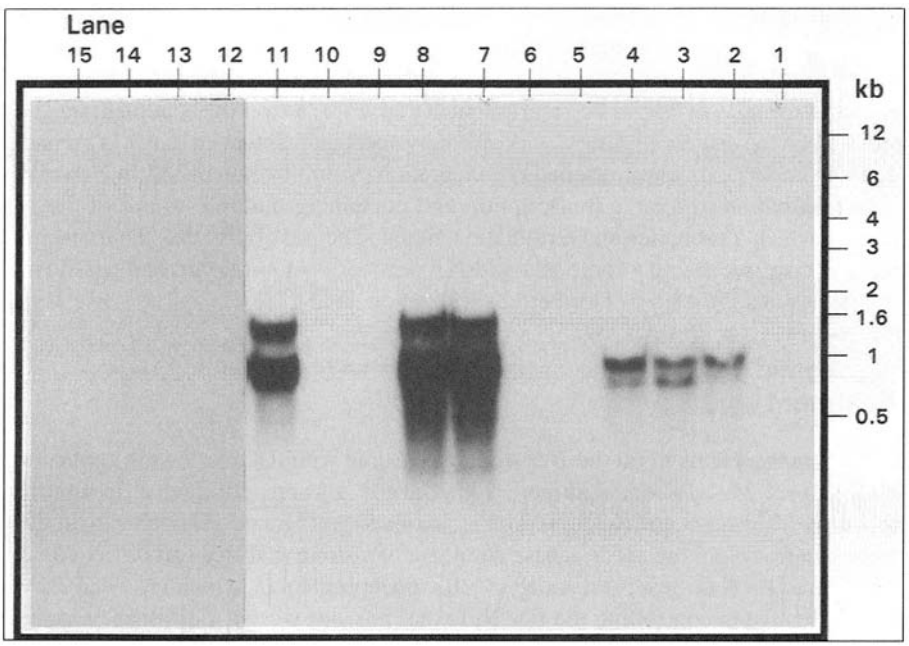

Fig. 2. Northern blot analysis of RNA from transgenic rice plants tested in the field. Total RNA (15 µg) was fractionated through 1.5% agarose gels, transferred to nylon membranes, and probed with ^{32}P-labeled *bar* gene DNA. Lanes 1-6 show RNA from the GFMT lines and lanes 7-15 RNA from the KOSH lines as follows: GFMT 517-1-R1 (lane 1), 517-2-R1(2), 517-3-R1(3), 517-5R1 (4), 517-7-R1 (5), 526-1 (6), KOSH 495-1-R1(7), 495-1-R2 (8), 496-1-R1 (9), 496-1-R2 (10), 496-2-R1 (11), 496-3-R1 (12), 496-3-R2 (13), 496-4-R1(14) and 496-4-R2 (15).

496-1-R1, KOSH 496-1-R2, KOSH 496-3-R1, KOSH 496-4-R2, and KOSH 496-4-R1, are likely to be untranslatable since no GUS activity was found in leaf material taken from five independent plants of each of these lines. These experiments are being pursued further to develop a better understanding of these phenomena. Interestingly, those plants from which genomic DNA revealed the greatest number of hybridization bands in Southern blot analyses with the *uidA* gene probe (KOSH 496-1-R1, KOSH 496-1-R2, KOSH 496-3-R1, KOSH 496-4-Rl, and KOSH 496-4-R2) also showed the presence of a very large, relatively low abundant transcript of more than 12 kb that hybridized to the *uidA* gene probe in Northern blot analyses. In addition, the lines exhibiting the simplest integration patterns of the *uidA* gene (KOSH 495-1-R1, KOSH-495-1-R2, and KOSH-496-2-R1) showed GUS expression more consistently across different plants of these lines. A large transcript hybridizing to the *bar* gene probe was detected in lines KOSH 496-1-R1 and KOSH 496-1-R2 that similarly demonstrated the more complex integration pattern for the *bar* transgene. These data suggest that there maybe a correlation between complexity of integration sites and reorganization of the input plasmid to produce a large transcription unit, perhaps containing multiple copies of the *uidA* or *bar* messages. How such transcripts are produced remains unclear and will be the subject of future work. We may speculate, however, that it is probably due to complex extrachromosomal preintegration recombination events, which result in the formation of a large transcription unit containing multiple copies of the *gus* gene between a promoter and termination signal. The possibility that the transcripts are polycistronic, being a composite of RNA sequences of *uidA, bar,* and possibly an *aphIV,* is not supported by Northern hybridization data.

Agronomic performance of transgenic material after herbicide treatment

All nontransgenic plants at the 3- to 4-leaf stage died within 8 d following application of 1.12 or 2.24 kg glufosinate ha^{-1} (J.H. Oard et al, pers. commun.). In contrast, transgenic Gulfmont and Koshihikari lines survived glufosinate treatments to produce fertile, normal-looking seeds. These results demonstrate that rice can be genetically engineered for field-level resistance to glufosinate herbicide, which has been shown to be effective in controlling red rice and other noxious weeds. Gulfmont transgenic lines displayed no visible injury from herbicide treatments, but all Koshihikari lines exhibited initial yellowing and some stunting 2-3 d after glufosinate application. The yellowing disappeared 7 d after treatment, however, stunting for some lines was observed at harvest. Significant differences among the Gulfmont- and Koshihikari-derived transgenic lines for grain yield were detected both at the 1.12 and 2.24 kg ha^{-1} rates. The grain yield of transformed Gulfmont lines varied between 1.8 and 1.7 t ha^{-1} for 1.12 and 2.24 kg ha^{-1} herbicide rates, respectively. A similar trend was observed within Koshihikari lines that displayed significant differences in yield, but with greater ranges (2.2 and 4.1 t ha^{-1}) at the two herbicide rates. Under no herbicide conditions, a few transformed Gulfmont and Koshihikari lines yielded below the untransformed parental cultivars, but 80% of the lines yielded equal or better than the controls. Mean

grain yields across herbicide treatments were not statistically significant within Gulfmont-derived lines, which indicates that glufosinate applications did not significantly alter performance of this material. In contrast, grain yields for 33% of the Koshihikari lines were significantly reduced with increasing glufosinate rates. Variation for grain yield among transgenic lines may be explained by variations of BAR expression. As with grain yield, significant differences among transgenic lines for mature plant height were observed for each herbicide rate. A 6- to 7-cm range in height was observed for Gulfmont lines while Koshihikari lines displayed an 11 - to 12-cm range within treatments. Mean height values across treatments were not statistically different among Gulfmont lines while a majority (67%) of Koshihikari lines showed significant reductions in plant height with increasing glufosinate rates. Plant height 14 d after spraying of 1.12 kg glufosinate ha^{-1} showed a slight decrease of 2.4 cm among transgenic lines when compared with the no-herbicide treatment in 1993 experiments. Most Gulfmont and Koshihikari lines (55-67%) displayed no significant differences in days to 50% heading across herbicide treatments. Moreover, heading values were nearly equal or only slightly greater with no-herbicide treatments when compared with the untransformed controls. For those lines where statistical differences in heading were observed, the 2- to 3-d delay in maturity would probably have minimal effect on agronomic performance of the transgenic material.

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Production and testing of insect-resistant transgenic rice plants

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Suspension cells or calli from three varieties of rice (*Oryza sativa* L.) were transformed, by microprojectile bombardment, with plasmids that contain the bacterial phosphinothricin acetyl transferase (PAT) gene (*bar*), and the potato protease inhibitor II (PINII) gene or the cowpea trypsin inhibitor (CpTi) gene. Fourteen transgenic lines were grown in an isolated experimental field at Zhejiang Agricultural University in Hangzhou. Rice plants were tested for their resistance to herbicide (1% Basta^{TM}) and two species of stem borer (*Chilo suppressalis* and *Sesamia inferens*). The leaves of most transgenic rice plants were not affected by the herbicide, but the leaves of all nontransformed control plants became yellow. Infestation of the rice plants by the stem borers caused severe damage to nontransformed control plants and resulted in dead panicles (commonly known as whitehead). In contrast, transgenic rice plants showed much less damage and fewer dead panicles. Thus, introduction of an insecticidal proteinase inhibitor gene into rice plants appears to be a useful strategy for controlling insect pests.

Rice is one of the most important crops in the world. The loss of rice yield due to direct insect damage is estimated to cost at least several billion dollars (Heinrichs et al 1985). It is also known that several devastating diseases in rice, such as rice tungro disease and yellow dwarf disease, are caused by viruses and transmitted by insects (Ling 1972). Conventional control of insect pests in rice cultivation often depends upon the use of chemically synthetic insecticides, which already cause public concern on food safety and environmental pollution. Recent progress in rice transformation technologies has made it possible to produce new rice cultivars with improved resistance to insect pests, diseases, and environmental stress by genetic engineering (Wu et al 1993).

Bacillus thuringiensis (*Bt*) insecticidal crystal protein genes are now widely used to develop insect-resistant transgenic crops (Gaser and Fraley 1992). Additional can-

didate genes for insect resistance, such as genes for proteinase inhibitors, are beginning to receive more attention (Ryan 1989, 1990). Insects use diverse proteolytic or hydrolytic enzymes in their digestive guts for digestion of food proteins and other food components (Wolfson and Murdock 1990, Masoud et al 1993). Plant-derived proteinase inhibitors are of particular interest because they are part of the natural plant defense system against insect predation. When foreign plant proteinase inhibitor genes were introduced into tobacco, transgenic tobacco plants expressing the proteinase inhibitor proteins at relatively high levels are resistant to typical tobacco insect pests (Hilder et al 1987, Boulter et al 1989, Johnson et al 1989).

As the first step in testing the usefulness of proteinase inhibitors for genetic engineering of insect resistance and producing insect-resistant rice, we set out to introduce two proteinase inhibitor genes into rice using our established rice transformation system. In this study, we introduced into rice the gene encoding a potato protease inhibitor II (PINII) and the gene encoding a cowpea trypsin inhibitor (CpTi). To warrant effective protection of transgenic rice against rice insect pests, the constitutively active and strong promoter of the rice actin 1 gene (*Acr1*) (Zhang et al 1991), or the wound-inducible *Pin2* promoter followed by the *Act1* intron, were used to drive the expression of the *Pin2* gene or *CpTi* gene in transgenic rice plants.

Materials and methods

Plasmids for rice transformation

The plasmid pTW (Fig. la) contains the *Pin2* promoter, the rice *Act1* 5' intron, the *Pin2* coding and 3' regions, the 35S promoter, the *bar* coding region and the *nos* 3' region. The plasmid pDM402 (Fig. 1b) contains the *Actl* promoter, the *CpTi* coding region, and the *nos* 3' region. The plasmid pDM307 (Fig. 1b) contains the 35S promoter, the *bar* coding region, and the *nos* 3' region.

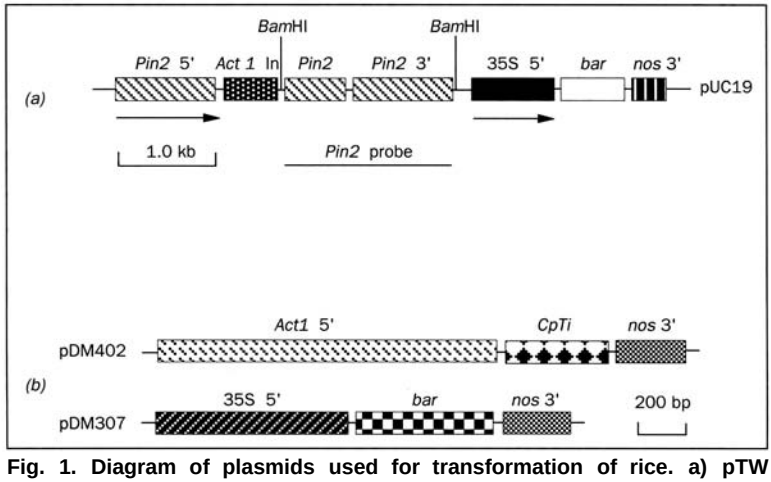

Fig. 1. Diagram of plasmids used for transformation of rice. a) pTW plasmid. b) pDM402 and pDM307: two plasmids used for cotransformation.

Production of transgenic rice plants

Suspension cultures were established from embryogenic calli induced from immature or mature embryos of rice (*Oryza sativa* L.) of three japonica varieties: Taipei 309, Tainung 67, and Pi4. Taipei 309 protoplasts were cotransformed with pDM307 and pDM402 (Zhang and Wu 1988). Suspension cells of Tainung 67 and Pi4 were bombarded with tungsten particles coated with the plasmid pTW as previously described (Cao et al 1992). One week later, the rice cells on filter papers were transferred to KPR medium (Kao 1977) containing 6 mg ammonium glufosinate L^{-1} (Crescent Chemical Co., Hauppauge, NY), and 5-6 wk later, resistant calli were obtained. Plants were regenerated in MS regeneration medium (Murashige and Skoog 1962) containing 3 mg ammonium glufosinate L^{-1}. Plants regenerated from the same resistant callus were regarded as clones of the same line. Regenerated plants were grown to maturity in a greenhouse (32 °C day/22 °C night, with a supplemental photoperiod of 10 h).

Analysis of transgenic rice plants

DNA blot analysis of transgenic rice plants. Integration of the transferred genes into the rice genome was analyzed by DNA blot hybridization using a DNA fragment containing the *CpTi* coding region or *Pin2* coding and 3' regions as the probe. Rice genomic DNA was isolated from leaves as described previously (Zhao et al 1989). For DNA blot hybridization analysis, 10-15 µg of DNA from each sample were digested by a suitable restriction endonuclease, separated on 1.0% agarose gel, transferred onto a nylon membrane, and hybridized with the ^{32}P-labeled *CpTi* or *Pin2* probe as previously described (Cao et al 1992).

Wounding treatment of transgenic rice plants and analysis of PINII protein. Plants or young tillers at the 4- to 6-leaf stage were used for wounding induction experiments and PINII protein analysis. Each plant (tiller) was wounded by making small cuts along both edges of one or two leaves using scissors. Effort was made to achieve uniform extent of wounding for each plant. The wounded leaves, nonwounded upper leaves, roots, and stem were sampled at 20 h after wounding and analyzed for the PINII protein levels. PINII protein levels in each part of a plant were analyzed in parallel in a nonwounded tiller from the same plant.

Field test of transgenic rice plants for insect resistance

The fourth generation (R_3) transgenic plants from two independent *Pin2*-transgenic lines were used for the field test under natural conditions. Both the first (R_0) and second generation (R_1) plants from which these R_3 plants were derived contained the *Pin2* gene in DNA blot hybridization and produced PINII protein, but the homozygosity of the R_1 plant was not confirmed. Thus, these R_3 plants are likely to represent a population that consists of homozygous and heterozygous transgenic plants, and segregated nontransgenic plants. Similarly, R_2 generation transgenic plants from a *CpTi*-transgenic line that produced a high level of *CpTi* protein were used for the field test.

For each transgenic line, transgenic plants as well as nontransformed control plants of the same rice variety were planted side by side in an isolated experimental

field of Zhejiang Agricultural University, Hangzhou, Peoples Republic of China. The ricefields in this area are often attacked by rice stem borers. The major rice insect pests in this area are striped stem borer (*Chilo suppressalis*) and pink stem borer (*Sesamia inferens*). In this field test, naturally occurring insect infestation began at the later flowering and milky stages during September 1994. During this period, infestation of rice plants by rice stem borers often causes hollow stem and dead panicle (commonly called whitehead). The insect damage symptoms were monitored and compared between transgenic plants and nontransformed control plants.

The field test was carried out according to the rules established in December 1993 by the State Science and Technology Commission of China.

Results

Efficient production and DNA blot hybridization analysis of fertile transgenic plants

Schematic maps of plasmids pTW, pDM402, and pDM307. used for rice transformation, are shown in Figure 1. Expression of the potato proteinase inhibitor II gene (*Pin2*) is regulated by its own promoter and 3' terminator sequence. The first intron of the rice actin 1 gene (*Act1*) (McElroy et al 1990) was inserted between the *Pin2* promoter and the *Pin2* coding region. In our previous study, the combination of the *Pin2* promoter and the *Act1* intron was demonstrated to confer high-level, wound-inducible expression of foreign genes in transgenic rice plants (Xu et al 1993). After transformation and regeneration, plants from more than 50 lines were analyzed by DNA blot hybridization; 73% of them were shown to contain the intact *Pin2* gene. More than 70% of the transgenic plants were fertile. In addition, 211 R_0 plants cotransformed with pDM402 plus pDM307 were regenerated. Of the 47 plants that were analyzed, 21 contained the intact *CpTi* gene.

Analysis of the levels of PINII protein or *CpTi* protein in transgenic plants

The levels of PINII protein in seven R_0 plants were determined. Twenty hours after wounding, the amount of PINII protein was between 0.6 and 1.9% of total soluble protein in different plants (without wounding, the PINII level was less than 20% of that in wounded samples). R_1 seeds from two transgenic lines, No. 6 (0.5% PINII) and No. 12 (0.6% PINII), were planted in China to produce R_2 seeds. More than 10 R_2 seeds from each R_0 line were separately grown to produce 10 R_2 and R_3 lines (e.g., 6-7-1, 6-7-2).

The level of CpTi protein in nine R_0 plants was determined. The amount of CpTi protein was between 0.3 and 2.7% of total soluble protein in different plants. R_1 seeds from line No. 2 (1.3% CpTi) were planted in China to produce R_2 seeds. Hundreds of R_2 seeds were produced from each of the R_1 plants and some were used for field tests.

As described in the "Materials and Methods" section, each subline of R_3 and R_2 seeds from the *Pin2*-containing and *CpTi*-containing transgenic plants represents a population that consists of homozygous and heterozygous transgenic plants, and segregated nontransgenic plants.

Small-scale field test of *Pin2*-containing transgenic rice plants for insect resistance

We used fourth generation (R_3) plants, which are from four R_2 plants of line No. 6 and four R_2 plants of line No. 12. These plants contained integrated copies of the *Pin2*-containing plasmid, pTW. The progeny of the same generation from transformation procedure-derived nontransformed plants were used as controls. Plants were grown in an experimental field in Hangzhou, China, a region that is routinely attacked by rice stem borers. Infestation of the rice plants by rice stem borers causes severe damage to the plants and often results in hollow stem and whitehead with no seed.

In the present field test under natural conditions, an apparent difference was observed between transgenic plants and nontransformed control plants in their response to the natural infestation of rice stem borers and development of whitehead symptom. The percentages of plants with whitehead symptom in the transgenic plant populations were much lower than that of nontransformed plant populations. More than 85% of the nontransformed plants showed the whitehead symptom, whereas an average of only about 40% (in line No. 6) and 16% (in line No. 12) of the transgenic plants showed the whitehead symptom (Table 1). In comparing the extent of damage to a single plant, it was often observed that insect infestation caused hollow stem or dead panicle in almost all tillers of a nontransformed plant, whereas no or only one tiller of a transgenic plant was damaged. At the time the R_3 seeds were planted for the field test, the homozygosity of these plants had not been confirmed. Thus, the R_3 plants used in the field test may represent a mixed population of homozygous and heterozygous transgenic plants together with segregated nontransgenic plants. Thus,

Table 1. Bioassay of R_3 transgenic rice plants harboring the *Pin2* gene for resistance to rice stem borers.

R_2 transgenic line	R_3 plants tested (no.)	Plants with whitehead symptoms[a] (no.)	Plants with whitehead symptoms(%)
6-7-1	16	8	50
6-7-2	24	16	67
6-7-3	42	16	38
6-7-4	36	11	31
Tainung 67 (Control)[b]	42	36	86
12-8-1	15	5	33
12-8-2	14	3	21
12-8-3	39	6	15
12-8-4	23	1	4
Pi4 (Control)[b]	33	29	88

[a] In scoring resistance/sensitivity to insect attack, if one shoot (tiller) on a plant was infested and showed the whitehead symptom, the whole plant was scored as sensitive (with whitehead). Only plants without infested shoots were scored as resistant. On average, each plant has eight shoots (or tillers). [b] Transgenic line no. 6 was derived from the rice variety Tainung 67, and transgenic line no. 12 was derived from the rice variety Pi4.

Table 2. Bioassay of R_3 transgenic rice plants harboring the *CpTi* gene for resistance to rice pink stem borer.

Rice plant (Taipei 309)	Tillers in 4 plants (no.)	Tillers with whitehead symptoms[a] (no.)	Tillers with whitehead symptoms (%)	Av weight of larvae[b] (mg)
Transgenic 2-3-4	36	8	22	66
Nontransformed	35	34	97	119

[a] Four larvae (second-instar, av weight = 25 mg) of pink stem borer were placed on each of four plants. The data on the number of tillers with whitehead symptoms were recorded 5 wk after insect infestation. [b] The weight of all larvae was measured 5 wk after insect infestation, and average weight of 16 larvae for each experiment is shown.

the observed differences in the extent of resistance/sensitivity phenotype in the transgenic plant population is likely to be associated with the segregation of the transgenes.

Small-scale greenhouse test of *CpTi*-containing transgenic rice plants for insect resistance

The R_3 plants from an R_2 line, 2-3-4, were tested in a greenhouse. Individual plants were grown in enclosed pots. Second-instar larvae of rice pink stem borer were applied to the rice plants at the early heading stage. The same generation of nontransformed rice plants (variety Taipei 309) derived from the transformation procedure was used as control.

We found that the transgenic plant population and the control plant population showed significantly different responses to insect infestation. All nontransformed control plants were severely damaged by the insect attack, and 97% of the tillers showed whitehead symptoms (Table 2). On the contrary, transgenic plants showed significantly increased resistance to infestation by the rice insects. In the transgenic plant population, only 22% of the tillers showed whitehead symptoms (Table 2).

Summary

We have introduced two serine-type trypsin inhibitor genes, one from potato, the other from cowpea, into rice. Expression of these dicot proteinase inhibitor genes derived by either the *Pin2* promoter followed by *Act1* intron, or by the rice *Act1* promoter results in high-level accumulation of functionally active inhibitor protein in transgenic rice. The transgenic rice plants showed increased resistance to two major rice insects in a small-scale field test or in a greenhouse test. Thus, proteinase inhibitor genes may be useful for genetic engineering of insect resistance in rice.

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The rice disease resistance gene, *Xa21,* encodes a receptor kinaselike protein

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A major goal of plant genome research is to develop and implement strategies for the isolation and engineering of agronomically important traits. The rice *Xa21* gene, which confers resistance to *Xanthomonas oryzae* pv. *oryzae* race 6, was isolated by map-based cloning. The sequence of the predicted protein, which carries both a leucine-rich repeat motif and a serine-threonine kinase-like domain, suggests a role in cell surface recognition of a pathogen ligand and subsequent activation of an intracellular defense response. Characterization of *Xa21* should facilitate understanding of plant disease resistance and lead to engineered resistance in rice.

Loci conferring disease resistance have been identified in virtually every plant species examined. Genetic analysis of many plant-pathogen interactions has demonstrated that plants often contain single loci that confer resistance against specific races of a pathogen containing a complementary avirulence gene (Flor 1971). Considerable effort has been directed at cloning plant genes conferring resistance to a variety of bacterial, fungal, and viral diseases and, recently, several disease resistance genes have been isolated from dicotyledonous species.

The deduced amino acid sequence of five of these genes reveals the presence of either a serine-threonine kinase (STK) or a leucine-rich repeat (LRR) domain (Staskawicz et al 1995). Kinases are important signaling molecules in diverse biological systems. LRR domains are found in a variety of proteins involved in signal transduction, cell adhesion, and various other functions. The tomato *Pto* gene conferring resistance to *Pseudomonas syringae* pv. *tomato* encodes a serine-threonine protein kinase, suggesting a role for *Pto* in a protein phosphorylation (Fig. 1). A second class of disease resistance genes all contain LRR motifs (Fig. 1). These include RPS2 (*Arabidopsis*), CF-9 (tomato), and N (tobacco) (Staskawicz et al 1995). Thus it appears that LRR- and STK-containing genes are widely conserved in virtually all plant species and clearly play a central role in resistance to diverse pathogens.

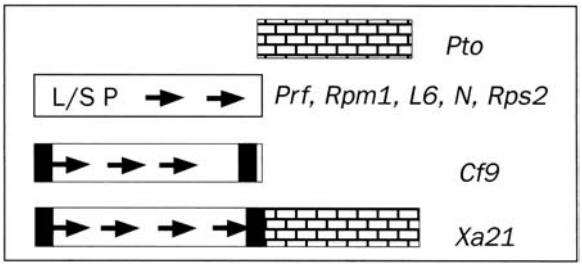

Fig. 1. Plant disease resistance genes: the predicted protein structure of the four known classes of disease resistance genes involved in cellular signaling. L/S = leucine zipper or signaling domain, P = nucleotide binding site, hatches = kinase domain, arrows = LRR do mains, black bar = signal peptide and transmembrane domains.

We have used a map-based cloning approach to isolate the resistance gene *Xa21* from the monocotyledonous species, rice. Compared with previously cloned genes, the structure of *Xa21* represents a novel class of plant disease resistance genes containing both STK and LRR motifs and supports a role for cellular signaling in plant disease resistance (Song et al 1995).

Map-based cloning in rice

Several genes have been isolated from *Arabidopsis* and tomato, using a map-based cloning strategy (Arondel et al 1992, Giraudat et al 1992, Martin et al 1993). Until recently, such techniques have been limited to dicotyledonous species.

Three members of the monocotyledonous family Poaceae (maize, rice, and wheat) provide most of the calories consumed by humans. Despite their agronomic importance, molecular genetic studies of monocots have been hindered by the large genome size of most of these plants. Recently, two high-density rice molecular genetic maps containing 722 and 1,500 markers and covering approximately 1,500 cM have been developed (Causse et al 1994, Shomura et al 1994, respectively). Many genes affecting important agronomic traits, such as disease resistance and drought tolerance, have been located on these maps. The saturated RFLP maps, the small DNA content (Arumunagathan and Earle 1991), the large percentage of low copy DNA (Deshpande and Ranjekar 1980), the availability of large insert genomic libraries (Wang et al 1995, Umehara et al 1994), and the ease of transformation (Hiei et al 1994) make rice a model monocot for molecular genetic studies and map-based cloning of agronomically important genes.

Bacterial blight disease of rice

Bacterial blight disease of rice, caused by *Xanthomonas oryzae* pv. *oryzae (Xoo),* provides an attractive system for studies of disease resistance because both the host and pathogen are amenable to molecular genetics. Races of *Xoo* that induce resistant or susceptible reactions on rice cultivars with distinct resistance *(Xa)* genes to the pathogen have been identified (Mew 1987). At the International Rice Research Institute, a new source of resistance *(Xa21)* was identified in the wild species *O. longistaminata* (Khush et al 1989). Unlike other *Xa* genes identified, the dominant resistant locus *Xa21* confers resistance to all Indian and Philippine races of *Xoo* tested (Ikeda et al 1990).

Isolation of *Xa21*

Construction of a high-resolution map of the *Xa21* locus

We previously identified six chromosomes and 11 markers linked to the *Xa21* locus (Ronald et al 1992; Williams and Ronald 1995, University of California, Davis, unpubl. data). One of these markers, RG103, hybridized with eight genomic DNA fragments in the resistant indica line, IRBB21, and cosegregated with the *Xa21* locus in 1,100 F_2 progeny. The deduced amino acid sequence of RG103 showed 20-30% identity to diverse proteins carrying LRR motifs.

Isolation of bacterial artificial chromosome and cosmid clones carrying the RG103-hybridizing sequences

The analysis described above suggested that the RG103-hybridizing DNA fragments in line IRBB21 may include the *Xa21* gene. To test this hypothesis, RG103 was used as a hybridization probe to isolate clones from bacterial artificial chromosome (BAC) and cosmid libraries (Song et al 1995, Wang et al 1995).

BAC libraries were first described by Shizuya et al (1992) and used to clone large DNA fragments of the human genome. This system utilizes an F factor-based vector and is capable of maintaining human genomic DNA fragments of >300 kb. Compared with yeast artificial chromosome (YAC) cloning, DNA can be cloned with high efficiency, manipulated easily, and stably maintained in *Escherichia coli.* Application of the BAC cloning system to plant species has greatly facilitated map-based cloning efforts.

Complementation analysis

Sixteen partially overlapping subclones representing seven of the RG103-hybridizing genomic fragments were transformed into the normally susceptible line, *O. sativa* ssp. japonica var. Taipei 309, using particle bombardment (Song et al 1995). For each subclone, approximately 15 independently transformed lines were generated. On average, six plants were subsequently propagated clonally from each independently transformed line. Four months after bombardment, 1,500 transgenic plants carrying 16 different subclones were inoculated with *Xoo* to test for resistance. Fifty plants,

arising from nine independently transformed lines containing a 9.6-kb *Kpn*I subclone showed a reduction in lesion length as compared with susceptible controls. Bacterial growth curve analysis demonstrated that growth of the pathogen was reduced 10- and 100-fold in transgenic 106-22 carrying the 9.6-kb *Kpn*I subclone as compared with IRBB21 and the recipient line Taipei 309, respectively. These results indicate that the transgenic lines containing the 9.6-kb subclone have gained resistance to *Xoo.* Because *Xa21* is the only known chromosome 11 locus that confers resistance to *Xoo* race 6, we have designated the gene contained on the 9.6-kb subclone *Xa21* (Song et al 1995).

To localize *Xa21* on the 9.6-kb *Kpn*I subclone, we carried out additional transformations with four partially overlapping DNA subclones from this region. These experiments delineated a 2.3-kb *Hind*III DNA fragment required for full resistance activity. Sequencing of the 9.6-kp *Kpn*I genomic fragment revealed a single large ORF of 3075 bp, interrupted by one intron of 843 bp. Sequencing of cDNAs indicates that the intron is processed as predicted in both IRBB21 and the transgenic line 106-17 (Song et al 1995). In Northern blot experiments of IRBB21, four bands hybridize with RG103. The largest band of approximately 3.1 kb is consistent with the size of the full-length cDNA isolated from the 106-17 (Song et al 1995).

Structure of *Xa21*

Analysis of the derived 1025 amino acid sequence of *Xa21* revealed a novel class of a plant disease resistance gene product with several regions with similarity to known protein domains (Fig. 1). The amino terminus encodes 23 hydrophobic residues characteristic of a signal peptide. The central core of *Xa21* consists of 23 imperfect copies of a 24-aa LRR followed by a 26-aa hydrophobic stretch that is likely to form a membrane spanning helix. The carboxyl terminal sequence encodes a putative intracellular protein kinase catalytic domain. This region carries the 11 subdomains and all 15 invariant amino acids diagnostic of protein kinases. The putative extracellular domain containing LRRs of *Xa21* revealed similarity to the tomato resistance gene *Cf9* (54.9% similarity; 32.5% identity) (Fig. 1). The STK domain of *Xa21* is most similar to the SRK-related proteins (56.3%; 33.7% to SRK-29), which is thought to mediate self-recognition between pollen and stigma during pollination (Stein et al 1991) and to the tomato *Pto* resistance gene product (56.5%; 30.6%) (Fig. 1). The overall structure of *Xa21* resembles a receptor kinase.

Receptor kinases

Receptor protein kinases mediate cellular signaling processes in diverse biological systems. In many animal systems, the binding of ligands to the extracellular receptor domain of these proteins activates a cytoplasmic tyrosine kinase domain. This interaction leads to a variety of cellular responses such as cell proliferation, differentiation, and survival (Fantl et al 1993). The few plant receptor kinase-like proteins that have been studied to date carry serine-threonine specificity in the kinase domain (Walker 1994). Based on models from mammalian systems, we hypothesize that *Xa21*

has a role in cell surface recognition of a pathogen ligand and subsequent activation of the intracellular STK leading to a defense response.

Future directions

Molecular studies of *Xa21* should provide clues regarding its mode of action and an understanding of the basis by which it confers broad-spectrum resistance. We have observed that *Xa21*-hybridizing sequences are clustered at the *Xa21* locus (Desphande and Ranjekar 1980). Similarly, the *Pto, M, Cf9,* and *N* resistance genes are all members of clustered gene families (Staskawicz et al 1995). In addition to the *Xa21* gene family, there are at least seven major genes and one quantitative trait locus encoding resistance to viral, bacterial, and fungal pathogens clustered within 30 cM on chromosome 11 (Mackill and Bonman 1992, Song et al 1995). Sequence comparison of the members of the *Xa21* gene family and linked disease resistance genes may lead to clues regarding evolution of plant disease resistance.

Ultimately, a better knowledge of the signal transduction pathway in plants should facilitate the designing of new approaches for disease control. For instance, intra- and intergeneric transfer of disease resistance genes may provide an additional tool for breeders in combating plant disease. Our results indicate that *Xa21* functions in diverse rice species and subspecies to reduce infection, and that genes encoding subsequent signal transduction components are conserved. Results from other laboratories suggest that intergeneric transfer of disease resistance genes may provide novel resistance to pathogens. For example, the tomato *Pto* and *Cf9* resistance genes function in the closely related genus tobacco to confer a defense response to strains of *Pseudomonas syringae* pv. *tabaci* and to the AVR9 peptide, respectively (B. Staskawicz, University of California, Berkeley, and J. Jones, Sainsbury Institute, Norwich, England, pers. commun.). Because the phytobacterial genus *Xanthomonas* infects virtually every crop species worldwide, future engineering and transfer of the *Xa21* resistance gene may help reduce loss to *Xanthomonas* infection in recipient species.

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Molecular analysis of the interaction between *Xanthomonas oryzae* pv. *oryzae* and rice

F.F. White, J.M. Chittoor, J.E. Leach, S.A. Young, and W. Zhu

Bacterial blight of rice, caused by *Xanthomonas oryzae* pv. *oryzae (Xoo),* is the most devastating bacterial disease of rice in Asia. Future genetic strategies to improve resistance will benefit from a better understanding of the molecular events that mediate resistance in the interaction of host and pathogen. We have characterized two avirulence genes, *avrXa10* and *avrXa7,* which trigger resistance in rice lines carrying the resistance genes *Xa10* and *Xa7,* respectively. The two *avr* genes are members of an avirulence gene family that appears to be exclusive to *Xanthomonas.* In efforts to further understand the modes of action of the genes, the interdependence of *avr* and *hrp* gene function is being investigated. Activity of *avrXa10* requires a functional *hrp* regulon. The dependence does not appear to involve *avr* gene regulation or lack of bacterial growth in *hrp* mutants, and the hrp genes cannot rescue avr activity when supplied in planta by mixed bacterial strain inoculation. The results suggest that *avr* activity requires that both type of genes be present in the same bacterial cell. The *hrp* secretory pathway may be required for avr protein secretion or for secretion of an elicitor whose synthesis is catalyzed by *avr* protein products. At the same time, peroxidase and related host genes that are induced during a resistance reaction are being characterized. Cationic peroxidase PO-C1 was localized to xylem lumen and parenchyma cell walls at the site of infection by an incompatible strain of *Xoo.* Peroxidase transcript levels appear to peak within 12 h of challenge with bacteria.

Bacterial blight, caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is the most devastating bacterial disease of rice in Asia (Ou 1985). Rice is a major target for crop improvement, and several strategies to improve disease resistance in rice through genetic engineering have been proposed (Toenniessen 1991). One approach involves the introduction of cloned disease resistance genes into rice cultivars (for review, see

Martin 1995). Blight has been controlled through the use of single-gene resistance introduced by traditional breeding methods. Genetically engineered resistance would expedite traditional breeding methods, facilitate the introduction of multiple resistance genes (pyramiding), and provide a wider range of sources for resistance genes. Although inexpensive and environment friendly, host-plant resistance has often been unstable. Instability of resistance to *Xoo* has been attributed to changes in the pathogen population as new virulent strains arise (Mew et al 1992). The use of genetic resistance will benefit from a better understanding of the molecular interactions between the pathogen and plant. Subsequent manipulation of the resistance genes may allow the design of more stable resistance.

Race specificity and *avr* genes

To identify critical factors involved in the elicitation of defense to bacterial invasion, we have been using molecular genetic analyses of both the pathogen and the plant. In interactions between *Xoo* and rice, resistance is governed by an interaction between single, dominant resistance genes (R genes) in rice and corresponding pathogen genes called avirulence (*avr*) genes (Mew 1987, Leach and White 1995). The products of *avr* genes control factors that elicit a plant resistance response. Races of *Xoo* are defined by the presence (or expression) of a unique combination of *avr* genes in the pathogen. Race 2, for example, should contain *avrXa10, avrxa5,* and *avrXa7.*

We have cloned and characterized two avr genes (*avrXa7* and *avrXa10)* from *Xoo* (Hopkins et al 1992). *Xoo* strains containing the cloned *avr* genes acquired the ability to elicit resistance when inoculated to rice cultivars with the corresponding resistance genes (*Xa7* and *Xa10*). The *avrXa7* and *avrXa10* genes are members of a gene family from *Xanthomonas* that are typified by the first cloned member of the family, *avrBs3* from *X. campestris* pv. *vesicatoria* (Bonas et al 1989). Strains of *Xoo* contain 12-14 copies of genes related to *avrXa7* and *avrXa10* (Hopkins et al 1992). To date, members of this gene family have been cloned from pathogens of such diverse hosts as pepper (Bonas et al 1989, Canteros et al 1991), cotton (De Feyter and Gabriel 1991, De Feyter et al 1993), citrus (Swarup et al 1991, 1992), and rice (Hopkins et al 1992). The gene family has not been identified in other genera.

The structure of the *avr* gene family from *Xanthomonas* is striking in that the middle third of the encoded protein of each member is a repeated sequence of 34 amino acids (Fig. 1). The number of repeats in an individual gene varies from 13.5 copies for *avrb6* from *X. campestris* pv. *malvacearum* to 25.5 copies in *avrXa7* (Bonas et al 1989, De Feyter et al 1993, Hopkins et al 1992), and are highly conserved with the exception of amino acids at positions 12 and 13. The sequence of positions 12 and 13 of each repeat is referred to as the variable region (Fig. 1). All members of the gene family encode relatively large polypeptides in the range of 120 kd. Although some intriguing structural features have been suggested based on the amino acid sequence (Yang and Gabriel 1995), no similarities to other proteins with known biochemical functions have been identified.

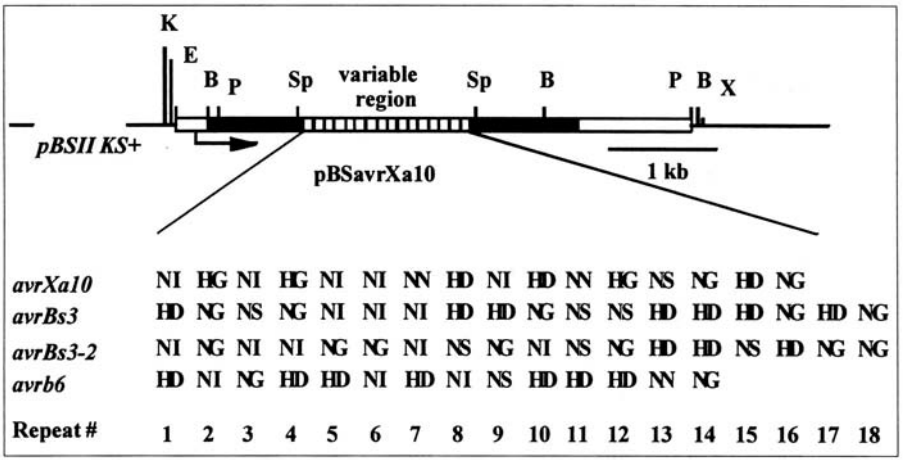

Fig. 1. *avrBs3* gene family structure. Map of pBS*avr*Xa10 is shown as representative of the family. Amino acid repeat structure of the variable region for four of the members is shown below the map. Only the highly variable amino acids of the 12th and 13th codon of each of the 34 amino acid repeats are shown. Single letter code is used for amino acids. B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; P. PstI, Sp, *Sph*I; X, *Xba*I.

The arrangements of the variable regions of all characterized genes vary and appear to be critical features for the race specificity of the proteins. Some deletions in repeats of *avrBs3* resulted in the loss of avirulence activity on pepper plants containing the *Bs3* gene for resistance and, concomitantly, gained new avirulence activity on tomato (Herbers et al 1992). Upon replacement of the repeat region of *avrXa10* with the region of *avrXa7* and *avrBs3,* the resulting phenotype was found in our laboratory to confer specificity that corresponds to the source of the repeat domain (Fig. 2). The *avrBs3* repeat domain in *avrXa10* conferred a resistant phenotype to *X.c.* pv. *vesicatoria* on pepper containing the *Bs3* gene, and a hypersensitive reaction (HR) response to *Xoo,* which normally does not give an HR on the nonhost plant pepper, when inoculated on pepper (Fig. 2). The *avrXa7* repeat in *avrXa10* conferred specificity for the resistance gene *Xa7* and lost specificity for *Xa10* (Fig. 2).

Some homologs of *avrBs3* appear to have a role in aggressiveness or fitness of the pathogen. This property was first recognized for the *pthA* gene of *X. campestris* pv. *citri* and has been described for other members of the gene family (Swarup et al 1991, De Feyter et al 1993). The *avrXa7* gene of *Xoo* also appears to act in this manner. If *avrXa7* is inactivated, the mutant strains are less aggressive on a susceptible cultivar (Fig. 3). These results may help explain the retention of *avr* genes, in general, and, more specifically, the presence of many copies of the genes in *Xoo.* The introduction of new plant genotypes to control the pathogen may select for new versions of the gene family that avoid recognition for resistance and retain functions for aggressiveness.

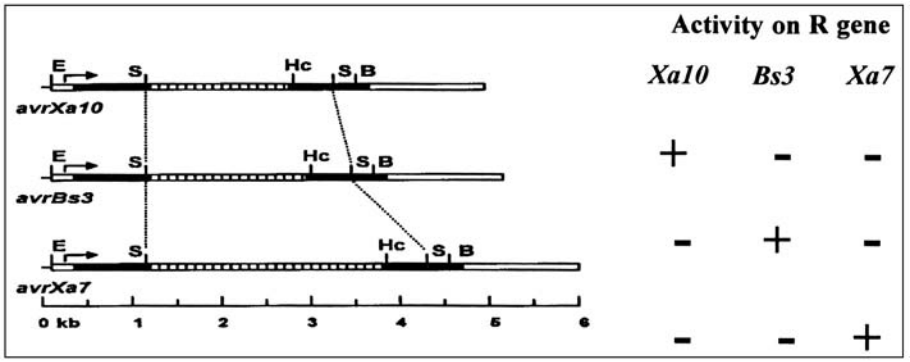

Fig. 2. Effect of repeat region on avirulence activity. The *SphI* fragments of avirulence genes *avrBs3* and *avrXa7* were substituted for the *SphI* fragment of *avrXa10,* introduced into *Xanthomonas* and tested on appropriate plant cultivars. The *avrBs3* construct was tested in both *X.c.* pv. *vesicatoria* and *Xoo. Xoo* without *avrBs3* is not pathogenic and does not elicit an HR on pepper. +, HR; -, pathogenic or no response.

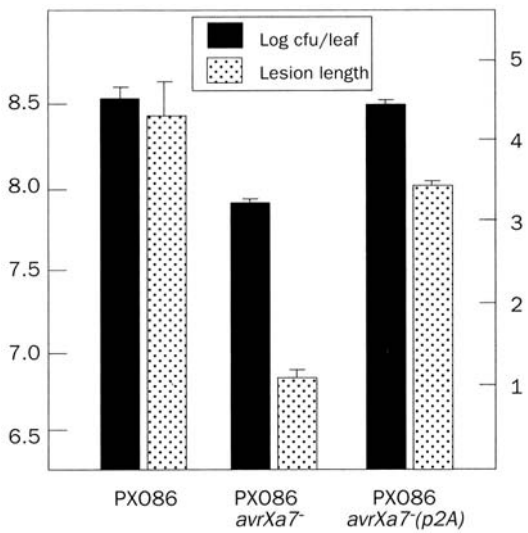

Fig. 3. Effect of loss of *avrXa7* activity in *Xoo.* Bacterial cell numbers (cfu; cell-forming units) and lesion lengths were measured after inoculation of rice cultivar IR24 with strain PX086 and derivatives. Plasmid p2A contains a copy of *avrXa7* and was used for complementation of *avrXa7* mutation.

Avirulence gene function

Several models for avirulence gene function have been put forward to explain the molecular basis for induction of resistance in race-specific interactions (Leach and White 1995). The model that predicts the avirulence gene product as the elicitor of resistance would appear to be the simplest explanation for the *avrBs3* family (Fig. 4, Model A). If this model operates in the *Xoo*/rice interactions, the products of the *avr* genes would be secreted outside the bacterial cells where they could interact with the plant cell. A consensus signal peptide has not been found at the amino terminus of any of the *avrBs3* family members. Nor has the product of the *avrXa10* gene, like that of *avrBs3* (Brown et al 1993), been shown to be located extracellularly (Young et al 1994). In addition, neither the *avrXa10* gene product, which was purified from *E. coli* or *Xoo* cells, nor the extracellular fluids of rice leaves undergoing an incompatible response elicited resistance in rice plants containing *Xa10* (Young et al 1994).

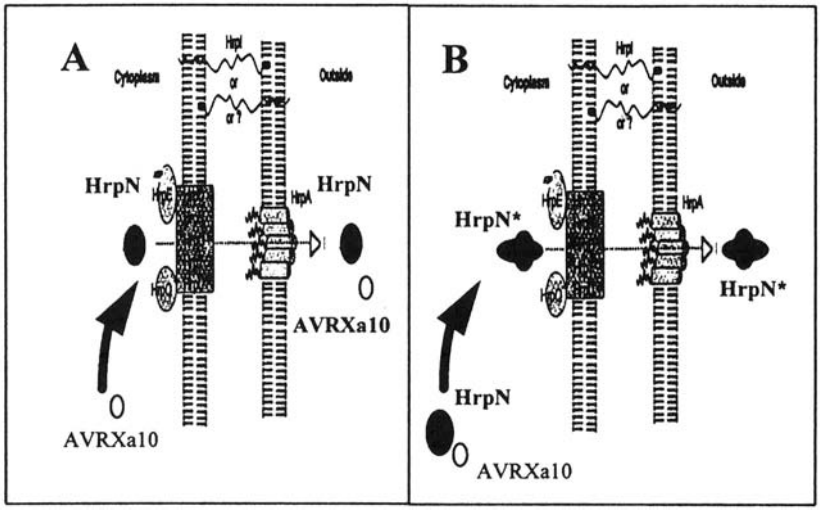

Fig. 4. Models for *avrXa10* and related gene function in avirulence. A, the *avrXa10* gene product or some processed form is secreted extracellularly via *hrp* secretory apparatus. B, the *avrXa10* gene product modifies a harpin-like protein, which is in turn secreted by *hrp* secretory apparatus. Schematic of *hrp* apparatus is modified from Van Gijsegem et al (1994).

The *hrp* gene cluster and avirulence activity

The evidence that *avrXa10*-like gene products do not function outside the bacterial cell, although compelling, remains inconclusive. Bacterial proteins can be exported through alternate secretion pathways without the need for signal sequences (Salmond and Reeves 1994), and some exported proteins require modification for activity (Hughes et al 1992). Thus, the *avr* gene products may function as elicitors and may not have been detected outside the bacterial cells. In future studies, purification of the race-specific elicitors may be facilitated by artificially induced growth conditions that mimic the *in planta* conditions.

One class of genes likely to be involved in the production of the race-specific elicitors from *Xoo* are the *hrp* (hypersensitive reaction and pathogenicity) genes. The *hrp* genes are known to be involved in the production of non-race-specific protein elicitors (elicitors not associated with race-specific or single gene resistance) (Bonas 1994). Three extracellular protein elicitors whose synthesis and secretion are directed by *hrp* gene clusters have been identified from *Erwinia amylovora* (Wei et al 1992), *Pseudomonas syringae* pv. syringae (He et al 1993), and *Pseudomonas solanacearum* (Arlat et al 1994). The first two proteins have been given the generic name harpin after the *hrpN* gene of *E. amylovora.* The *hrp* regions of *Xoo* (our unpublished results) and *X.c.* pv. *vesicatoria* are similar to the *hrp* regions of *P. solanacearum* (Bonas 1994).

The *hrp* genes also are required for avirulence gene function in *X.c.* pv. *vesicatoria* and *Xoo* (Bonas et al 1989, Knoop et al 1991, Schulte and Bonas 1992, our unpublished results). Mutations in the *hrp* loci, in general, result in the loss of avirulence activity on the appropriate cultivar. The reasons for the requirement are not clear. Avirulence genes from *P.s.* pv. *syringae* are typically coinduced with the *hrp* genes, and activity is, at least in part, dependent on *hrp* functions that are involved in *hrp* gene regulation. However, the *hrp* genes do not appear to be involved in the regulation of *avrBs3* (Knoop et al 1991) or *avrXa10* (our unpublished results). Another possibility for the dependence is that elicitor quantities may not be adequate to induce resistance due to poor *in planta* growth of strains with *hrp* mutations. However, our studies indicate that *hrp*-deficient strains of *Xoo* multiply to the same level as wild-type bacteria that elicit a resistance response in plants. In addition, avirulence function in *hrp*-deficient bacteria is not restored by growing the mutants in the presence of *hrp+* bacteria. Thus, inadequate bacterial multiplication does not account for the lack of elicitor activity, and *avrXa10* and related genes need to be in cells with a functional *hrp* cluster.

In general, the *hrp* genes are induced *in planta* (Bonas 1994), and we are attempting to characterize the transcriptional control mechanisms to induce *hrp* function *ex planta.* In *P.* solanacearum, *hrp* gene transcription is directly or indirectly under the control of the *hrpB* gene, which codes for a homolog of the AraC/XylS family of transcriptional regulators (Genin et al 1992). Mutations in *hrpB* eliminate expression of *hrp* genes in *P. solanacearum.* Independently, a homolog of the *hrpB* was found in *Xoo* and was termed *hrpXo* (Kamdar et al 1993). The involvement of *hrpXo* in the transcriptional regulation of *hrp* genes in *Xoo* was not determined. We

have constructed a b-glucuronidase fusion with the reporter of the *hrpC* operon of *Xoo* and are investigating the role of *hrpXo* in the regulation of *hrp* transcription. We hope that manipulation of culture components and/or constitutive expression of the transcriptional regulator will allow expression of the *hrp* genes in liquid culture and facilitate the control of conditions for elicitor purification.

Defense response of rice to *Xoo*

Xoo is primarily a vascular pathogen that enters the plant through hydathode water pores or wounds (Tabei 1977, Mew et al 1984). Antibacterial compounds have been isolated from healthy leaves of susceptible and resistant rice cultivars, some of which were oxidized lignin components with aldehyde and phenol groups (Horino and Kaku 1989). Lignin polymers accumulate in inoculated leaves during the resistant interaction between rice cultivars carrying the *xa5, Xa7,* and *Xa10* genes for bacterial blight resistance and strains of *Xoo* carrying the corresponding avirulence genes (Reimers and Leach 1991). Lignin and other phenolic polymers serve as physical bamers (Ride 1983) and, as such, probably would not be an effective defense against *Xoo,* a vascular pathogen, unless lignified materials prevented bacterial spread by blocking vessels. Peroxidases are the last enzymatic step in lignin biosynthesis, that is, the oxidation of hydroxy cinnamyl alcohols into free radical intermediates, which subsequently are coupled into lignin polymers. Although a role for peroxidases in defense responses has not been clearly demonstrated, increases in peroxidase activity have been correlated with infection in many plant species (Kolattukudy et al 1992).

Localization of the cationic peroxidase PO-C1

After infiltration of rice cultivars containing the *Xa10* gene for bacterial blight resistance with strains of *Xoo* containing *avrXa10,* total peroxidase activity increased and several changes in the peroxidase isoenzyme profile occurred, including the appearance of a cationic peroxidase and increased activities of two anionic peroxidases (Reimers et al 1992). PO-C 1 (pI 8.6, apparent M_r 43 kd) was further characterized. The enzyme was purified, partially sequenced, and domain-specific antibodies were generated to a synthetic peptide derived from a sequence of PO-C1 that was diverged from other plant peroxidases and shown to be specific for PO-C1 (Young et al 1995). Immunolocalization studies provided evidence that PO-Cl is located in the xylem vessels. First, PO-C1 accumulates in guttation fluids of resistant plants within 24 h of exposure to avirulent *Xoo* strains and was not detected in guttation fluids of plants undergoing susceptible interactions until a later time (48 h). Second, anti-PO-C1 antibodies were observed on the cell walls and lumen of xylem vessels within 24 h after inoculation with avirulent strains of *Xoo* (Fig. 5). Induced PO-C1 also was detected in the parenchyma cells adjacent to the xylem vessels, suggesting that the isoenzyme is expressed in neighboring cells and distributed to the anucleated xylem vessels. The accumulation and location of PO-C1 in xylem elements are consistent with a role for the peroxidase in rice defense responses against *Xoo.*

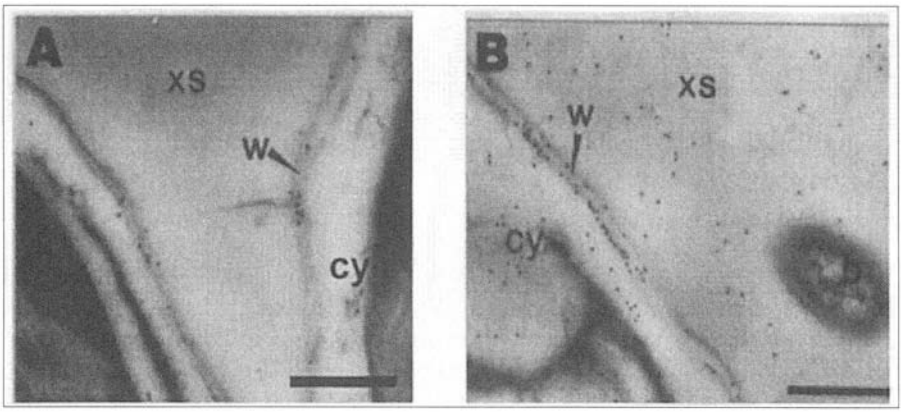

Fig. 5. Localization of PO-C1. Immunogold labeling of cationic peroxidase PO-C1 at 24 h after infiltration during (A) compatible and (B) incompatible reaction of rice and *Xoo*. w, cell wall; xs, extracellular space; cy, cytoplasm. Bar = 0.5 mm.

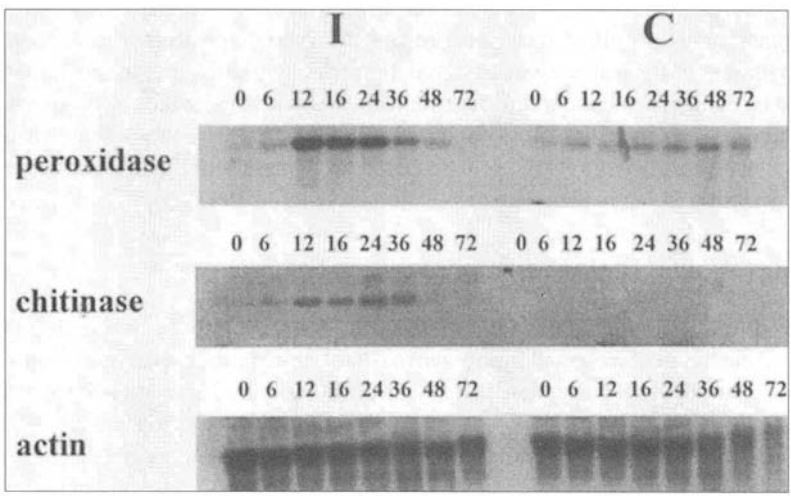

Fig. 6. Defense gene induction. mRNA was isolated from incompatible (I; resistant) and compatible (C; susceptible) interactions of *Xoo* and rice at the hours after inoculation as indicated above the lanes. The mRNA was subjected to electrophoresis, blotted, and hybridized with probes for peroxidase (3' end of OSPER, Reimmann et al 1992); chitinase (Anuratha et al 1992); and actin (McElroy et al 1990).

As part of the characterization of the peroxidase isozymes that are induced during the rice/*Xoo* interaction, cDNAs from resistant and susceptible interactions are being isolated. In conjunction with increases in peroxidase activity, mRNA corresponding to peroxidase increases to peak levels at 6 h in the incompatible (resistant) reaction. Peroxidase mRNA also increases in compatible reactions. However, peak activity levels are lower and occur at 36 h (Fig. 6). Messenger RNA of peroxidase and

other genes begins to degrade in the compatible reactions by 48 h. Chitinase (a defense-associated protein) mRNA levels increase up to 24 to 36 h, yet do not appear in the compatible response (Fig. 6). A variety of cDNAs that correspond to the mRNA in the different interactions have been or are in the process of being isolated. Hybridization analysis using the 3' end of each cDNA will allow further delineation of the expression pattern for each gene.

Summary and future directions

The avirulence genes that have been identified from *Xoo* are multicopy members of a gene family, and many questions remained regarding their role in the *Xoo*/rice interaction. Some homologs of the *avr* genes may correspond to R genes in rice other than *Xa10, Xa7,* or *xa5.* The *Xa21* gene recently cloned from rice contains features in common with other R genes (P. Ronald, University of California, Davis, 1995, pers. commun.), and is a particularly interesting case in point. The broad-spectrum resistance of the gene may be due to recognition of one or several of the *avrXa10* homologs. Identification of the avirulence gene or genes that correspond to *Xa21* will provide a better understanding regarding the stability and utility of the gene.

The mode of action of most avirulence genes including the *avrBs3* family remains unknown. Despite the results of the localization studies, it remains reasonable to speculate that *avrXa10* and related gene products are the elicitor molecules that interact with the resistance gene product in a signal transduction complex. Our current direction is to determine the interaction of the *avr* gene products with *hrp* genes, and whether some portion of the *avr* protein is externalized via the *hrp* secretory apparatus. An additional possibility is that the protein is directly transferred to the plant cell and never occurs in the external growth medium (Yang and Gabriel 1995). If supportive evidence for the hypothesis is found, physical alterations in the avirulence protein will be correlated with changes in elicitor activity, binding and, in the case of *avrXa7,* aggressiveness. An understanding of the interaction between elicitor and R proteins may allow the engineering of new and more stable resistance.

An alternative approach to introducing R genes for rice improvement involves the introduction or enhanced expression of genes whose products either inhibit pathogens directly (e.g., lysozyme, antimicrobial peptides, proteinase inhibitors) or are involved in the biosynthesis of toxic compounds (Toenniessen 1991). The success of these approaches will depend on expression and targeting the antimicrobial enzymes to the proper tissue. Perhaps even more important, new and more effective strategies will be evident as we clarify the physiological and molecular events of resistance. The characterization of peroxidase will provide both insight regarding the functions required for resistance, as well as information on how enzymes are targeted to the diseased tissue.

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Notes

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Exploring the application of molecular markers for improving resistance to bacterial blight

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Bacterial blight of rice, caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is endemic in many parts of Asia and periodic epidemics can cause severe yield losses. Although many genes for resistance to bacterial blight are known, few have been systematically used for rice improvement. To allow the judicious use of resistance, it is important to understand the population biology of the pathogen and to understand the genetics of resistance. Molecular marker technology has contributed to both of these objectives. A series of molecular markers was developed for analysis of *Xoo* populations, including four transposable elements, a family of avirulence genes, and a set of polymerase chain reaction-based markers. One of these primers can be used for DNA fingerprinting of pathogen cells oozed directly from infected leaves. To shed light on the diversity and distribution of *Xoo,* a hierarchical analysis of variation was conducted. Pathogen populations from different Asian countries were distinct. Intensive sampling was conducted in the Philippines, revealing population differentiation between regions, sites, and even fields within a site. Certain DNA fingerprint types (haplotypes) were widely distributed (even between countries), providing evidence of pathogen movement. By subsampling the dataset, an optimized sampling strategy could be recommended for subsequent studies. Resistance genes for bacterial blight have been tagged and mapped in several laboratories. Using restriction fragment length polymorphism-based methodology, even genes with minor effects on disease (putative quantitative trait loci) have been detected. Based on inoculation data, gene combinations likely to be effective for disease reduction in the field were designed. Several rice lines carrying multiple genes have been produced by marker-assisted selection. In some cases, such as in the *Xa4* + *xa5* line, plants carrying pairs of resistance genes show

greater resistance than expected based on the performance of the individual genes. Experiments on the deployment of resistance genes are being conducted in Laguna, Philippines. These studies are making use of near-isogenic lines carrying different resistance genes, as well as mixtures and pyramid lines. Analysis of pathogen subpopulations sampled from different treatments indicated which genes are functionally distinct in this environment. Lines carrying *xa5,* expected to be resistant, showed low levels of disease due to selection of a rare pathogen lineage. Now that powerful and inexpensive methods are available for analyzing pathogen populations and for identifying resistance loci, it will be possible to deploy resistance more effectively.

Bacterial blight (BB), caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is endemic in many parts of Asia. The disease became prominent in the 1960s, when new high-yielding varieties were first developed and introduced (Mew 1987). Varietal resistance is the main strategy for controlling the disease. The *Xa4* gene for BB resistance was introduced from TKM6 into improved varieties and has provided durable resistance to BB in China for 2 decades (Bonman et al 1992). In Southeast Asia, however, compatible races have developed. Over the 10-yr period following the release of varieties carrying the *Xa4* gene for resistance in the Philippines, pathogen races able to overcome the gene became increasingly frequent in the pathogen population (Mew et al 1992).

Perhaps because of the substantial quantitative resistance incorporated into most of the improved varieties (Koch and Parlevleit 1991a), and in part due to residual resistance conditioned by the *Xa4* gene (Koch and Parlevleit 1991b), the disease has rarely reached epidemic proportions in recent years. However, severe epidemics still occur periodically. For instance, the rice crop in the Punjab region of India was badly damaged by BB in 1995. Now, particularly as new rice types are being developed to meet the food requirements of increasing populations, there is a need to devise strategies for the deployment of our finite genetic resources for BB resistance.

Although many genes for resistance to *Xoo* are known, few of these genes have been systematically deployed in improved rice cultivars. Molecular marker technologies have been applied to the BB pathosystem, leading to greater knowledge about both pathogen and host. Many BB resistance genes have been mapped on the rice chromosomes, and several have been tagged with molecular markers, and one has recently been cloned. These advances should make it possible to deploy resistance genes more efficiently and effectively. This paper reviews recent research progress that could be of value in designing strategies for the deployment of resistance to BB.

Progress in analysis of populations of *Xoo*

Virulence analysis

To determine the pathotypic diversity of Philippine populations of *Xoo* and to assess differentiation of the pathogen over time and across regions, extensive surveys of the BB pathogen were conducted in the Philippines in the 1970s and 1980s (Mew et al 1992). To allow pathogen virulences to be determined, a set of differential cultivars was developed, including varieties and lines carrying *Xa4, Xa10, xa5, Xa7,* and *Xa18* (Mew and Vera Cruz 1979, Mew et al 1982, Vera Cruz and Mew 1989). Based on these studies, six strains of the pathogen were selected to represent the six races detected, and these have been widely used to represent the diversity of the pathogen in the Philippines, at IRRI and elsewhere. Strong spatial and temporal differentiation among pathogen collections was observed in the Philippine pathogen population (Mew et al 1992).

Although pathotypic analysis has provided a clear picture of race diversity and distribution, it does not allow the evolutionary relationships among pathotypes to be discerned. Inoculation tests require considerable time, space, and labor. In an effort to learn more about race evolution of *Xoo,* and in the hopes of developing efficient techniques for monitoring pathogen populations, molecular markers were utilized to characterize pathogen collections (reviewed by Leach et al 1995).

Development and utilization of DNA markers for *Xoo*

Underlying initial efforts at DNA fingerprinting of *Xoo* was the hope that the groups of strains distinguished by molecular markers would correspond closely with the races distinguished by inoculation tests. The relationship between phylogeny (as inferred from DNA fingerprinting analysis) and pathotype has not turned out to be that simple. The first study of DNA fingerprint of *Xoo* was based on the repetitive element pJEL101, cloned from the genome of the pathogen itself (Leach et al 1990). Leach et al (1 992) used this element to analyze a subset of the Philippine collection developed by Mew et al (1992), which included strains representing the six recognized races. Five groups of strains were distinguished at the 75% similarity level. Some groups contained strains from more than one race, and some races were found in more than one group.

To further investigate the relationship between phylogeny and virulence, Nelson et al (1994) isolated four of transposable elements from *Xoo,* one of which proved homologous to the element used in the study of Leach et al (1992). In addition to the transposable elements, an avirulence gene probe was also utilized as a hybridization probe for population analysis. This probe, cloned by Hopkins et al (1992), carried the gene *avrXa10,* a member of a family of avirulence genes in *Xoo* and other xanthomonads. Based on the results of bootstrap analysis, we found that some probes gave much more stable phenograms than others. The avirulence gene probe did not provide more useful information than the "random" fingerprinting probes. Relatively stable phenograms were derived from the restriction fragment length polymorphism (RFLP) datasets obtained using *IS1112* (the pJEL101 homolog) and *IS1113.* The phenograms derived from these probes gave relatively robust phenograms, and results from the two were fairly consistent.

From analysis using these probes, we were able to make inferences about the evolution of the Philippine races of *Xoo.* Race 1 was predominant in Central Luzon prior to the wide cultivation of varieties carrying *Xa4,* and was later replaced by race 2, which could overcome *Xa4.* Strains of race 2 were found to more closely resemble strains of race 5 than those of race 1. Race 2 was thus apparently not derived by mutation from race 1, but rather was derived from race 5 by mutation and migration. Race 3, which can overcome *Xa4* and which has become prevalent in parts of Luzon (Ardales et al 1996), is closely related to race 1.

Discrimination of "new" races based on DNA fingerprinting

For three of the races, strains designated as belonging to the same race were found in distinct clusters based on DNA fingerprint analysis. Strains belonging to the same race group but representing different lineages were tested on rice varieties and near-isogenic lines carrying previously untested resistance genes. For two of the "split" races, a new pathotype could be distinguished based on reaction on the *Xa14* gene, with the "new" pathotype corresponding to one of the groupings defined by DNA fingerprinting. For race 3, however, consistent differences in reactions were not obtained by Nelson et al (1994).

To clarify the genetic and phenotypic structure of race 3, Finckh and Nelson (IRRI, unpubl. data) analyzed a larger collection of strains (120 strains of race 3, plus representatives of other races) previously classified as race 3 by Mew et al (1992). For each of the strains analyzed, DNA fingerprints were obtained using *IS1113* and *avrXa10.* Based on their DNA fingerprints, the 43 haplotypes of race 3 strains were divided into three groups, and 77 of the race 3 isolates were inoculated on expanded sets of differential cultivars. Statistically significant differences in aggressiveness on IR-BB7 carrying *Xa7* were detected between the two major groups of race 3 strains.

Pathogen variation in Asia

Adhikari et al (1995) analyzed 308 strains of *Xoo* provided by scientists from seven rice-growing countries in Asia using the probes *IS1112* and *avrXa10.* They detected evidence indicating both regional differentiation (many groups of similar strains were specific to one country), and for pathogen migration between countries (some groups of the pathogen were common to more than one country). Similar observations were made by scientists in the national rice research programs of the Philippine Rice Research Institute (PhilRice) and the Central Research Institute of Food Crops (CRIFC) in Indonesia. For instance, the *IS1113* haplotype that predominates in parts of both Indonesia and the Philippines is identical (Ardales et al 1993; PhilRice, CRIFC, and IRRI, 1995, unpubl. data). This may be related to the popularity of IR64 in both countries.

Hierarchical analysis of pathogen diversity in the Philippines

To determine the extent and distribution of pathogen diversity among agroecosystems, sites, and fields, we conducted a hierarchical analysis of pathogen variation along a transect on Luzon in the Philippines (Ardales et al 1996). More than 1,200 strains

were collected systematically along a 310-km transect spanning an indigenous rice-growing area in the mountainous region and the modern agroecosystem in the lowlands of Luzon. Each strain was characterized by DNA fingerprinting with *IS1113* and restriction enzyme analysis (REA) using *Pst*I, and by inoculation on a set of differential cultivars.

Nineteen haplotypes, grouping into three robust clusters, were defined using *IS1113*. *Pst*I fingerprinting revealed greater diversity (46 unique haplotypes defined), but the derived phenogram was not robust. Within a site, there was a near-perfect correspondence (>99%) between lineage or haplotype and race. Hierarchical analysis of genetic variation showed population substructuring at all levels. A high degree of genetic differentiation was seen among ecosystems and sites. On this backdrop, there was also evidence of pathogen migration: five *IS1113* haplotypes were present in more than one site and one of these haplotypes was found at 10 sites located in all six provinces sampled in the study. A subsampling exercise using the datasets obtained in this study suggested that pathogen populations could be sampled less intensively by decreasing the number of samples collected per field, without considerable loss of diversity.

Development of PCR-based markers

To allow greater efficiency for future studies on the BB pathogen, we decided to develop polymerase chain reaction (PCR)-based markers that could be used to obtain robust DNA fingerprints from bacteria leached from lesions collected directly from the field. We experimented with various PCR-based methods, including randomly amplified polymorphic DNA (RAPD) sequence, ligation-mediated PCR, and PCR (George et al 1995; M.L.C. George, IRRI and Kansas State University, 1995, unpubl. data), and REP, ERIC and BOX, as well as PCR with various other primers (C.M. Vera Cruz, T. Adhikari, and J.E. Leach, IRRI and Kansas State University, 1995, unpubl. data). Findings in both the Kansas State laboratory and IRRI indicate that primers amplify the regions of the bacterial genome between *IS1112* elements (primers designed from the ends of the *IS1112* element). The fingerprints obtained using this method were consistent between experiments, even from bacteria oozed directly from infected leaves. The groups of strains identified based on this primer were the same as those obtained using RFLP.

Progress in the analysis of resistance to BB

Over the last several years, substantial progress has been made in understanding the genetic basis of resistance to BB. Nineteen resistance genes have been reported (Kinoshita 1991). Near-isogenic lines (NILs) carrying individual resistance genes have been developed in three genetic backgrounds, providing superb differential sets suitable for different environments (Ogawa et al 1991, Ogawa 1993). The availability of NILs makes it possible to systematically characterize the resistance spectra of the genes in relation to pathogen strains.

Inoculation of these NILs with a range of Philippine pathogen strains revealed that the available resistance genes do not have simple complementary resistance spectra. Most of the NILs were susceptible to races 4 or 6. Among the NILs tested, only IRBB21 gave resistance to the strains of races 4 and 6; this line, carrying *Xa21,* was resistant to all of the strains tested. Under these circumstances, it might be desirable to utilize a broad-spectrum resistance gene, such as *Xa21,* in combination with other resistance genes. Because the *Xa21* gene conditions resistance to all the isolates tested from South and Southeast Asia (Ronald et al 1996), it would not be possible to combine this gene with other resistance genes by phenotypic selection. Lines carrying multiple resistance genes could, however, be selected through the use of molecular markers closely linked to resistance genes. Through the work of researchers in several laboratories, many BB resistance genes have recently been mapped relative to molecular markers (e.g., Ronald et al 1992, Yoshimura et al 1992a,b; 1995a,b).

For example, Bordeos and Nelson (IRRI, 1994, unpubl. data) tagged a resistance gene derived from the wild rice species, *Oryza minuta.* A BB-resistant BC_2 progeny of the *O. sativa/O. minuta* cross was identified (Amante-Bordeos et al 1991). To obtain molecular markers linked to the resistance gene, we conducted bulk segregant analysis for resistant and susceptible plants selected from a BC_2F_3 population developed from the resistant line. Pooled DNA extracts of the resistant and susceptible BC_2F_3 lines, or DNA extracts of a resistant line and the *O. sativa* parent, were analyzed with 493 10-base primers. To determine whether a band observed in the resistant bulk or individual was associated with resistance, putative positives were reassayed using four resistant and four susceptible lines and the parents. and finally using the segregating population of 60 individuals.

Bands derived from *O. minuta* and cosegregating with resistance were amplified using primers OPACl1 (5'-CCTGGGTCAG-3') and OPLl3 (5'-ACCGCCTGCT-3'). $OPAC1l_{700}$ was located 4.7 cM from the resistance locus, while $OPL13_{700}$ was located 11.3 cM from the gene. Efforts to generate RFLP markers based on the RAPD markers have not been successful. Both RAPD fragments, when used as probes, hybridized with repetitive elements in the rice genome. Due to the presence of *Xa4* and perhaps other genes affecting the reaction to the BB pathogen in the recurrent *O. sativa* parent, the resistance spectrum of the gene from *O. minuta* cannot be characterized in detail at present. Crosses have been made to transfer the resistance to the IR24 genetic background.

With the availability of several resistance genes tagged with molecular markers, it has become feasible to select lines carrying a desired combination of genes. The utility of the gene pyramiding strategy would depend on the phenotype of lines canying multiple genes, and on the ability of pathogen populations to adapt to such host genotypes. To assess the interactions between major genes, Yoshimura et al (1995b) tagged *Xa3, Xa4, xa5,* and *Xa10* and used a combination of molecular markers and inoculation assays to select lines carrying pairs of resistance genes. They compared the lesion lengths produced for diverse pathogen strains, on NILs carrying one or two genes in the IR24 genetic background. In some cases, epistatic interactions were

detected, so that lesions developed more slowly on the pyramid line than on either parental line for some isolates. For instance, lesion lengths for PXO71 (race 4) were shorter on the *Xa4 + xa5* pyramid than on either the *Xa4* or *xa5* line at the maximum tillering stage (Yoshimura et al 1995b). Such effects were also seen for some strains of race 6.

In rice breeding programs at IRRI, and in national rice improvement programs in the Philippines, Indonesia, and India, *Xa4, xa5, Xa7,* and *Xa21* are being transferred to commercially important rice varieties (L. Sebastian, PhilRice, 1995, pers. commun.; M. Bustamam, CRIFC, 1995, pers. commun.; N. Huang, IRRI, 1995, pers. commun.). *Xa21* has recently been isolated by the University of California, Davis laboratory (Ronald et al 1996). This will make it possible to deploy the gene more easily, by direct gene transfer into varieties with various genetic backgrounds.

Genes for quantitative resistance to BB were mapped in two populations. A preliminary analysis of quantitative resistance was conducted for a recombinant inbred (RI) population derived from the cross Asominori/IR24 (A. Yoshimura, T. Mew, and R. Nelson, Kyushu University and IRRI, 1994, unpubl. data). Asominori is considered to have a high level of horizontal resistance to BB in Japan. RFLP data at 115 marker loci were obtained in Japan for the 67 lines in the population. Isolates PXO71 (race 4) and PXO99 (race 6) were inoculated to each of the RI lines at IRRI. For both isolates, a continuous distribution of lesion lengths was observed for the population. The general linear model procedure of SAS was used to examine the association between marker loci and effects on lesion length. For both of the isolates used, the known BB resistance locus *Xa1,* and markers linked to it, had a significant effect ($P>0.01$) on lesion length, though even the more resistant group had long (susceptible-type) lesions. For each of the isolates, three other loci were also identified with effects significant at the 5% level. Among these, only marker Xnpb333, on chromosome 10, showed significant effects for both of the isolates tested.

The IR64/Azucena doubled haploid (DH) mapping population was also used for mapping of quantitative trait loci (QTLs) affecting reaction to *Xoo* (M. Baraoidan, R. Nelson, S. McCouch and N. Huang, IRRI and Cornell University, 1995, unpubl. data). RFLP data at 135 loci were available for this population from previous work done in the IRRI and Cornell laboratories. Three inoculation experiments were conducted on this population. Based on the first two experiments, putative QTLs were identified. The third experiment was conducted to test hypotheses established in the preceding experiments. Lines with and without the putative QTLs, and not carrying *Xa4,* were inoculated with 15 diverse strains of the pathogen. Based on general linear modeling, a large number of significant locus by isolate interactions were detected. We were most interested, however, in those loci affecting most or all of the pathogen strains tested. When loci with significant effects (at $P<0.05$) on at least 10 of the strains were considered, 18 markers defining 7 chromosomal regions on 5 chromosomes were identified. These loci, which did not coincide with regions previously known to carry major genes for BB resistance (Causse et al 1994), were associated with reductions in lesion length of 3-7 cm. Regions of chromosomes 5, 9, and 12 were associated with

significant effects on all 15 of the strains tested. Most of the alleles associated with reductions in lesion length were from IR64, but the putative QTL on chromosome 5 was derived from Azucena.

Experiments on the deployment of resistance to BB

The experiments described above have been conducted to explore the interactions between resistance genes and individual strains of the pathogen. Although these strains were selected in an effort to represent the known diversity of the pathogen (based on inoculation experiments and DNA fingerprinting), it is probably impossible to accurately represent the actual genetic diversity of the natural pathogen population. To determine the interactions between populations of the rice host and the pathogen in the field, we have undertaken experiments on the deployment of resistance to BB. BB-infected rice leaves were collected from each of the four replications of experiments conducted in Calauan and Mabitac, Laguna. The nine host genotypes planted were a pure stand of IR20 *(Xa4);* three pure stands of NILs—IR-BB4 *(Xa4),* IR-BB5 *(Xa5),* and IR-BB10 *(Xa10);* two pure stands of pyramids—IR-BB4/10 *(Xa4* and *Xa10)* and IR-BB4/5 *(Xa4* and *Xa5)* and three mixtures—IR-BB4+10, IR-BB4+5, and IR20 + IR-BB10. The plots were naturally infected with BB. Twenty isolates were analyzed from each plot. DNA fingerprinting was done by RFLP using *IS1113* and avr*Xa10* as hybridization probes for a total of 811 isolates from the two sites. There was a strong correspondence between the *IS1113* and avr*Xa10* haplotypes, and between lineages and pathotypes.

There was little difference in structure of the pathogen collections taken from IR-BB4 and IR20, carrying the *Xa4* in different genetic backgrounds. Surprisingly, the collections taken from lines carrying *Xa4* and *Xa10* were also similar, although these genes are functionally distinct for strains of races 2 and 3 in greenhouse inoculation tests. The collections taken from *xa5* were, however, quite different from those taken from *Xa4* and *Xa10.* The *xa5* gene was expected to give resistance against the pathogen population, but a low level of disease was observed. Our results indicate that this unexpected susceptibility of lines carrying *xa5* was probably due to the presence of initially rare subpopulation of lineage E, rather than to a mutation arising within the predominant lineages.

Conclusions

The effectiveness and durability of varietal resistance depend upon the host, pathogen, and environment, The BB pathogen can change to overcome host resistance, the primary method for disease control. Therefore, it is important to manage host resistance carefully. Useful molecular markers and NILs carrying single and multiple resistance genes are available to allow efficient characterization of pathogen populations, to allow careful selection of resistance genes and design of suitable resistance genotypes and deployment strategies. Field experiments and modeling studies are now

under way to determine the potential utility of using host diversity for disease management (C. Mundt, 1993-95, IRRI, pers. commun.). Resistance based on both major genes and minor genes is available and reasonably well characterized, and both types of resistance should be utilized in resistance breeding. The identification of molecular markers closely linked to resistance genes, and the cloning of such genes, should allow the efficient exploitation of major gene resistance. The availability of cloned resistance genes should make it easier to maintain both major and minor genes in breeding programs.

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Molecular analysis of repeated DNA sequences from the rice blast fungus *Magnaporthe grisea*

P. Kachroo, S.A. Leong, and B.B. Chattoo

Genome organization and distribution of repeated DNA sequences were studied in the rice blast fungus *Magnaporthe grisea.* The analysis led to the identification of an inverted repeat transposon and a short interspersed nuclear element. Both of these elements were isolated as insertions into unrelated repeated DNA sequences, a process that appears to be of common occurrence in the genome of *M. grisea.* The repeated DNA sequences were characterized with respect to their fingerprinting patterns in rice and nonrice isolates of *M. grisea,* dispersion in the genome, copy number, and sequence characteristics. The close physical association of diverse repeated DNA sequences and the genome rearrangements caused by them indicate that they may also contribute to genome variability seen among isolates of *M. grisea.*

Rice blast, caused by the filamentous ascomycete *Magnaporthe grisea,* is one of the most devastating diseases of cultivated rice. This pathogen is classified into various pathotypes or races based on the infection pattern obtained on a set of differential rice cultivars and, like most other phytopathogenic fungi, shows a high level of variability (Latterell 1975). The variable nature of the pathogen often leads to breakdown of host resistance leading to widespread epidemics. Thus, breeding for durable blast resistance is one of the major challenges faced by rice breeders (Ou 1985).

Various models and hypotheses have been proposed to explain the genetic variability among isolates of *M. grisea* (Yamasaki and Niizeki 1965, Giatgong and Frederiksen 1969, Ou 1985). However, an understanding of this phenomenon at a molecular level is still lacking. We have initiated an analysis of the genomic architecture of this fungus in an attempt to study the basis of variability. As a first step, we began an analysis of repeated DNA sequences with respect to their genome organization, since these sequences have been demonstrated to mediate DNA rearrangements in a variety of organisms (Fedoroff 1979, Petes 1980, Coen et al 1982, Rothstein et al

1987). Here, we report on a detailed analysis of three different repetitive elements from *M. grisea* and demonstrate how these may mediate genomic rearrangements in this fungus.

Results and discussion

A repeat element of 1.3 kb was cloned from the genome of *M. grisea* and studied with respect to its fingerprinting pattern among rice and nonrice isolates. Southern hybridization analysis of genomic DNA from 36 isolates representing different geographical regions, when digested with *Pst*I and probed with a 1.3-kb repeat, showed an intense band at 1.3 kb and several higher molecular weight bands (Fig. 1). The repeat was present at a high copy number (approximately 100) in both the subgroups of *M. grisea.* Contour-clamped homogeneous electric field (CHEF) analysis carried out with both rice and nonrice isolates showed that the repeat was dispersed in nature and was also present on B chromosomes (Fig. 2).

Several members of this repeat family were cloned and found to have heterogeneity in their restriction maps. Spot sequence analysis also showed heterogeneity at the sequence level, which was due to point mutations, transitions, and transversions. The heterogeneity and the presence of several higher molecular weight bands in a Southern blot containing the genomic DNA digested with *Pst*I and probed with the 1.3-kb repeat were indicative of this repeat being prone to genome rearrangements. This hypothesis was investigated by cloning and characterization of 5.1- and 3.2-kb *Pst*I fragments from isolates B101 and B157, respectively.

Analysis of the 5.1-kb repeat

Restriction mapping and Southern analysis of the 5.1-kb fragment showed that it contained at least two other repeated DNA sequences in addition to the 1.3-kb element. These flanked the 1.3-kb region (Fig. 3a) and also showed a different fingerprinting pattern. Sequence analysis of the 5.1-kb fragment revealed that the 1.3-kb repeat was truncated at the 5' end by 395 bp and that the *Pst*I site. which marked the end of 1.3-kb repeat, had undergone a point mutation resulting in the loss of the site. Sequence analysis of the 2.7-kb region (designated as Mg-RT; Fig. 3a) upstream of the 1.3-kb repeat showed features typical of retroelements that include the presence of TG at the insertion site, 4-bp inverted repeats that mark the start and end of the long terminal repeat (LTR), and homology to the gag and pol regions. It is, therefore, likely that an insertion element, represented by a partial length of 2.7 kb in the 5.1-kb fragment, had inserted within the 1.3-kb repeat, thereby displacing the 395 bp at the 5' end.

The 1.4-kb region downstream of the 3'- *Pst*I site of the 1.3-kb repeat had a DNA fingerprint similar to that of the 1.3-kb repeat and showed an intense band at 1.4 kb in the genomic digests and 4-12 higher molecular weight bands. A similar intensity/ pattern of the hybridization signal obtained with 1.3- and 1.4-kb repeat probes and their association in the 5.1-kb *Pst*I fragment prompted us to analyze if the presence of the two elements in the 5.1 kb was a chance occurrence or whether they were parts of a larger repeat element. Southern analysis of genomic DNA digested with 27 restriction

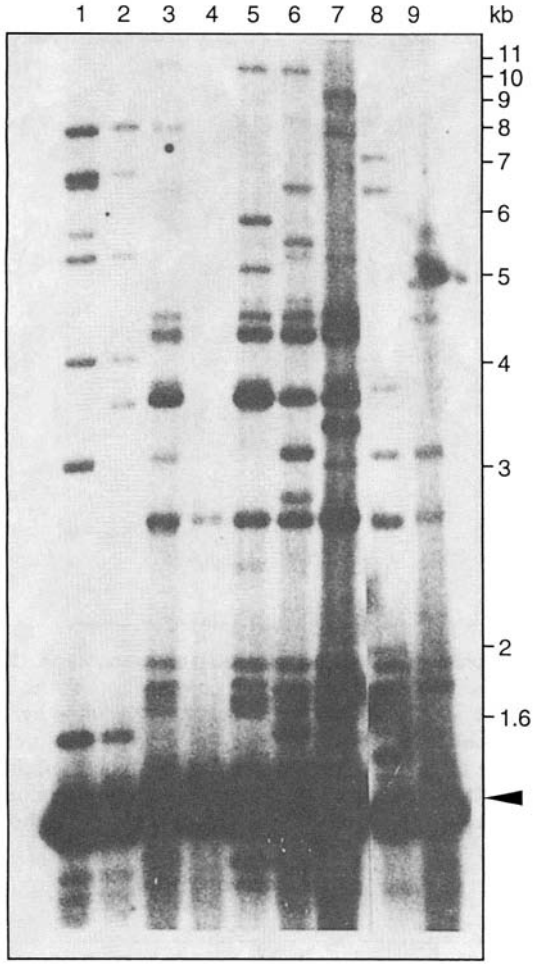

Fig. 1. Southern hybridization of genomic DNA of rice and nonrice pathogens of *M. grisea* probed with a 1.3-kb *Pst*I repeat element. Genomic DNA (2 g) was digested with *Pst*I, electrophoresed through a 0.8% agarose gel, transferred to a nylon membrane, and hybridized with radiolabeled pBC157.3 (1.3-kb *Pst*I fragment from isolate B157 cloned into vector pUC19). The lanes contain DNA from Pr342 (nonrice pathogen, Philippines), lane 1; Pr886 (nonrice pathogen, Philippines), lane 2; Lc454 (nonrice pathogen, Philippines), lane 3; Pg 3393 (nonrice pathogen, India), lane 4; Cb334 (nonrice pathogen, Philippines), lane 5; Ec552 (nonrice pathogen, Philippines), lane 6; Ei476 (nonrice pathogen, Philippines), lane 7; 91-A-58 (rice pathogen, USA), lane 8; 4360-17-1 (rice pathogen, USA), lane 9. The conserved 1.3-kb band is marked by an arrowhead.

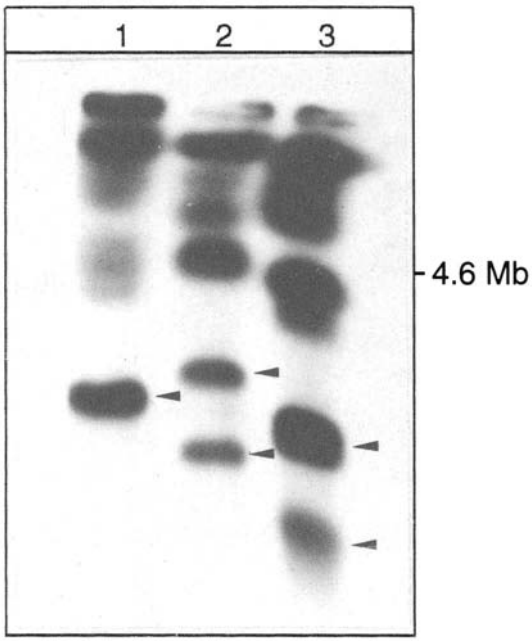

Fig. 2. Southern hybridization analysis of *M. grisea* chromosomes. Chromosome-sized DNA molecules were separated by CHEF-gel electrophoresis as described earlier (Kachroo et al 1994), blotted onto nylon membrane, and hybridized with the radiolabeled 1.3-kb repeat (pBC157.3) probe. The lanes contain chromosomal DNA from isolate A (nonrice pathogen), lane 1; B157 (rice pathogen), lane 2; B101 (rice pathogen), lane 3. Molecular size 4.6 Mb corresponds to chromosome 6 of isolate Guy11 (Skinner et al 1993). Arrowheads show hybridization of the 1.3-kb repeat to the B chromosomes.

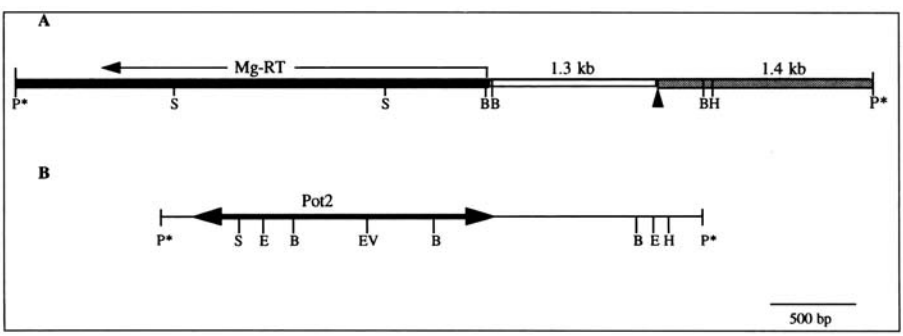

Fig. 3. Restriction map of 5.1-kb (A) and 3.2-kb (B) derivatives of the 1.3-kb *Pst*I repeat element. Arrowhead in A denotes the 3' *Pst*I site of 1.3 kb, which is lost due to a point mutation. Mg-RT in 5.1 kb is a partial length clone. Triangles in B represent the inverted repeats of Pot2. Restriction sites shown are P, *Pst*I; B, *Bam*HI; S, *Sal*I; E, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII. Asterisks denote the cloning site in vector pUC19.

enzymes and hybridized to the 1.3-kb repeat revealed a prominent 2-kb *Bam*HI and two less intense *Hin*dIII fragments of molecular weights 4 and 5.2 kb. Since both 1.3 kb and MGR583 (Hamer et al 1989) hybridized to a conserved 2-kb *Bam*HI band in the genomic DNA, the 1.3/1.4-kb repeat was used as a probe against MGR as well as other available repeats (Skinner et al 1993, Kachroo et al 1994, Leong et al 1994, Kachroo et al 1995; P. Kachroo and B.B. Chattoo, M.S. University, India, unpubl. data). The probe hybridized strongly to MGR583 and the regions showing homology were further narrowed down to 1.3- and 1.4-kb *Pst*I fragments within the MGR583. Our recent analysis has shown that both the 1.3- and 1.4-kb repeats are present as a contiguous piece in the genome and are parts of the LINE-like region of MGR583. Since LINE sequences require the activity of reverse transcriptase for their retroposition, which has a lower fidelity rate as compared with DNA polymerase, it also explains the sequence heterogeneity seen among members of the 1.3-kb repeat family.

Pot2: an inverted repeat transposon

Pot2 (*Pyricularia oryzae* transposon) was isolated as an insertion within a member of the 1.3-kb repeat family. Restriction mapping and sequencing of a 3.2-kb derivative showed that the increase in size was due to the presence of an additional 1.8-kb DNA within the 1.3-kb repeat element (Fig. 3b). This extra piece of DNA (named Pot2) had 43-bp perfect inverted repeats at the end and 16-bp direct repeats within each inverted repeat (Kachroo et al 1994). Computer analysis showed the presence of a single open reading frame (ORF) coding for a putative protein of 535 amino acids. Analysis of the empty site, which corresponded to the insertion site of Pot2 within the 3.2-kb repeat, showed a duplication of dinucleotide TA at the target site. A target site analysis carried out by sequencing 12 random insertions revealed that TA dinucleotide was common to all. The insertion sequence Pot2 resembled the inverted repeat transposons from *Drosophila* (Mariner) and *Caenorhabditis elegans* (Tcl) in showing inverted repeats at the ends, duplication of dinucleotide TA at the target site, and the presence of a large OFR.

Data base comparison of the putative protein from Pot2 showed 40% identity to Fot1, an inverted repeat transposon from *Fusarium oxysporum.* The identity was maximum toward the middle of the protein. The two elements showed a high level of organizational similarity (Table 1). At the DNA level, a high degree of similarity was seen between the inverted repeats of Pot2 and Fot1, which were aligned into three domains of perfect identity. Both of these elements showed the presence of direct repeats within the inverted repeats, which appears to be a unique feature of this group of elements.

A very high level of conservation seen between Pot2 and Fotl at the protein level and within TIRs indicate the presence of a new family of transposable elements that may be dispersed among fungi. The distribution of transposable elements among closely related or diverse species could be explained on the basis of vertical or horizontal transmission of genetic material. These mechanisms are not mutually exclusive and could account for the high level of sequence identity. This may represent parallel

Table 1. Comparison between the inverted repeat transposons Pot2 and Fot1.

	Pot2	Fot1
Size of the element	1.861 kb	1.928 kb
ORF, size of putative protein	535 aa	542 aa
Inverted repeats	43 bp	44 bp (imperfect by 1 bp)
Direct repeats	16 bp	13 bp
Copy number	~100	~100
Amino acid homology	39.3% identity to Fot1	39.3% identity to Pot2
Target site	Duplicate TA at target site	Duplicate TA at target site

evolution of the two species, and in this context, it will be interesting to see if this group of elements is present in other closely related genera.

Isolation and characterization of another inverted repeat transposon, Impala, from *F. oxysporum* has revealed that, like retroelements, the inverted repeat transposons may also form multiple families in the filamentous fungi. Interestingly, Impala does not show any significant similarity with Fot1 either at the nucleotide level, particularly in the termini of the TIRs or at the amino acid level. This element also differs from Fotl in a number of structural features although it appears to follow a similar mechanism of transposition leading to duplication of TA at the target site. In yet another study, another inverted repeat transposon has been recently characterized from *M. grisea,* which unlike Fotl and Impala, shows a number of structural features similar to Pot2 (Farman et al 1996a).

Mg-SINE: short interspersed nuclear element

Mg-SINE (*M. grisea* short interspersed nuclear element) was identified as an insertion within the inverted repeat transposon Pot2 (Kachroo et al 1995; Fig. 4a). Sequence analysis revealed the presence of both A and B box sequences with a perfect match with the tRNA polymerase III consensus. Mg-SINE showed the characteristic features of a generic SINE element that includes the presence of an RNA polymerase III consensus binding site, duplication of the target site, and an A-rich 3' end (Fig. 4b). The target site duplication of Mg-SINE elements was found to vary from 1 to 16 bp, as is also seen in the mammalian SINE elements. The A-rich region of Mg-SINE was represented by from five to nine copies of trinucleotide repeat TAC. Data base comparison showed a significant homology to tRNA of *Drosophila* and yeast, which was predominantly due to the conserved A and B box sequences.

Southern hybridization analysis of 35 isolates (Fig. 5; data shown for 12) obtained from different geographical regions showed that Mg-SINE is present in both rice and nonrice pathogens of *M. grisea.* Mg-SINE represents the third highly repetitive element characterized in the present study (including Pot2 and 1.3-kb repeats), which is ubiquitous in its distribution among different host-specific forms of *M. grisea.* This

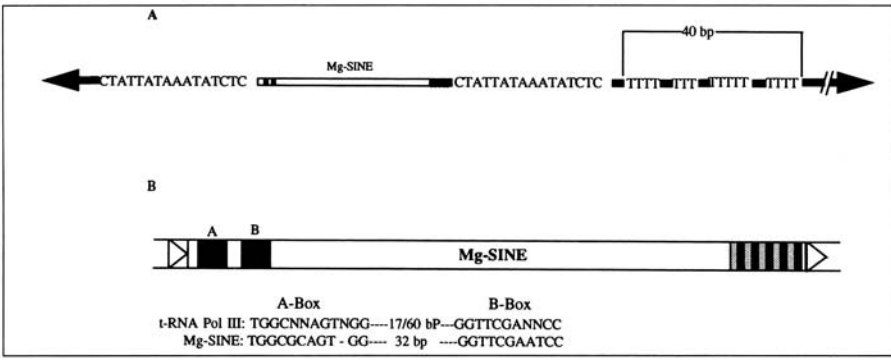

Fig. 4. Schematic representation of Mg-SINE insertion in Pot2 (A) and the structure of the element (B). The sequence in A represents the 16-bp target site, which undergoes duplication upon retroposition of Mg-SINE. The target site is A/T rich consisting of 13 A/T residues. The sequence flanking the target site contains four consecutive rows of three or more Ts in a span of 40 bp, which can serve as potential terminators of RNA polymerase III. The open triangles in B represent direct repeats formed as a result of target site duplication. Dark boxes represent A and B box RNA polymerase III consensus sequence. Shaded box with vertical bars represents trinucleotide repeat region of Mg-SINE, each bar representing a single repeat unit.

result further strengthens our hypothesis of a common origin among rice and nonrice isolates. CHEF and quantitative dot blot analysis revealed that Mg-SINE. like Pot2 and 1.3-kb repeat elements, is dispersed at approximately 100 copies per haploid genome in both rice and nonrice isolates.

Both in vivo and in vitro studies, carried out with Alu and other related SINE families from the mammalian genome, have shown that the SINE elements are transcribed into an RNA by RNA polymerase III (Fuhrman et al 1981, Slagel and Deininger 1989). As proposed earlier (Deininger 1989), the retroposition of SINE elements depends on synthesis of cDNA by reverse transcriptase and, since these elements do not possess any ORFs, the reverse transcriptase function should be available in trans. Mg-SINE appears to be a functional element as judged by the presence of a transcript of approximately 0.5-kb size observed in Northern blot hybridization analysis. The presence of a functional retroelement Maggy in the genome of rice-infecting isolates has already been shown (Farman et al 1996b; M.H. Lebrun, B. Valent, F. Chumley, V. Shull, J.E. Hamer, unpubl. data) and it is likely that Mg-SINE uses the reverse transcriptase function of this retroelement to generate a copy of cDNA. The retroelement Maggy is present in all the rice-infecting pathogens analyzed and has a variable copy number in nonrice-infecting pathogens of *M. grisea* (Farman et al 1996b). A low copy number of Maggy in most nonrice pathogens makes it very likely that these pathogens possess an additional source of reverse transcriptase function, which would have assisted in amplification and dispersion of Mg-SINE. The presence of a retroelement Grh in *Eleusine* pathogens of *M. grisea* and a partial sequence of a repetitive DNA from rice pathogens, which shows identity to

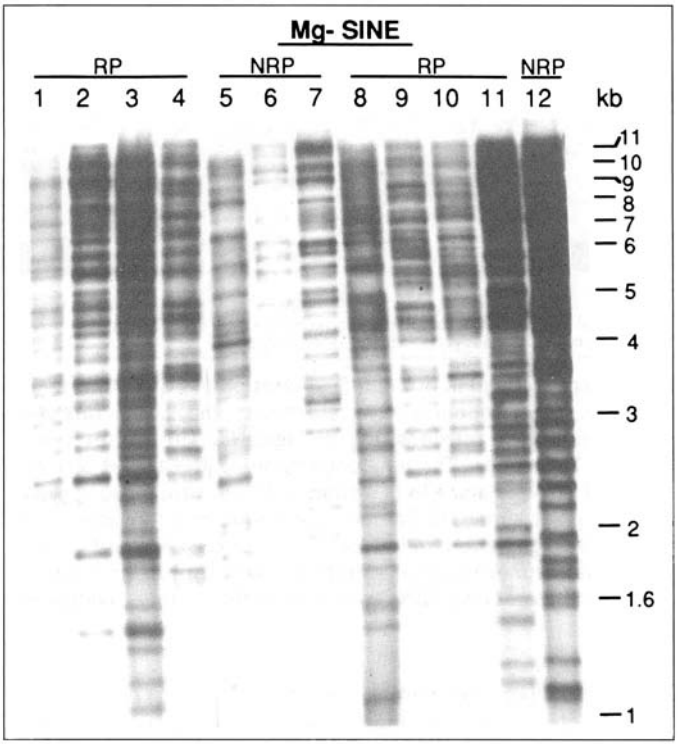

Fig. 5. Southern hybridization analysis of genomic DNA of rice and nonrice pathogens of *M. grisea* probes with Mg-SINE. Genomic DNA (2 g) was digested with *Eco*RV, electrophoresed through a 0.8% gel, transferred to MSI-nylon membrane, and hybridized with Mg-SINE. *Eco*RV does not have any site within Mg-SINE. The lanes contain DNA from: B101 (India), lane 1; B157 (India), lane 2; F (India), lane 3; 102 (Philippines), lane 4; Pr342 (Philippines), lane 5; Pr886 (Philippines), lane 6; Pg3393 (India), lane 7; 4360-17-1 (USA), lane 8; JMB840610 (Philippines), lane 9; 104 (Philippines), lane 10; B (India), lane 11; Ei 476 (Philippines), lane 12. RP and NRP designate rice pathogens and nonrice pathogens, respectively.

retroelements, is supportive of the above hypothesis (Dobinson et al 1993, Sone et al 1993).

The consensus transcription terminator of RNA polymerase III transcription is a sequence of four or more consecutive Ts, which have been shown to reside in the sequence flanking the SINE elements (Jagadeeswaran et al 1981, Deininger 1989). Analysis of the sequence flanking the Mg-SINE insertion in Pot2 revealed the presence of four blocks of Ts in a span of 40 bp downstream of the trinucleotide repeat at the 3' end (Fig. 4A). However, since different Mg-SINE elements would have a different

sequence flanking the 3' end, it is possible that these elements would result in transcripts of different lengths. This, in turn, would depend upon how far the poly T stretch is from the 3' end of Mg-SINE. Sequence analysis of various Mg-SINE elements revealed a high level of sequence conservation, suggesting that these may be generated by transcription of a master element as has been hypothesized for Alu members (Deininger et a1 1992). Alternatively, various heterogeneous transcripts generated by Mg-SINE elements distributed throughout the genome may be processed to a uniform size followed by cDNA synthesis and integration within the genome. The self-priming reverse transcription process hypothesized for Alu elements (Jagadeeswaran et al 1981, Deininger 1989) cannot be used to explain the cDNA synthesis of Mg-SINE and other similar SINE elements whose 3' end sequence consists of di tri or tetra nucleotide repeats (Deininger 1989). However, its also likely that the poly U sequence of the RNA Pol III transcript forms a partial loop upon base pairing with A and G residues (Wyatt et al 1989) of trinucleotide repeats of Mg-SINE. The position where the loop formation takes place could also result in generation of variable number of trinucleotide repeats as seen in Mg-SINE.

The secondary structure analysis of Mg-SINE showed a tRNA related, a tRNA unrelated, and an AT-rich region that is typical of mammalian SINE elements (Fig. 6). The 73 bp at the 5' end of Mg-SINE folds into a tRNA-like structure that terminates with nucleotides CCA, a characteristic feature of tRNA. The predicted secondary structure folds into D and TC loops corresponding to the A and B boxes of Mg-SINE. while the anticodon loop forms the middle arm. suggesting that Mg-SINE originated from a precursor tRNA. The absence of a short arm between the anticodon loop and the B box could be explained by sequence divergence. However, it appears likely that the precursor tRNA belongs to class I tRNAs. which have five nucleotides in the short arm as compared with class II which have a long extra loop region (Sakagami et al 1994).

Analysis of various cosmid clones, which hybridized to Mg-SINE, revealed that the ones hybridizing weakly to the probe contained a chimeric structure (Ch-SINE), which showed sequence homology to a 242-bp region at the 3' end of Mg-SINE while the 452-bp region at the 5' end was dissimilar to Mg-SINE. Alignment of the Ch-SINE and Mg-SINE sequence showed point mutations, insertions, and deletions of one or more bases in the SINE-like region of Ch-SINE, which have contributed to sequence divergence. The type II Galgo SINE family resembles Ch-SINE in being a chimeric element composed of sequences related to monomer family in its left half and sequences identical to Alu in the right half and has been proposed to have arisen as a result of fusion between a monomer family member and an Alu family member. The Ch-SINE does not contain the A and B box consensus sequence of RNA polymerase III promoter or direct repeats at the ends although it possesses the trinucleotide repeats at their 3' ends. Southern hybridization analysis carried out with DNA of both rice and nonrice pathogens of *M. grisea* shows that Ch-SINE is a moderately repetitive element present in both subgroups. Sequence analysis of two Ch-SINE elements showed that both contained a variable number of trinucleotide repeats at their 3' end. Whether this could be due to many such fusion events taking

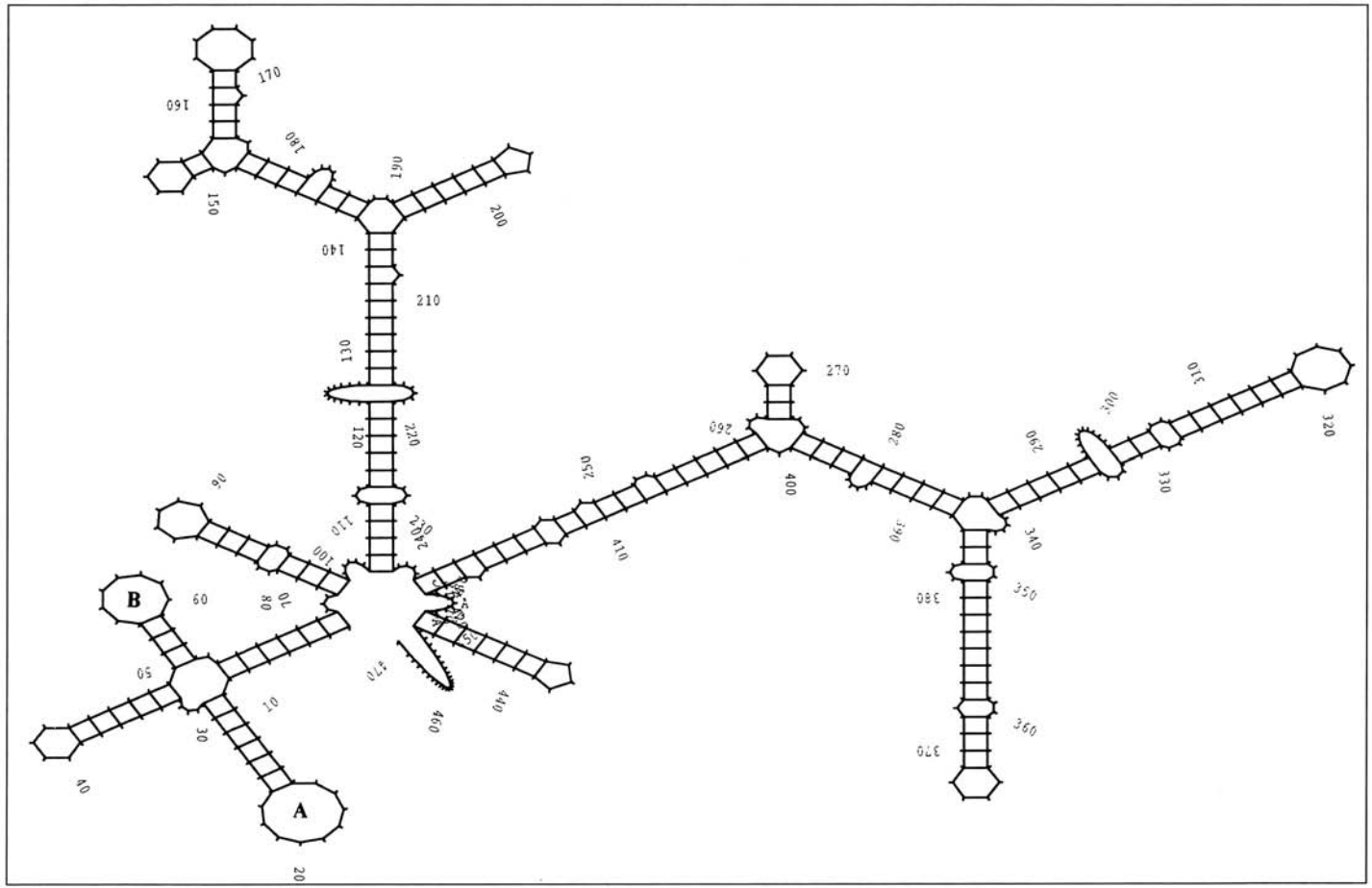

Fig. 6. Secondary structure analysis of Mg-SINE. The structural analysis was carried out using Squiggle and Fold programs of the University of Wisconsin Genetics Computer group programs (Devereux et al 1984). Numbers are given from the 5' end of Mg-SINE. A and B designate the conserved polymerase III consensus promoter sequence. The 1-73-bp region shows the cloverlike structure typical of tRNA while the rest of the structure is unrelated to tRNA.

place in the genome followed by their amplification or this element having a function similar to the SINE element, which generates a variable 3' end during their retroposition process, still remains an open question. Our recent studies have shown the presence of yet another LINE-like element in the genome of *M. grisea* which was isolated as an insertion into Mg-SINE and Ch-SINE elements indicating that such events may be of common occurrence in the genome of *M. grisea* (P. Kachroo, M. Ahuja, S. Leong, B. Chattoo, unpubl. data).

SINE elements occupy an important place among all the known groups of transposable elements. In addition to causing insertions and genomic rearrangements, these have been speculated to have a variety of other functions including organization of chromatin structure, RNA processing/stability, and a role in replication and transcription (Deininger 1989). Their ability to mobilize other sequences in the genome by undergoing fusion with them is also a unique feature known only for SINE elements (Weiner et al 1986). Most studies on SINE elements have been carried out in mammalian systems although their presence has been shown in tobacco (Yoshioka et al 1993), rice (Mochizuki et al 1992) and *Drosophila* (Weiner et al 1986). Our present study clearly demonstrates that these elements share a high level of structural similarity, irrespective of their phylogenetic position. The presence of repetitive DNA having SINE-like properties in obligate parasite *Erysiphe graminis* (Rasmussen et al 1993) further indicates that SINE elements may be of common occurrence in the genome of phytopathogenic fungi. A detailed study involving elucidation of their role in genome rearrangements will therefore be helpful in understanding the molecular basis of genome variability among phytopathogenic fungi.

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Notes

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Sequence-tagged sites and low-cost DNA markers for rice

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Sequence-tagged sites (STSs) facilitate the conversion of a genetic map into a physical map, provide a common basis for the comparison of diverse types of mapping data, are stored and disseminated as electronic data, and are amplified from genomic DNA by polymerase chain reaction (PCR). STSs find application as DNA markers in breeding programs and germplasm management because they offer speed, convenience, reliability, and low cost in genomic analysis, but these applications are currently limited by the small number of STSs available. We report here the terminal sequencing of 354 DNA markers of the Cornell-IRRI genetic map of rice and the conversion of 100 of them into STSs by synthesis of pairs of PCR primers. PCR was used to amplify the corresponding loci from genomic DNA of IR36 (indica), Taichung 65 (japonica), and *Oryza longistaminata* (AA genome wild species). More than half of the RZ clones amplified DNA segments that were 0.1-2.0 kbp larger than expected, presumably because of the presence of introns. Amplicon length polymorphisms were detected between *O. sativa* and *O. longistaminata* for about one quarter of the clones. The applications of STSs are illustrated by reference to 1) DNA marker-aided selection for pyramiding of bacterial blight resistance genes, 2) breeding for gall midge resistance, 3) monitoring the inheritance of transgenes, and 4) analysis of genetic variation of AA genome wild species.

Marker-aided selection can increase the efficiency of rice breeding for traits that are difficult to phenotype. Although interest in this approach has been stimulated recently by the construction of genetic maps saturated with DNA markers (Cause et al 1994, Kurata et al 1994), its adoption by breeders will depend on cost, convenience, and reliability when it is applied on the scale of typical breeding. Depending on the dissimilarity of the parental lines, F_2 populations normally range in size from 2,000 to 6,000 plants—large numbers for routine application of most molecular techniques.

Breeders may of course opt to delay DNA marker-aided selection for certain traits until the F_4-F_5 generations, when population sizes decline to several hundred plants, but, in general, DNA marker technology should be expected to cope with thousands of plants per cross and many crosses per season. Furthermore, breeders will need the results by the end of the 3-4 mo breeding cycle and perhaps even more quickly. DNA marker technology based on sequence-tagged sites (STSs) offers a way of meeting these requirements.

Sequence-tagged sites

The STS concept was introduced by Olson et al (1989). In assessing the likely impact of the polymerase chain reaction (PCR) on human genome research, they recognized that single-copy DNA sequences of known map location could serve as markers for the genetic and physical mapping of important genes along the chromosomes. They also recognized that, if the human genome were to have enough markers to be useful in such studies, enormous libraries of mapped DNA clones would have to be stored, amplified, checked, and distributed to interested researchers. Partial sequencing of these clones would, however, provide enough information for the "recovery" of any desired marker by means of PCR. Instead of storing biological material, scientists would store electronic information that could easily be augmented, sorted, and disseminated. These electronically stored markers were called sequence-tagged sites (STSs). The application of STSs in the development of physical maps from genetic maps has indeed gone ahead, not only with the human genome projects but also with other animals and with plants, including rice (Inoue et al 1994).

STS-based PCR and DNA marker technology

DNA maps were originally constructed through the use of restriction fragment length polymorphism (RFLP) analysis and DNA blotting (McCouch et al 1988). Currently, maps are constructed more quickly by random amplification of polymorphic DNA (RAPD) and amplicon fragment length polymorphism (AFLP) analysis (Kurata et al 1994, Vos et al 1995), two techniques that use PCR. PCR is also a more suitable technique than DNA blotting for marker-aided selection: it requires DNA of lower quantity and quality than DNA blots and can therefore tolerate the simplified DNA extraction protocols that are unavoidable with large sample sizes, it is highly automated, and it does not use radiochemical or complex biochemical detection systems (Zheng et al 1995a). STS-based PCR has the further advantage of producing a simple and reproducible pattern on agarose or polyacrylamide gels. The pattern is easily recorded and interpreted, and, being codominant in most cases, these markers allow heterozygotes to be distinguished from the two homozygotes. The equipment needed for STS-based DNA marker technology is relatively inexpensive and robust and requires little bench space or training (Zheng et al 1995b).

STS-based markers have two disadvantages: 1) they require suitable sequence data for each locus, and 2) they are not as polymorphic as some other types of DNA markers, such as microsatellites (Wu and Tanksley 1993). The first disadvantage is

rapidly diminishing in importance, as DNA sequences from rice and other cereals accumulate in the international data bases. In the next section, we report on our contribution to this effort.

The importance of the second problem—low frequency of polymorphism—varies with each locus and each cross. It is, at present, impossible to predict whether STS-based polymorphisms will be found between any two parental lines for any given locus, but in a later section, we look at some of the issues.

Sources of STSs suitable for DNA marker-aided selection

The first set of STSs for rice was identified by Williams et al (1991) who sequenced the ends of 30 of the mapped clones that had been used to prepare the Cornell-IRRI genetic map (McCouch et al 1988). They showed that PCR primer pairs for eight of these loci amplified genomic DNA to yield single bands of the expected sizes. An additional 63 STSs were reported by lnoue et al (1994) for clones mapped by the Rice Genome Research Project in Japan (Kurata et al 1994). In these two cases, clones were first mapped and then partially sequenced.

It is also possible to derive STSs by sequencing clones first and then mapping them. This order of events is obligatory for microsatellites and is widely followed for other types of cloned DNAs that are converted into STS, such as cDNAs, cloned genes, and cloned PCR products. The large number of expressed tagged sites (ETSs) obtained from rice cDNA clones by single-pass sequencing and mapping (Kurata et al 1994) also constitute STSs. Single-pass automated sequencing provides information (350-450 bases) from only one end of each clone. This is enough information for ETSs to be used in the development of a physical map or for similarity searches in data bases, but the PCR amplicons derived from ETSs are generally too short to reveal polymorphism between parental lines by amplicon length polymorphism (ALP) or PCR-based RFLP (Ghareyazie et al 1995). It is possible, however, to detect polymorphisms between such short amplicons through special electrophoretic procedures that detect single-stranded conformational polymorphism (Fukuoka et al 1994). Microsatellites form another class of STS marker: the ends of the clones are unique even though the satellite sequence appears in multiple locations around the genome (Wu and Tanksley 1993).

Terminal sequencing of RFLP markers from the Cornell-IRRI map

To increase the number of STS markers available for rice, we sequenced both ends of 354 of the 726 RFLP markers placed on the Cornell-IRRI genetic map by Causse et al (1994). Some of the markers (136 clones of the RZ series) were cDNA clones derived from mRNA of etiolated leaves of IR36. Other markers (183 clones of the RG series) were random *Pst*I clones of genomic DNA of IR36. Of the remaining 35 STSs, 27 were derived from oat cDNA library clones (CDO series) and 6 were from barley cDNA clones (BCD series).

The choice of about 350 loci as targets for sequencing was dictated by the need to achieve a useful density of markers at an affordable cost. This number of markers

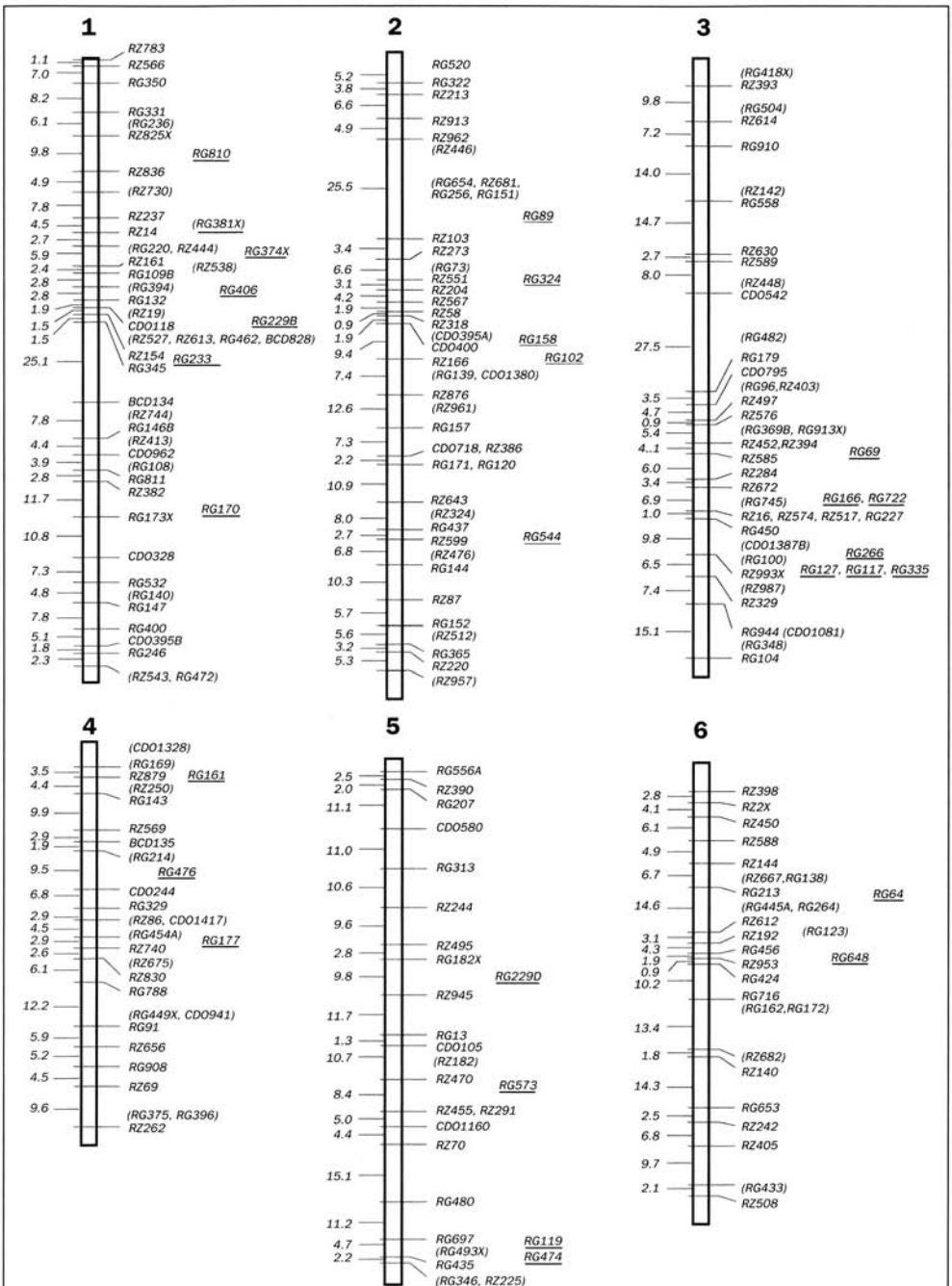
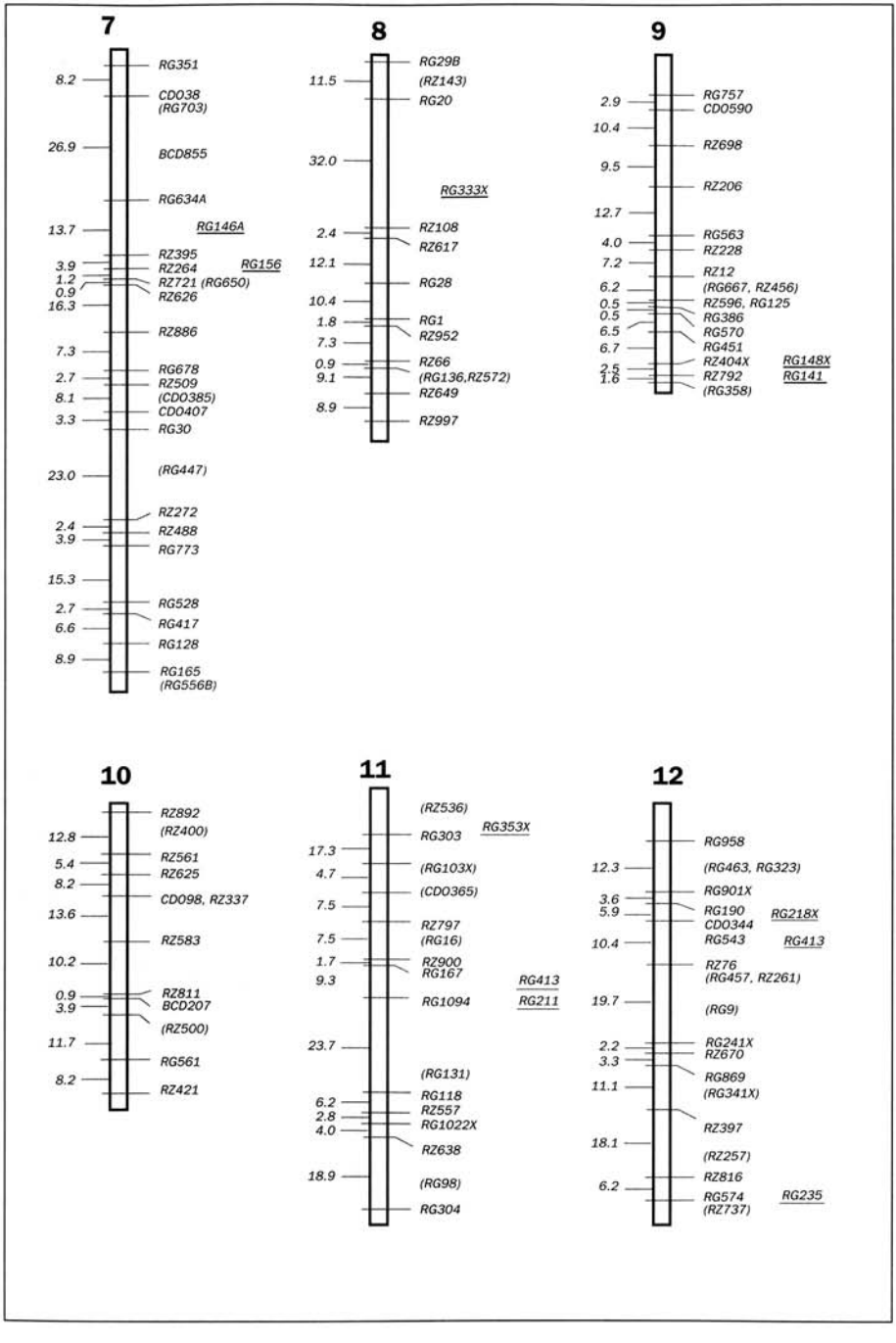


Fig. 1. Sequence-tagged sites on the Cornell-IRRI genetic map of rice. Cloned markers were mapped at Cornell University (McCouch et al 1988, Causse et al 1994) and sequenced at IRRI and Texas A&M University.


7
8.2 RG351
CD038
(RG703)
26.9 BCD855
RG634A
13.7 RG146A
RZ395
3.9 RZ264 RG156
1.2 RZ721 (RG650)
0.9 RZ626
16.3
RZ886
7.3
RG678
2.7 RZ509
8.1 (CD0385)
3.3 CD0407
RG30
23.0 (RG447)
RZ272
2.4 RZ488
3.9 RG773
15.3
RG528
2.7 RG417
6.6 RG128
8.9 RG165
(RG556B)
8
11.5 RG29B
(RZ143)
RG20
32.0
RG333X
2.4 RZ108
RZ617
12.1
RG28
10.4
1.8 RG1
RZ952
7.3
0.9 RZ66
9.1 (RG136,RZ572)
RZ649
8.9 RZ997
9
2.9 RG757
CD0590
10.4
RZ698
9.5
RZ206
12.7
4.0 RG563
RZ228
7.2
RZ12
6.2 (RG667, RZ456)
0.5 RZ596, RG125
0.5 RG386
6.5 RG570
6.7 RG451
2.5 RZ404X RG148X
1.6 RZ792 RG141
(RG358)
10
RZ892
12.8 (RZ400)
5.4 RZ561
8.2 RZ625
CD098, RZ337
13.6
RZ583
10.2
0.9 RZ811
3.9 BCD207
(RZ500)
11.7
RG561
8.2 RZ421
11
(RZ536)
RG303 RG353X
17.3
4.7 (RG103X)
(CDO365)
7.5
RZ797
7.5 (RG16)
1.7 RZ900
9.3 RG167 RG413
RG1094 RG211
23.7
(RG131)
6.2 RG118
2.8 RZ557
4.0 RG1022X
RZ638
18.9 (RG98)
RG304
12
RG958
12.3 (RG463, RG323)
3.6 RG901X
5.9 RG190 RG218X
CDO344
10.4 RG543 RG413
RZ76
(RG457, RZ261)
19.7
(RG9)
2.2 RG241X
3.3 RZ670
RG869
11.1 (RG341X)
RZ397
18.1
(RZ257)
RZ816
6.2 RG574 RG235
(RZ737)

would give an average spacing of about 5 cM for a genome that displays a total genetic length of 1,200-2,000 cM, depending on the relatedness of the parents of the mapping population (McCouch et al 1988, Causse et al 1994, Kurata et al 1994). Our goal was to be able to identify a pair of flanking markers less than 5 cM distant from each gene of interest. This proximity would give a 99.75% chance of avoiding false positives and false negatives in DNA marker-based selection.

STS map of rice

A map of the sequences is provided in Figure 1. Manual sequencing was successful for 294 clones, but the remaining 60 clones could be sequenced manually from only one end. Automated sequencing at Texas A&M University was able to provide the missing sequences. The sequence data from the 354 loci were used for 1) blast searches with the aim of identifying clones with homologues in the international data bases (Benson et al 1993) and 2) the design of PCR primers to amplify the corresponding loci from genomic DNA. The information obtained from blast searches will be provided in detail elsewhere.

PCR primer synthesis for recovery of STSs

The PCRPlan program of the PC/Gene software package was used to design suitable primers for amplification of the corresponding genomic loci. The two terminal sequences for a given clone were combined in inverse orientation and treated as a single sequence for computer analysis. More than 300 primer pairs have now been synthesized and validation has been completed for the majority. The validation test requires that each pair of primers should amplify:

- a single product of the expected size when the sequenced plasmid clone is used as template, and either
- a single product of the same size when genomic DNA from IR36 is template, or
- for RZ clones only, a single product of a larger size if the genomic DNA contains introns missing from the corresponding RZ clone.

Of 100 loci (50 RZ and 50 RG) analyzed in detail (Table 1), all RG clones and 42% of RZ clones satisfied the first and second criteria, while 58% of RZ clones satisfied the first and third criteria.

Amplicon length polymorphisms (ALPs)

Of the 100 primer pairs referred to in Table 1, 70 pairs amplified bands of identical size from IR36 (indica), Taichung 65 (japonica), and *O. longistaminata* (AA genome wild species). However, at 30 loci, ALPs were seen among these three DNAs. Figure 2 shows the amplifications obtained for five representative RG and RZ loci. RG369B and RZ452 were monomorphic, amplifying a product of the same size from plasmid DNA and the three genomic DNAs. With RG100 and RG450, the amplicon from *O. longistaminata* was different from the other three amplicons. RZ142 showed evidence of an intron but the intron length was different in IR36 compared with

Table 1. Comparison of amplicon lengths among IR36 (indica), Taichung 65 (japonica), and *Oryza longistaminata* at 100 sequence-tagged sites.[a]

Amplicon lengths for IR36, Taichung 65, and *O. longistaminata*	Type of marker				
	RG	RZ +intron	RZ -intron	Total RZ	Total
Same length	33	16	21	37	70
O. longistaminata different	14	12	0	12	26
IR36 different	0	1	0	1	1
T65 different	2	0	0	0	2
All three different	1	0	0	0	1
Total	50	29	21	50	100

[a]The sites correspond to 50 RG and 50 RZ markers (see text). The RZ markers were divided into two groups based on whether the primer pairs amplified a band from IR36 genomic DNA that was larger than (+intron) or equal in size to (-intron) the amplicon from the corresponding RZ clone.

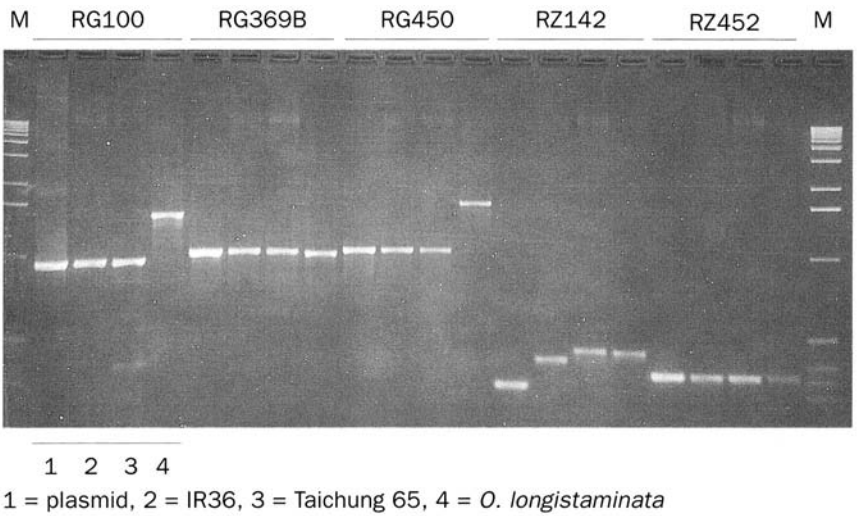


Fig. 2. Comparison of amplicon lengths obtained for plasmid DNA and genomic DNA from IR36, Taichung 65, and *O. longistaminata* at five sequence-tagged sites (RG100, RG369B, RG450, RZ142, and RZ452).

Taichung 65 and *O. longistaminata.* The additional length of amplicons attributable to introns varied from 0.1 to 2.0 kbp.

Most of the ALPs were between *O. longistaminata* and the two *O. sativa* cultivars. Only 4% of the loci showed ALPs between IR36 and Taichung 65. The percentage of ALPs was higher for RZ loci with introns (45%) than for RG loci (34%). No ALPs were found for RZ loci that lacked introns between the primer annealing sites. This result reflects the greater evolutionary constraints placed on coding sequences compared

with introns in respect of the insertions and deletions required to register an ALP. We are now studying the frequency of RFLPs obtained by digestion of these amplicons with a range of restriction endonucleases.

Applications of STSs

We turn now to four applications of STSs: two examples of DNA marker-aided selection, the use of multiplexing in the search for transgenes, and germplasm analysis within the AA genome wild species.

Pyramiding of major genes for durable resistance to bacterial blight

Durable pest and disease resistance continues to be a major goal of rice breeding (Khush 1995). Although it is recognized that major resistance genes are unlikely to offer durability when used singly, it is possible that combinations of major genes could achieve this goal. To test this hypothesis, IRRI staff are pyramiding major genes for blast and bacterial blight resistance using DNA marker-aided selection. These diseases are found in most countries and ecosystems where rice is grown but are especially prominent under rainfed cultivation. The pyramiding process was initiated with RFLP/DNA blotting, but now about half of the markers have been converted into STS markers. Table 2 shows the current situation with four genes for bacterial blight resistance. STS markers are available for *xa5* and *Xa21* but DNA blotting is still required for *Xa4* and *xa?* (the latter on chromosome 8). For a population of 200 lines, STS marker-aided selection for two loci is accomplished in less than a week, but selection by DNA blotting takes up to 2 mo and consumes more leaf material, more chemicals, and more labor.

Breeding for gall midge resistance

Gall midge is the major dipteran pest of rice. It is found from South China to Sri Lanka and in parts of tropical Africa. Asian rice gall midge (*Orseolia oryzae*) exists as a number of biotypes. For some of these biotypes, excellent sources of major gene resistance are known, but changes in biotype distribution and the evolution of new biotypes pose a threat to rice production in certain areas. The absence of gall midge

Tabl 2. Sequence-tagged sites for pyramiding genes for bacterial blight resistance.

Gene (chromosome)	Marker	Genetic distance (cM)	RFLP/DNA blot or STS	Restriction endonuclease
Xa4 (11)	Npb181	1.7	RFLP	*Hind* III
xa (5)	RG556	0.5	STS	*Dra* I
Xa? (8)	RG136	3.8	RFLP	*Dra* I
	RZ28	5.1	RFLP	*Xho* I
Xa21 (11)	pTA248	1.2	STS	ALP[a]
	RG103	0	STS	*Mva* I

[a] *ALP* = amplicon length polymorphism.

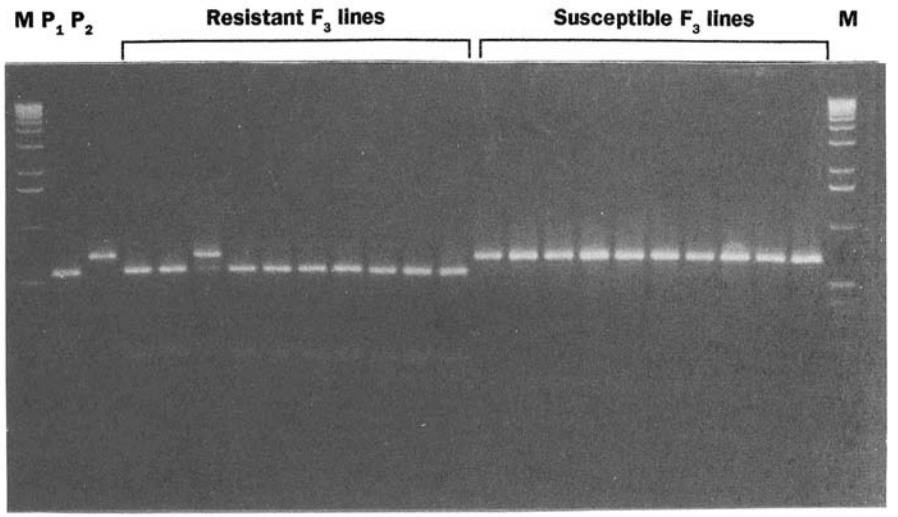


Fig. 3. Cosegregation of *Gm6(t)* with STS marker RG476. DNA was extracted from the two parental lines and from 10 F_3 lines breeding true for resistance and 10 F_3 lines breeding true for susceptibility. M = marker DNA, P_1 = Duokang #1 (resistant parent), P_2 = Feng Ying Zhan (susceptible parent). Note a single recombination event giving a heterozygote in the third resistant F_3 line.

from the Philippines, the existence of biotypes, and the sporadic occurrence of the high-humidity conditions favoring the insect have created difficulties for IRRI in developing a breeding program for gall midge resistance. DNA markers linked to resistance genes offer a way forward, not only for IRRI's breeders but also for breeders in affected countries wishing to pyramid genes for more durable resistance. Figure 3 shows the use of STS marker-aided selection for the *Gm6(t)* gene that provides resistance to gall midge biotypes 1-4 in southern China (Tan et al 1993). The marker 476 is located on chromosome 4, about 4 cM from the *Gm6(t)* gene. Note in Figure 3 the single heterozygote, representing one recombination event among the 20 lines breeding true for either resistance or susceptibility.

Multiplexing for studies on the inheritance of transgenes

PCR-based methods are used to follow the inheritance of genes inserted into plants by transformation. In most cases, it is easier to follow the inheritance of these "transgenes" by PCR than by any other method. Of course, transgenes may be amplified directly: it is not necessary to identify flanking markers for them. Transgenes are therefore usually not mapped and they do not constitute STSs. However, in all other respects, they may be analyzed as if they were STSs.

Figure 4 illustrates the application of the method to a backcross population. The transgene in this example is a synthetic *CryIA(b)* gene acquired from Plantech in Japan in a Nipponbare background (Fujimoto et al 1993). The backcross was part of

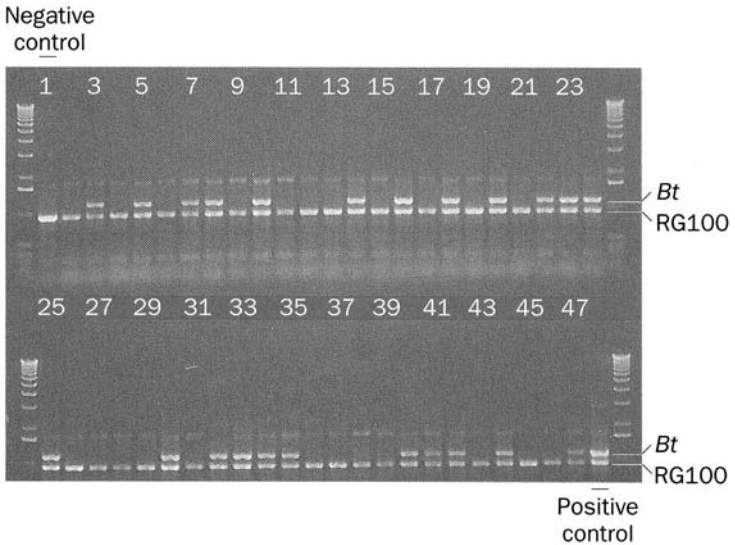


Fig. 4. Segregation of synthetic *CryIA(b)* gene in BC_2 population. *Bt* rice from Fujimoto et al (1993) crossed and backcrossed with line A as recurrent parent. PCR with primer pairs specific for *Bt* gene and RG100. End lanes: DNA markers, lane 1: negative control (DNA from nontransgenic plant), lane 48: positive control (DNA from nontransgenic plant plus DNA of plasmid *Bt* 1291 (Fujimoto et al 1993) equivalent to one gene copy per haploid rice genome, lanes 2-47: DNA from BC_2 plants.

a program to introduce the gene into the IRRI breeding line A3049. As expected, the *CryIA(b)* gene segregated 1:1 (PCR positive:PCR negative) in the BC_1 population.

One way of reducing costs and increasing the convenience of DNA marker technology is to use duplexing or multiplexing: the mixing of two or even more pairs of primers to give more than one amplicon per incubation. Of course, this is only possible when the amplicons differ in mobility and do not require digestion with restriction endonucleases to detect polymorphisms. We use duplexing in the PCR analysis of transgenic plants (Fig. 4). The purpose here is to provide a positive control for the DNA extraction and the PCR reaction. One primer pair corresponds to the transgene, the other to an endogenous rice DNA sequence (usually RG100 from chromosome 3). This control is a useful safeguard when rapid minipreps (Zheng et al 1995b) are employed for extraction of leaf DNA: the DNA is used for PCR without quality check or quantity check. Using duplexing for quality control is cheap, fast, and convenient.

Application of STS markers to the study of relations within the AA genome species

We are using STSs to develop rapid procedures for the analysis of AA genome species of the genus *Oryza*. Table 3 shows that, with just 14 STS primer pairs and three

Table 3. Evolution of a DNA marker-aided selection program.

Stages in marker-aided selection program	Location of stage[a]	
	Present	Future
1 Priority setting and identification of breeding objective	B	B
2 Identification of donor and recipient varieties	B	B
3 Development of mapping populations	B	B
4 Phenotyping of mapping populations	B	B
5 DNA extraction from mapping population	M	B
6 RAPD/bulk segregant analysis to tag gene	B	
7 Mapping of gene	M	M
8 Identification of closest flanking markers (<5 cM)	M	M
9 Identification of polymorphisms between donor and recipient varieties	M B	B B
10 Development of breeding populations	M	B
11 Application of DNA marked-aided selection		

[a]B = breeding station, M = specialist DNA marker laboratory.

accessions of each species, it is possible to infer that *O. meridionalis* is the AA genome species most distantly related to *O. sativa,* with *O. longistaminata* the second most distantly related. Wang et al (1990) came to the same conclusion about affinities among AA genome species through an extensive study using RFLP/DNA blots. Table 3 shows also that *O. longistaminata* is the AA genome species with the greatest intraspecific genetic diversity, a result consistent with its outbreeding habit (Vaughan 1994).

From such studies on AA genome species, non-AA genome species, and the cereals in general, we shall form a data base that will simplify the molecular characterization of these materials. The data base will identify STS markers that are appropriate to use within taxa of different degrees of divergence. The results in Table 3 indicate that STSs will simplify the study of gene flow between *O. sativa* and weedy AA genome relatives found in Asia, such as *O. nivara* and *O. rufipogon.* Such studies will be relevant to the current debate on biosafety of transgenic rice.

Conclusions

Molecular breeding with STS markers as a multidisciplinary activity

Zheng et al (1995a) describe in detail the methodology of PCR-based DNA marker-aided selection using STS. Here, we emphasize the multidisciplinary nature of molecular breeding. Table 4 presents a breakdown of molecular breeding into 11 steps. About half of these steps are routine for the expert staff of rice breeding stations. Relatively few steps require the expertise and infrastructure of a specialized DNA marker laboratory.

Agreement on step 1 is important for effective collaboration between the breeding station and the DNA laboratory and may require considerable multidisciplinary activity in itself. The staff of the breeding station would certainly be able to accomplish steps 2-4 and step 10 with conventional resources. Initially, the staff of the DNA marker

Table 4. PCR-based comparison of the eight AA genome species of the genus *Oryza* at 14 sequence-tagged sites (STSs).

Species	Number of STSs showing species-specific ALP[a]	Number of STSs showing Intraspecific ALP
O. sativa	1	3
O. rufipogon	0	4
O. barthii	0	2
O. nivara	0	7
O. glaberrima	0	5
O. glumaepatula	1	4
O. longistaminata	3	11
O. meridionalis	5	4

[a]ALP = amplicon length polymorphism.

laboratory would have to accomplish steps 5-9 and collaborate with the breeding station on step 11.

If step 11 involves PCR-based STS analysis, this step would be one of the first steps transferred to the breeding station, along with step 5. Step 6 would be transferred next, followed perhaps by step 9. Because of their resource requirements and their difficulty, steps 7 and 8 would probably remain the responsibility of the DNA marker laboratory. Nevertheless, over a period of about 5 yr, it should be possible for major rice breeding stations in Asia to develop basic skills in DNA marker technology and experience the benefits of the approach.

Maximizing the probability of finding intraspecific STS polymorphisms

The frequency of STS polymorphisms between and within the indica and japonica subspecific groups of *O. sativa* will determine the utility of STS-based markers for rice breeding. ALPs will be uncommon (Table 1) and it will be necessary to use RFLP analysis on the STS amplicons. In their study of STS polymorphisms between ecotypes of *Arabidopsis thaliana,* Konieczny and Ausubel (1993) used up to 83 restriction enzymes to look for polymorphisms. This reliance on restriction endonucleases can become very expensive and is not certain to be effective.

Strategies are needed for maximizing the probability of detecting STS polymorphisms and for deciding when to move on to alternative approaches. These strategies will emerge as experience with STS markers and other markers increases. For this reason, it is desirable that an international STS data base be set up to record the successes and failures that are encountered in attempts to find polymorphisms for STS markers between various cultivars.

STS data base

A data base has been initiated at IRRI to record the results of STS marker studies in a systematic manner. Data base entries will draw attention to a range of helpful points.

For example, Table 1 suggests that cDNA clones for genes lacking introns should be avoided when searching for ALPs and probably also PCR-based RFLPs. The data base will indicate whether RZ clones (and other cDNA clones) correspond to gene segments with or without introns. The data base will also identify which restriction enzymes have been used to digest each amplicon and how many bands are produced. It should be possible to correlate these data with the lineages of breeding programs to understand how ancestral genes have been inherited by elite breeding lines. Inclusion of these data in the RiceGenes relational data base (Paul et al 1994) would be highly desirable.

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Marker-assisted prediction of agronomic traits using diverse rice germplasm

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A set of 47 diverse accessions of Asian rice was grown in a single trial at IRRI. The number of culms per plant and the time to 50% flowering were measured. DNA was then extracted and a set of 63 randomly amplified polymorphic DNA (RAPD) markers that were polymorphic across the 47 accessions was scored. Multiple regression analysis was carried out to determine whether associations between the presence/absence of individual markers and performance for the two quantitative traits could be detected. The results showed that 85% of the variation for culm number and nearly all the variation in flowering time could be explained by regression models using sets of RAPD markers. Isozyme analyses were then carried out on the same 47 accessions yielding a set of 39 polymorphic markers. Multiple regression analysis revealed that 70% of the variation in culm number and 49% of the variation in flowering time could be explained using this more limited set of markers. Analyses were performed using RAPD and isozyme data together to determine the best regression models and for culm number an improvement of 5% in r^2 was achieved. Using these models for the remaining 46 accessions, predictions were made of performance for the two traits for each of the 47 accessions individually. Only four of the 94 performance predictions significantly differed from the observed performance.

Biodiversity offers opportunities to the research scientist and a portfolio of varying characters to the breeder. Crop biodiversity, in the form of plant genetic resources, is actively conserved in genebanks around the world. Centers of the Consultative Group on International Agricultural Research (CGIAR) maintain about 500,000 accessions of more than 30 crops and the U.S. Plant Germplasm System stores 380,000 samples of more than 8,000 plant species. The International Rice Genebank in the Genetic

Resources Center at IRRI holds more than 80,000 samples of rice germplasm (Jackson and Huggan 1993, Jackson 1994). Because of their size, the management of such collections can be very difficult. Recent work has shown that molecular marker techniques can be valuable as parts of protocols designed to measure diversity and facilitate the organization and prioritization of germplasm resources (Virk et al 1995a,b).

A further problem for management of large germplasm collections is the identification of the most appropriate material to distribute for use in basic and applied research programs. Within the rice collection held at IRRI, a large number of accessions have been characterized for easily measured quantitative traits such as culm height, culm number, and days to flowering, among others as a result of an ongoing program of field evaluation. However, it is not possible to characterize all of the accessions for the large range of traits that may be of interest to pathologists, physiologists, agronomists, geneticists, and even social scientists. Performance for many important traits such as stress tolerance requires much more carefully controlled conditions than can be achieved in routine field tests, and it is not usually possible to apply such evaluations to more than a small proportion of the whole collection. The ability to predict performance for quantitative traits of germplasm collections would be extremely valuable as it would greatly improve the efficient provision of appropriate genetic material to plant breeders and research scientists.

In this study, we have tested the hypothesis that, using a diverse range of rice germplasm, we can detect associations between individual randomly amplified polymorphic DNA (RAPD) markers and performance for two quantitative traits using multiple regression analysis. Further studies were then carried out to discover whether such associations could be detected using isozyme markers. The data obtained have been used to demonstrate that performance for the traits can be predicted for rice accessions using molecular marker data and the regression models that have been constructed.

Materials and methods

Quantitative evaluation

A set of 200 rice accessions from South and Southeast Asia maintained in the International Rice Genebank at IRRI was selected at random. These 200 accessions were then evaluated at Los Baños, Philippines, in a randomized plot experiment with two replicate blocks, which were grown during the dry season (Nov 1993-May 1994). The quantitative data were collected on 10 representative plants of each accession and each plant was scored for 10 traits: culm number, culm length (cm), culm diameter (mm), grain length (mm), grain width (mm), leaf length (cm), leaf width (cm). days to 50% flowering, panicle length (cm), and seedling height (cm), essentially following the IBPGR-IRRI descriptors for rice (IBPGR-IRRI Rice Advisory Committee 1980).

To assemble material that represented the range of diversity found within the initial 200 accessions, the quantitative data standardized to zero mean and a unit variance were subjected to cluster analysis. Agglomerative hierarchical clustering was performed, on the squared Euclidean distance matrix utilizing the unweighted pair

group method using arithmetic averages (UPGMA) (Rohlf 1992). This then led to the selection of 47 accessions (Table 1), whichwere extracted from the resultant dendrogram (not shown) by stratification.

Table 1. Countries from which rice accessions were originally collected, their observed and predicted performances for days to 50% flowering and culm number.[a]

Accession	Country	Days to 50% flowering		Culm number	
		Observed	Predicted	Observed	Predicted
25840	Bangladesh	74.3	75.9	34.5	33.9
25851		71.7	70.1	36.5	39.9
25868		74.3	77.4	30.3	25.5
64789		71.2	66.5	37.3	44.6
64792		76.8	74.7	22.7	22.1
64793		77.0	79.9	42.1	40.2
66787		**83.5**	**77.9**	18.3	25.6
66791		77.0	76.1	32.4	34.4
66817		75.8	76.9	40.9	38.3
77210		88.5	86.0	71.7	64.4
77264		82.2	79.5	57.0	57.1
77272		57.5	57.7	24.5	25.7
77279		91.2	89.9	41.0	45.6
64887	Bhutan	57.8	61.4	30.4	23.2
64890		67.0	66.8	19.7	16.4
64913		57.3	55.7	24.6	23.9
67848		72.7	75.6	34.7	25.8
67480	India	65.4	67.8	41.0	33.8
74716		59.4	61.0	43.4	36.6
74720		56.0	56.3	**23.0**	**34.0**
74773		73.5	69.9	40.7	37.5
66540	Indonesia	108.1	108.2	33.5	32.5
66603		94.4	93.4	38.1	36.9
66612		118.5	114.4	28.5	22.8
66669		124.4	128.6	24.0	29.3
66678		73.6	75.4	31.4	36.9
71493	Malaysia	90.1	87.9	19.0	24.8
71501		92.5	94.6	22.0	19.3
71515		80.3	81.6	23.6	19.1
71517		95.6	96.2	26.0	23.4
71537		109.5	110.8	21.3	17.5
71545		75.5	76.2	30.7	31.1
71578		101.1	105.6	39.9	40.5
71596		91.2	90.5	22.5	24.3
71646		106.0	102.3	20.5	24.6
73090	Pakistan	88.0	84.2	54.3	63.2
67436	Philippines	104.0	106.4	**23.2**	**35.3**
66513	Sri Lanka	121.6	119.3	38.2	36.6
66529		93.0	94.9	19.9	19.6
78245	Thailand	72.7	72.4	26.9	34.0
78250		82.0	79.7	34.0	41.7
78253		84.6	86.9	46.9	38.6
78259		76.6	74.5	17.8	17.5
78270		96.4	101.5	**43.4**	**31.2**
78275		74.5	76.5	11.2	14.5
78276		67.4	65.4	31.7	31.7
78357	Vietnam	90.9	89.5	47.3	44.2

[a]Figures in boldface differ at the 5% level.

DNA extraction and PCR analysis

Seedlings of the 47 accessions were raised in jiffy pots for 2-3 wk to obtain leaf material for DNA extraction. Two milligrams of fresh leaf were taken from each of 10 randomly selected seedlings and then mixed before co-extraction to obtain a DNA sample representative of each accession (Virk et al 1995a). For the RAPD analysis, the total reaction volume was 25 µl containing 5 ng DNA, 200 µM of each dNTP, 0.4 µM decanucleotide primer (supplied by Operon), 1 unit of *Taq* polymerase, 2.25 mM $MgCl_2$ and 1X (16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8), 0.1% Tween 20) reaction buffer. The mixture was overlaid by 45 µl of mineral oil and the amplification was performed in a thermocycler (Hybaid-Omnigene) programmed as follows: 1 cycle of 95 °C for 2 min, 2 cycles of 95 °C for 30 s, 37 °C for 1 min and 72 °C for 2 min, 2 cycles of 94 °C for 30 s, 37 °C for 1 min and 72 °C for 3 min, 41 cycles of 94 °C for 30 s, 35 °C for 1 min and 72 °C for 2 min, and finally 1 cycle of 72 °C for 5 min. Ten microliters of the amplified products were subjected to electrophoresis on a 1.4% agarose gel cast in 1XTBE and run in 0.5X TBE at 200 V for 2.5-3.0 h. The electronic image of the ethidium bromide-stained gel was captured using a Flowgen IS500 imaging system and the bands were scored from the image displayed on the monitor. The seven Operon primers used in the RAPD analysis were C-03, C-06, C-08, C-10, C-14, F-13, and K-11. These primers were selected from a survey of more than 100 decamers screened with six diverse accessions of rice, only on the basis that they revealed a large number of polymorphic bands.

Isozymes

Isozyme variation was surveyed using 22 enzyme loci following Glaszmann et al (1988). For enzyme extraction, 10 coleoptiles were used. As the monomorphic allelic variation was not used in the analyses, the data from 15 enzymes were considered (Table 2).

Data analysis

Associations between the RAPD or isozyme markers and the phenotypic means of accessions for the various quantitative traits were established using the multiple regression approach. Each quantitative trait was treated as a dependent variable and the various molecular marker genotypes (scored 1 for presence and 0 for absence) as independent variables. The analysis was based on the model:

$$Y = a + b_1m_1 + b_2m_2 + \ldots b_jm_j \ldots b_nm_n + d + e$$

which related the variation in the dependent variable (Y = accession means for a quantitative trait) to a linear function of the set of independent variables m_j, representing the RAPD markers. The b_js are the partial regression coefficients that specify the empirical relationships between Y and m_j; d represents the between accessions residual which is left after regression; e is the random error of Y that includes environmental variation. This method provided maximum likelihood solutions of relationships between individual quantitative traits and various markers, which were then

Table 2. Key to polymorphic isozyme and RAPD markers and the oligonucleotides used in the present study.

RAPD markers						Isozyme markers		
Primer	Key	Size	Primer	Key	Size	Locus	Key	Allele
OPC-03	1	325	OPC-14	41	530	Pgi-1	64	1
	2	350		42	600		65	2
	3	375		43	650	Pgi-2	66	1
	4	475		44	950		67	2
	5	775		45	1150		68	3
	6	2500		46	1500		69	4
OPC-06	7	340		47	1350	Amp-1	70	0
	8	355	OPF-13	48	360		71	1
	9	460		49	400		72	2
	10	600		50	420		73	3
	11	1020		51	340	Amp-2	74	1
	12	1600		52	530		75	2
OPC-08	13	280		53	690	Amp-3	76	1
	14	340		54	880		77	2
	15	350		55	1000		78	3
	16	500	OPK-11	56	220		79	4
	17	625		57	480	Amp-4	80	1
	18	950		58	520		81	2
	19	1050		59	650	Sdh-1	82	1
	20	1000		60	1200		83	2
	21	2200		61	1500	Icd-1	84	1
OPC-10	22	350		62	2100		85	2
	23	400		63	2800	Cat-1	86	1
	24	500					87	2
	25	520				Est-1	88	0
	26	600					89	1
	27	625				Est-2	90	0
	28	700					91	1
	29	750					92	2
	30	720				Est-9	93	1
	31	950					94	2
	32	490				Adh-1	95	1
	33	1450					96	2
	34	2200					97	3
	35	2800				Pgd-1	98	1
OPC-I4	36	325					99	3
	37	390				Pgd-2	100	1
	38	410					101	2
	39	460						
	40	520						

used to identify the most significant components (markers) of the best fitting multiple regression equation and test its goodness of fit following Mather and Jinks (1982) and Draper and Smith (1981). The maximum r^2 improvement (MAXR) option of the PROC REG (SAS 1990) was used to determine the most appropriate model. Initially, one-variable (= marker) models were assessed and the marker with the highest r^2 value was identified. Then the second variable was added and the best two-variable model selected using the usual criteria of the largest r^2. The model fitting was continued until

all significant variation in Y was exhausted. A further condition was imposed on the model, whereby a new variable remained in the model only if it was significant at the 5% level.

The multiple regression analyses were carried out on 47 accessions. The predictive power of the model utilizing the information that is available from the data can be validated with the use of the "leave out one at a time" method where multiple regression analysis is applied to 46 of the 47 accessions at a time and the 47th is predicted. This procedure allowed for the fitting of 47 models for each trait, one for each accession, and the variance of the 47 residual deviations provided a measure of the standard deviation of the predictions.

Results

Forty-seven accessions of *Oryza sativa* were selected to represent the diversity found in South and Southeast Asia (see Table 1). RAPD data were generated for these 47 accessions using seven decamer primers and this revealed 63 polymorphic markers (Table 2).

Multiple regression analysis was used to detect associations between presence/absence of RAPD markers and performance for two quantitative traits, days to 50% flowering and culm number. Nearly all (99.8%) of the variation for days to flowering was explained by a subset of 29 RAPD markers. One marker (49 = OPC-14-400) explained 23.2% of the total variation. Similarly, 85.2% of the variation in performance for culm number could be explained using nine RAPD markers with one marker (14 = OPC-08-340) explaining 48.4% of the total variation (Tables 3 and 4).

To test whether such associations could be detected using other types of molecular markers, isozyme analysis was carried out. The presence/absence pattern of 46 alleles was recorded, and 39 were found to be polymorphic across the 47 accessions (Table 2). Multiple regression analysis was carried out using this more limited set of data. Three isozyme markers together explained 48.7% of the variation in performance for flowering time and 11 markers explained 70.2% of the variation in culm number.

Further multiple regression analyses were carried out using the same quantitative trait data but with the combined dataset containing both RAPD and isozyme markers. For flowering time, a set of 29 markers had already explained 99.8% of the variation among the accessions so that addition of the isozyme marker data had no effect on the regression model. However, for culm number the combined set of markers improved the model from explaining only 85.2% (RAPD markers only) or 70.2% (isozyme markers only) to explaining 90.0% of the variation. This improvement was the result of the combined effect of seven RAPD and six isozyme markers (Table 3).

Predictions for the performance for every accession for each of the two traits were made based upon their marker profiles. In each case, the data for the remaining 46 accessions were used to estimate the parameters of the regression model for each trait. Hence, 94 predictions were possible and of these only 4 differed significantly from the observed score (see Table 1).

Table 3. Regression coefficients, Fisher's test of goodness of fit of the model, and r^2 value for culm number.

Marker	Regression coefficient		
	RAPDs	Isozymes	RAPDs + Isozymes
5	4.03		
14	-13.29		-11.68
26	-7.39		-4.98
33	-10.11		-12.22
37	-6.45		
41	17.67		13.33
46	-21.13		-21.25
53	7.50		7.89
59	-3.69		-4.42
66		10.45	
71		15.07	9.63
77		13.36	
78		-15.31	-6.32
82		33.70	4.40
83		34.03	
86		10.80	
88		6.42	
91		10.78	5.35
93		9.32	7.63
99		-10.52	-4.98
Intercept	69.87	-33.03	56.63
F	0.78 (37,46)	1.66 (35,46)	0.59 (33,46)
r^2	85.2%	70.2%	90.0%

[a]See Table 2 for key to markers; each regression coefficient is significant at the 5% level.

Table 4. Regression coefficients, Fisher's test of goodness of fit of the model, and r^2 value for days to 50% flowering.

RAPDs or RAPDs + isozyme marker[a]	Regression coefficient	Isozyme marker	Regression coefficient
2	-6.35	71	19.22
3	-7.91	78	-22.89
5	-7.90	87	16.84
9	-10.24		
10	12.73		
16	-18.43		
17	5.38		
18	4.48		
19	16.68		
21	-11.94		
22	25.57		
24	-4.06		

continued on next page

Table 4 continued

RAPDs or RAPDs + isozyme marker[a]	Regression coefficient	Isozyme marker	Regression coefficient
25	-14.88		
26	-28.31		
27	31.32		
28	6.77		
31	-3.96		
32	-5.89		
33	11.65		
34	-7.10		
37	-22.45		
39	14.58		
42	8.81		
45	-13.88		
49	22.57		
54	-19.81		
55	-21.73		
57	-30.33		
61	-23.64		
Intercept	152.78		72.34
F	0.34 (17,46)		30.41 (43,46)
r^2	99.8%		48.7%

[a] See Table 2 for key to markers; each regression coefficient is significant at the 5% level.

Discussion

Our results demonstrate that molecular markers can be used to predict rice phenotypes within germplasm collections. Associations have been found between performance for two quantitative traits and both RAPD and isozyme markers. In other work, we have shown associations between RAPD markers and four further quantitative traits in rice (leaf length, grain width, panicle length, and culm length); in total, we have looked at six traits and have detected such associations in all cases (Virk et al 1995c).

We have also tested our approach by using a set of 26 accessions of wild *Beta* germplasm. Using 64 polymorphic RAPD markers, associations were found with days to flowering (the only quantitative trait evaluation data available to us). Predictions of flowering time were made using the regression model and none of the 26 predictions significantly differed from the observed value.

One possible reason for the associations between markers and performance for quantitative traits reported here could be linkage disequilibrium involving chance associations due to correlated allele frequencies in small samples. However. genetic linkage between markers and quantitative trait loci (QTLs) is the most likely explanation for these results. If true, it appears that linkage between alleles at QTLs and at marker loci has been conserved throughout the period of diversification of rice

germplasm in South and Southeast Asia. To test for such linkage, mapping work is currently being carried out on a segregating population of rice using the markers identified above.

Around one-third of the variation for culm number was accounted for by *Cat-1* (allele 1), which is located on chromosome 6 (Kinoshita 1993). Interestingly, the most important RAPD marker for this trait (marker number 14; Table 2) was found to be strongly associated ($c^2 = 32.75$ (1 *df*) $P < 0.001$) with this isozyme marker. This suggests that for culm number, QTLs with a large effect along with the *Cat-1* and RAPD marker under question are situated on chromosome 6. Our initial mapping also places this RAPD marker on chromosome 6.

If genetic linkage is the main cause of the associations, then one benefit of obtaining information about molecular markers and quantitative traits could be for the efficient selection of parents for producing populations to map QTLs for a particular trait. For example, for mapping QTLs for days to 50% flowering, two accessions (IRGC 66669 and IRGC 74720) represent phenotypic extremes for this trait and are also polymorphic for the RAPD bands that are informative with respect to performance for this trait. Also, this procedure will provide an initial screening method for the identification of QTLs. The established method for this is the selection of two parents that differ markedly in a particular character, and then the determination of associations between markers and that character in F_2 or backcross progeny. The apparent advantages of using diverse germplasm instead are 1) that this could allow the detection of QTLs that vary across a wide spectrum of biodiversity rather than just between two parental lines, and 2) that QTLs for any quantitative trait can be studied in the same investigation.

Regardless of the underlying causes of the associations that we have detected, the use of molecular markers such as RAPD, which are more or less randomly distributed across the genome (Kurata et al 1994), coupled with the multiple regression analysis, could substantially change the way that crop biodiversity is used in the future. The combination of techniques will allow us to predict what a plant will look like in a determined environment in terms of quantitative agronomic traits prior to elaborate field trials. Furthermore, if a diverse test array of germplasm of a species is scored for important traits requiring specialized assessment conditions (such as stress tolerances), then marker data can provide an efficient means of predicting the value of additional germplasm for such characteristics, and even to identify suitable material among germplasm in situ.

Our results therefore demonstrate the value of ex situ plant germplasm collections not just as repositories of useful genes, or as sources of information about phenotypic characters. One of the major criticisms regularly leveled at genetic resource conservation programs over the last 40 yr has been that they have frequently been unable effectively to provide material for crop improvement. However, with appropriate organization and the application of current DNA-based technology, genebanks can more easily fulfill this role and become much more valuable interfaces between the activities of conservationists on the one hand and those wishing to exploit germplasm for the benefit of mankind on the other.

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Notes

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Relationships between molecular marker polymorphism and hybrid performance in rice

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This study investigated the relationship of molecular marker heterozygosity with hybrid performance and heterosis in rice in an attempt to make use of molecular markers in predicting hybrid performance. Four sets of diallel crosses, 147 in total, were made using parents encompassing a wide range of germplasm, including 1) a set of 28 crosses using 8 elite parental lines commonly used in hybrid rice production in China, 2) a set of 36 crosses using 9 indica varieties representing a wide range of germplasm from three countries, 3) a set of 55 crosses using 11 japonica varieties also with a wide range of representation from three countries, and 4) a set of 28 crosses using 8 long-grained rice lines from the southern United States. All F_1s and their parents were evaluated for agronomic performance in replicated field trials. The parental lines were surveyed for DNA polymorphisms using more than 100 molecular markers including restriction fragment length polymorphisms and simple sequence repeats. The marker genotypes of the F_1s were deduced from the genotypes of their parents. The results showed that there are extensive genetic polymorphisms among the parental lines of each diallel set. High levels of heterosis are common in yield and yield component traits in crosses of all four diallel sets. The correlations between marker genotype divergence and hybrid performance varied greatly from one data set to another depending on the materials studied. The highest correlation was observed between hybrid yield and general heterozygosity in the set of southern US. long-grained varieties. A high level of correlation was also observed between specific heterozygosity and heterosis in the set of Chinese hybrid rices, whereas the correlations were much lower in the sets of indica and japonica mixtures. Detailed characterization of the rice germplasm and in-depth understanding of the genetic basis of heterosis are needed for molecular markers to have practical utility in predicting hybrid performance.

Recent advances in genome research have generated considerable interest in predicting hybrid performance using molecular markers. Hopefully, the availability of molecular marker-based linkage maps will provide an effective means for predicting heterosis, which would speed up the selection process.

A prerequisite in using molecular markers is a strong correlation between marker distance between the parents and F_1 performance (heterosis). Studies conducted in maize to investigate the relationship between marker distance and hybrid performance have produced mixed results (Lee et al 1989, Smith et al 1990, Godshalk et al 1990, Melchinger et al 1990, Dudley et al 1991, Boppenmaier et al 1993). In rice, Zhang et al (1994, 1995) studied the relationship of molecular marker heterozygosity with hybrid performance and heterosis in a number of characters using eight elite parental lines of hybrid rice in China. Their analyses showed correlations to be mostly low between general heterozygosity, which was based on all the markers included in the study, and F_1 performance and heterosis. In contrast, very high correlations were detected between mid-parent heterosis and specific heterozygosity, which was based only on the markers that detected significant effects on each trait, for most of the traits. They suggested that such a high level of correlation might have a practical utility in predicting heterosis.

Here, we report on the results of our studies on correlations between molecular marker polymorphisms and hybrid performance using four sets of rice varieties. The objective was to investigate the extent of such correlations in different rice germplasm.

Materials and methods

Genetic materials and field experiments

We used four sets of rice lines, collectively representing a wide range of rice germplasm (Table 1). The first set consisted of eight lines representing elite indica hybrid rice germplasm in China, which include the parents of several of the best performing hybrids in China as well as the parents of some newly released hybrids (Lin and Ming 1991). The second set consisted of nine indica varieties from three Asian countries and included landraces, primitive cultivars, historically important cultivars, modern elite cultivars, and parents of superior hybrids. The third set consisted of 11 japonica varieties from three countries, also representing a wide range of japonicas. The eight varieties in the fourth set were long-grained rices adapted to the southern United States and used extensively as parents there.

All rice lines within each set were intermated in all possible nonreciprocal combinations to form four sets of diallel crosses, resulting in 28 crosses each in sets 1 and 4, 36 in set 2, and 55 in set 3. All progeny and respective parental lines were examined for agronomic performance in replicated field trials. Field tests for sets 1, 2, and 3 were conducted at two locations in Hubei Province, China; field tests for set 4 were conducted at two locations in Arkansas, USA (Gravois 1994).

Markers and laboratory assay

Two classes of markers were used to survey DNA polymorphism among the parental lines: restriction fragment length polymorphisms (RFLPs) and simple sequence repeats

Table 1. Genetic materials used in this study.

	Name	Description	Source
Set 1: hybrid parents	Ce 64 (IR9761-19-1-64)	Restorer	China (IRRI line introduced in China)
	Guang B	Maintainer	China
	Maxie	Maintainer	China
	Minghui 63	Restorer	China
	Qingsiai	Maintainer	China
	Teqing	Restorer	China
	Xiangai	Maintainer	China
	Zhenshan 97	Maintainer	China
Set 2: indica mixture	Nanjing 11	Cultivar	China
	Teqing	Cultivar (hybrid parent)	China
	Zhaiyeqing 8	Cultivar	China
	Aijiao Nante	Cultivar	China
	Shengli Xian	Cultivar	China
	Minghui 63	Cultivar (hybrid parent)	China
	Indonesia paddy rice	Landrace	Indonesia
	Yuchi 231-8	Cultivar	China
	Dular	Landrace	India
Set 3: japonica mixture	Taichung 65	Cultivar	China (Taiwan)
	Mudanjiang 8	Cultivar	China
	Taihu Wanjing	Landrace	China
	Zaoshajing	Cultivar	China
	Balilla	Cultivar	Italy
	Yaso	Cultivar	Japan
	Nongken 58	Cultivar	China
	Akihikari	Cultivar	Japan
	C57	Cultivar (hybrid parent)	China
	02428	Cultivar (hybrid parent)	China
	Ketan Nangka	Landrace	Indonesia
Set 4: long-grained lines	Jasmine 85 (IR841)	Cultivar	USA (IRRI line introduced in the USA via Thailand)
	Katy	Cultivar	USA
	Lemont	Cultivar	USA
	L202	Breeding line	USA
	Millie	Cultivar	USA
	Newrex	Cultivar	USA
	RU8601136	Breeding line	USA
	Lebonnet	Cultivar	USA

(SSRs). The markers were selected from a published molecular marker linkage map (Tanksley et al 1992, Wu and Tanksley 1993), the details of which are described in Zhang et al (1994). The first set of parental lines was surveyed for RFLPs with each of 140 RFLP probes, and for SSRs with 12 pairs of published primer sequences (Wu and Tanksley 1993, Yang et al 1994). DNA polymorphisms of parental lines in the second and third sets were assayed with 96 RFLP and 10 SSR markers. A total of 108

markers, including 82 RFLPs and 26 SSRs, were used in the fourth set. The majority of the markers assayed were common across the four sets of parental lines.

Data processing and statistical analysis

The molecular marker data were scored and processed following the schemes described by Zhang et al (1994). Briefly, banding patterns of the RFLPs, resolved by a majority of probe/enzyme combinations (PEs) and SSRs that are in accord with single-locus variation, were scored as genotypes. RFLPs and SSRs, which did not agree with single-locus variation (multiple variable bands), were scored by the presence or absence of individual bands. In cases where redundant data were obtained within a probe among different enzymes, data from only one of the enzymes were used to avoid redundancy. The same data processing principle also applies to those PEs and SSRs whose variation was scored by bands. The marker genotype of an F_1 hybrid was deduced according to the genotypes of the parents, and F_1 heterozygosity was measured as the percent difference of marker genotypes between the two parents.

Results

Molecular marker polymorphisms of the parents

Table 2 shows data pertaining to the level of polymorphisms of each set of parental lines. A "marker" refers to RFLP and SSR variation detected by a single probe or a pair of primers. A polymorphism is calculated as the percent difference of marker genotypes (or bands) between two parental lines based on the polymorphic markers within each set of rice lines.

The set of long-grained rices from the southern United States showed the highest percentage of polymorphic markers. Most of the polymorphism in this set is due to the large difference between cultivar Jasmine 85 and the remaining seven lines. The proportions of polymorphic markers for the sets of hybrid parents and mixture of indica varieties are on the same order of magnitude, while the proportion of the polymorphic markers in the mixture of japonica varieties is lightly lower (Table 2).

The average levels of polymorphisms of the four sets are divided into two groups (Table 2): hybrid parents and indica mixture, with about equal levels of polymorphisms (44.2 and 43.0%, respectively), are in the first group; the japonica mixture and the

Table 2. Molecular marker polymorphisms among the parental lines and grain yield heterosis of the F_1 hybrids in the four sets of diallel crosses. (See text for definition of polymorphic markers.) The average and range of polymorphism are based on polymorphic markers.

Material	Polymorphic markers (%)	Polymorphism (%)		Mid-parent heterosis (%)	
		Av	Range	Av	Range
Hybrid parents	69.1	44.2	24.8–51.9	57.0	-8.5–130.0
Indica mixture	69.8	43.0	19.7–67.2	62.7	-64.4–146.6
Japonica mixture	66.0	37.2	4.7–61.7	90.7	-6.8–237.0
Long-grained lines	88.0	38.8	20.6–82.5	36.1	2.6–106.2

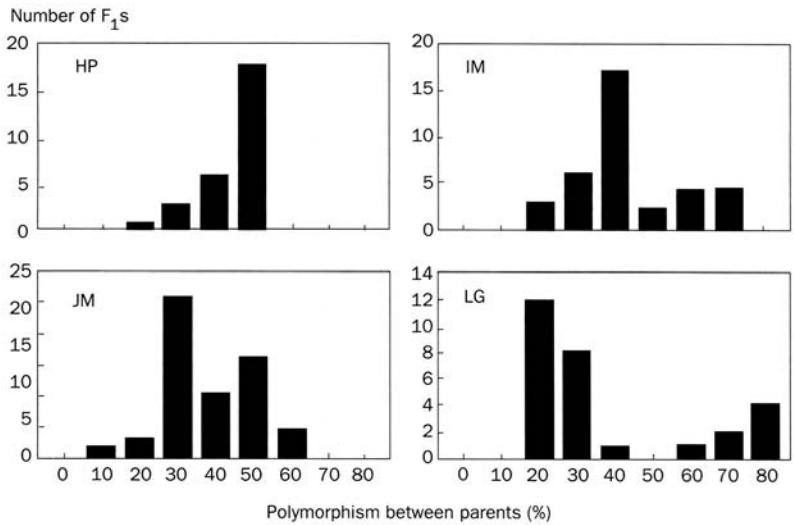

Fig. 1. Distribution of molecular marker polymorphisms in the four sets of rice varieties. HP = hybrid parents; IM = indica mixture; JM = japonica mixture; LG = long-grained varieties.

long-grained rices, also with about equal levels of polymorphisms (37.2 and 38.8 %), are in the second group. Polymorphisms in the japonica mixture and long-grained sets showed much wider distribution than did the sets of hybrid parents and the indica mixture (Fig. 1).

Heterosis

Yield and three yield component traits (number of tillers per plant, number of seeds per panicle, and seed weight) were examined in sets 1, 2, and 3. In all three data sets, yield demonstrated the highest level of heterosis, which is not surprising since yield is determined multiplicatively by the three component traits. The traits examined in the fourth set were total rough rice (equivalent to yield in the other three sets), total head rice, and percentage of head rice. About equal amounts of heterosis were observed in total rough rice and total head rice, but little heterosis was observed in head rice percentage.

The distribution for the amounts of mid-parent heterosis for yield is illustrated in Figure 2; average and range statistics are in Table 2. The level of heterosis varied widely from one cross to another within each diallel set, and also differed greatly among the four sets. The widest range of variation in heterosis was observed among the crosses in the mixture of japonica varieties followed by the mixture of indica varieties. The distribution range of heterosis was much narrower in the sets of hybrid parents and long-grained lines.

Crosses with high heterosis (>l00%) were observed in all four diallel sets. The japonica mixture showed the largest proportion of highly heterotic crosses. However,

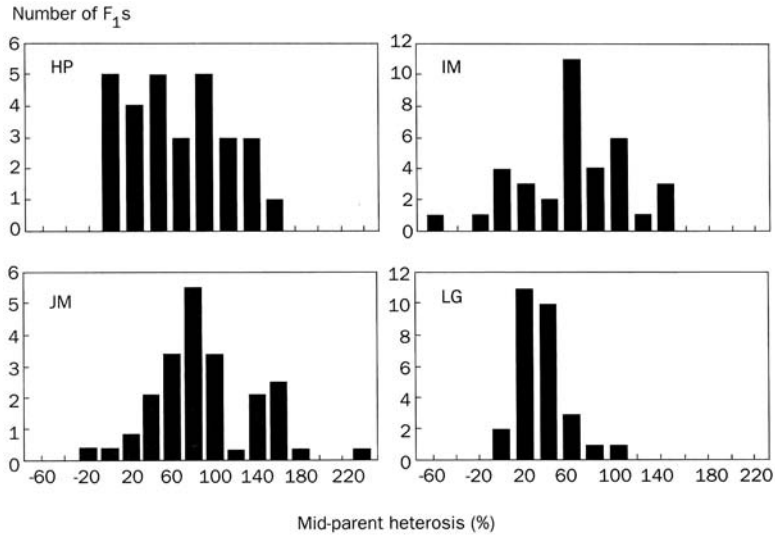

Fig. 2. Distribution of mid-parent heterosis in the four sets of diallel crosses. HP = hybrid parents; IM = indica mixture; JM =japonica mixture; LG = long-grained varieties.

there was a highly significant negative correlation between heterosis and mid-parent values among the crosses within this set, indicating that much of the high heterosis resulted from hybrids between poor-performing parents. Only one cross expressed more than 100% heterosis in the long-grained set, and the overall level of heterosis in this set was much lower than those of the other three sets.

Correlations of marker heterozygosity with hybrid performance and heterosis

Two measurements of heterozygosity were adopted in calculating the correlations of molecular marker heterozygosity with hybrid performance and heterosis: general heterozygosity and specific heterozygosity. General heterozygosity of an F_1 refers to the percentage of difference between the two parents detected by all the markers included in each set. Specific heterozygosity of a particular trait of an F_1 hybrid refers to the percentage of difference between the two parents based only on the markers that, using a one-way analysis of variance, detected highly significant effects on that trait.

In the hybrid parent set, the correlations between general heterozygosity and performance were mostly low for yield and yield component traits, whereas intermediate correlations were observed between general heterozygosity and heterosis (Table 3). Correlations of specific heterozygosity with hybrid performance and heterosis were substantially different from those calculated using general heterozygosity. The most noteworthy incidences were the large increases in the correlations of F_1 seed weight, and heterosis of seeds per panicle and yield, resulting in high levels of correlations between specific heterozygosity and these three attributes of performance and heterosis (Table 3).

Table 3. Correlations of general (GH) and specific (SH) heterozygosity with hybrid performance and heterosis in three sets of diallel crosses. $r_{0.05}$ and $r_{0.01}$ refer to the critical correlation values at the 0.05 and 0.01 probability levels, respectively.

	Tillers per plant	Seeds per panicle	Seed weight	Yield per plant
Hybrid parents ($r_{0.05}$ = 0.374, $r_{0.01}$ = 0.478)				
GH & performance	0.132	0.126	0.527	0.355
GH & heterosis	0.487	0.539	0.301	0.561
SH & performance	0.097	0.179	0.702	0.479
SH & heterosis	0.349	0.714	0.250	0.773
Indica mixture ($r_{0.05}$ = 0.325, $r_{0.05}$ = 0.418)				
GH & performance	-0.135	0.468	0.365	0.425
GH & heterosis	0.165	0.236	0.140	0.338
SH & performance	0.265	0.570	0.610	0.443
SH & heterosis	0.299	0.323	0.541	0.445
Japonica mixture ($r_{0.05}$ = 0.273, $r_{0.01}$ = 0.354)				
GH & performance	0.118	0.422	-0.083	0.484
GH & heterosis	0.167	0.117	-0.037	0.165
SH & performance	0.515	0.683	0.260	0.585
SH & heterosis	-0.029	0.125	-0.050	0.093

Table 4. Correlations of F_1 heterozygosity with hybrid performance and heterosis of the three traits in the diallel set of U.S. southern long-grained rice. Critical values for significant correlations are 0.374 and 0.478 at the 0.05 and 0.01 probability levels, respectively.

Trait	Hybrid performance	Heterosis
Head rice percentage	-0.610	0.198
Total head rice	0.856	0.577
Total rough rice	0.873	0.462

In the indica mixture set, correlations of general heterozygosity with both performance and heterosis were low or intermediate (Table 3). Unlike in the hybrid parent set, correlations of general heterozygosity with hybrid performance appeared to be higher than those with heterosis. There were some increments in all the correlation coefficients when specific heterozygosity was used in the calculation.

In the japonica mixture set, little correlation was detected between heterosis and heterozygosity (either general or specific). Intermediate correlations were observed between hybrid performance and heterozygosity (both general and specific), and the correlations calculated using specific heterozygosity were higher than those using general heterozygosity for all the traits (Table 3).

In the long-grained rice set, very large positive correlations were detected between general heterozygosity and hybrid performance for total rough rice and total head

rice; an intermediate negative correlation was detected between general heterozygosity and head rice percentage (Table 4). Correlations of general heterozygosity with heterosis were much lower than those with hybrid performance. Specific heterozygosity was not used in the calculation because a majority of the markers appeared to be positive on total rough rice and total head rice due to the very different marker genotypes of Jasmine 85 coupled with the higher performance of the F_1 hybrids derived from this line.

Discussion

The genetic materials used in this study encompassed a broad spectrum of the cultivated rice gene pool. The molecular marker-based analysis of the four diallel sets totaling 147 crosses revealed some important features of molecular diversity and heterosis in rice.

The analysis clearly showed extensive genetic diversity within each set of rice lines at the DNA sequence level. It is remarkable that the set of eight hybrid parents contains an amount of genetic polymorphisms approximately equal to the mixture of indica varieties, which was carefully selected to represent a wide range of germplasm. The southern U.S. long-grained cultivars, which have been considered to have a rather narrow genetic base (Dilday 1990), harbored about the same level of genetic polymorphisms as the mixture of japonica varieties that was likewise carefully selected to represent a very wide range of germplasm.

It is clear from the data that a considerable proportion of the crosses expressed large amounts of heterosis, indicating that heterosis is commonly high in rice. Both the highest level and widest range of heterosis occurred in japonica set, which stands in contrast to the lowest level of molecular polymorphisms among the parents. This suggests the complexity of the genetic basis of hybrid performance and heterosis.

The most important objective of this study was to investigate the relationship of molecular marker polymorphisms with hybrid performance and heterosis. Our analysis showed that the correlations varied greatly from one data set to another depending on the materials studied. The highest correlation was observed between hybrid yield and general heterozygosity in the southern U.S. long-grained varieties. A high level of correlation was also observed between specific heterozygosity and heterosis in the Chinese hybrid set whereas the correlations were much lower in the two sets of variety mixtures. These results seem to suggest that correlations between heterozygosity and hybrid performance are higher in improved germplasm than in primitive germplasm.

In summary, the relationships between molecular marker heterozygosity and hybrid performance are complex because of the diversity of the rice germplasm (Oka 1988) as well as the complexity of the genetic basis of hybrid performance and heterosis. For molecular markers to have practical utility in predicting hybrid performance, there is a great need for detailed characterization of the rice germplasm and for in-depth understanding of the genetic basis of heterosis. Such knowledge will certainly enhance the understanding of the relationship between marker heterozygosity

and hybrid performance, and provide strategies for utilizing molecular markers in hybrid rice breeding programs.

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Notes

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Dominance as the major genetic basis of heterosis in rice

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A set of 194 F_7 lines derived from a subspecific rice cross showing strong F_1 heterosis was backcrossed to the two parents to produce BC_1F_7 families. The 388 BC_1F_7 families and the 194 F_8 lines along with the two parents and their F_1 were evaluated for 12 quantitative traits. Thirty-seven significant quantitative trait loci (QTLs), with a log of odds $\geq$ 2.0, were detected in the BC_1F_7 populations through 141 restriction fragment length polymorphism markers covering approximately 95% of the rice genome. Twenty-seven (73%) QTLs were detected in only one of the two backcross populations. In 82% of these cases, the heterozygotes were superior to the respective homozygotes. The remaining 10 (27%) QTLs were detected in both backcross populations. In all of these cases, the heterozygote had a phenotype falling between those of the two respective homozygotes and in no instances were the heterozygotes found to be superior to either of the homozygotes. These results suggest that dominance complementation (including partial dominance) is the major genetic basis of heterosis in rice.

Heterosis, or hybrid vigor, is a widely documented phenomenon in diploid organisms that undergo sexual reproduction. The genetic basis of heterosis has been debated for more than 80 yr and is still not resolved. Two major hypotheses have been promulgated to explain this phenomenon: the dominance hypothesis and the overdominance hypothesis. The dominance hypothesis, proposed by Davenport (1908), Bruce (1910), and Keeble and Pellew (1910), and later elaborated by Jones (1917), supposes that heterosis is due to the canceling of deleterious recessives contributed by one parent by dominant alleles contributed by the other parent in the heterozygous F_1. The overdominance hypothesis, proposed by East (1908), assumes that the heterozygous combination of the alleles at a single locus is superior to either of the homozygous combinations of the alleles at that locus. In the past, geneticists have found it difficult to experimentally solve the dominance versus overdominance controversy.

The recent advent of molecular linkage maps has made it possible to detect and individually analyze the loci that underlie heterosis. Using molecular markers, Stuber et al (1992) were able to detect quantitative trait loci (QTLs) that contribute to hybrid vigor in maize. Their results showed that the heterozygotes of most QTLs detected for grain yield had higher phenotypic values than those of either respective homozygotes, suggesting that overdominance is the principal factor controlling heterosis in this open-pollinated crop species.

Mainly due to China's great success in hybrid rice, IRRI resumed its research on hybrid rice in 1979. Scientists in India, Indonesia, the Philippines, South Korea, Japan, Malaysia, Thailand, USA, Brazil, Mexico, and Vietnam have launched hybrid rice breeding programs; India released its first commercial hybrids in 1993.

Currently, the highest yielding hybrids in rice involve crosses between the indica and japonica subspecies. The goal of the study was to use the molecular map of rice to determine the genetic basis of heterosis in one of the highest yielding indica/japonica hybrids.

Materials and methods

Development of experimental populations

Elite homozygous lines, indica parent 9024 (I) and japonica parent LH422 (J), which has wide compatibility gene(s), were crossed, using I as the female. The F_1 hybrid yields, on average, 40% more than either parent (Lo and Yuan 1987). From the F_1, 194 F_7 lines were developed through six consecutive selfing generations with each F_7 line tracing to a different F_2 plant. No conscious selection was made in any generation. A single plant from each of 194 F_7 lines was randomly chosen and: 1) backcrossed to each of the two parental lines to generate two BC_1F_7, families, 2) selfed to produce F_8 progeny.

Phenotypic evaluation

The 388 BC_1F_7 backcross families, 194 F_8 inbred lines, along with the two parental lines and their F_1, were laid out in a field in a randomized complete block design with two replications (plots) for phenotypic evaluation in the summer season of 1992 at the Hunan Hybrid Rice Research Center, China. Twenty-seven plants (three lines × nine plants line^{-1}) were planted at a density of 300,000 plants ha^{-1} in each of 1,170 plots. The middle five plants in the central line of each plot were used for data collection. The 12 quantitative traits investigated were plant height (cm), panicle length (cm), days to heading, days to maturity, panicles plant^{-1}, spikelets panicle^{-1}, grains panicle^{-1}, 1000-grain weight (g), spikelets plant^{-1}, grains plant^{-1}, percent seed set, and grain yield (t ha^{-1}). Means over replications, for each trait, for each of two backcross populations, were used for QTL and other analyses.

RFLP linkage map construction

Seedlings of 30-40 selfed seeds (F_8) from a single plant of each F_7 line were used for bulk DNA extraction. A subset of 141 polymorphic restriction fragment length

polymorphism (RFLP) markers were selected from the rice high-density molecular map (Causse et al 1994) to construct the linkage map of the recombinant inbred (RI) population. Because few heterozygotes (theoretically 1.5625%, in reality 3.23%, averaged over 141 markers) for each marker were possible, the genotype heterozygous at a particular marker was treated as missing data. Recombination fractions between pairs of linked markers were calculated using both MAP MANAGER version 2.5 (Manly 1993) and MAPMAKER (Lincoln et al 1992a) computer programs. Both estimates were in good agreement. The RI-derived RFLP map reported here was constructed using MAPMAKER/EXP. version 3.0 (Lincoln et al 1992a). All RFLP markers were allocated to linkage groups by pairwise analyses with a threshold of a log of odds (LOD) score of 4.0. The framework of the map was established by analyses of highly informative and well-spaced markers. The order of RFLP markers on each linkage group (frame) had an at least 1000-fold higher likelihood (LOD score 3.0) compared with any alternative order. The remaining markers were assigned to their corresponding linkage groups with an LOD score ≥ 2.0. The final order of markers on each linkage group was reconfirmed using 'ripple' command with an LOD score $\geq$ 2.0.

QTL analyses

For simplicity and other purposes, such as phenotypic comparison between heterozygous and homozygous genotypes, the allele at the nth locus from I is designated as *In, Jn* for the allele from J. The analyses of QTLs linked to markers for each trait in each of the two BC_1F_7 populations were performed using both single-point analysis (Tanksley et al 1982) and interval mapping (Lander and Botstein 1989). Single-point analysis for detecting the association of a marker with a QTL lying at or close to the marker in this study was tested using one-way ANOVA from Data Desk 4.0 with each marker considered as a treatment with two levels and the phenotype of each trait as the dependent variable. This analysis involved comparing, for each trait and each RFLP marker, the phenotypic means of heterozygous and homozygous classes (*InJn* vs *Inln* or *JnJn*) of BC_1F_7 families, for the two BC_1F_7 populations. The difference between the phenotypic means of heterozygous and homozygous marker classes was used as an estimate of the phenotypic effect of different marker genotypes. The proportion of the total phenotypic variation explained by each marker associated with a QTL was calculated as an sR^2 value (sR^2 = ratio of the sum of squares explained by the marker locus to the total sum of squares). Interval mapping developed by Lander and Botstein (1989), which is able to define the most likely position of a QTL and precisely estimate the phenotypic effect of the QTL if it does not lie exactly at the marker locus, was also employed for QTL analysis for each trait. An LOD score threshold of 2.4 would be needed to test at the $P = 0.001$ level of significance per test, or $P = 0.05$ for the entire rice genome (Lander and Botstein 1989). To reduce type II errors, an LOD score of 2.0 was chosen as the threshold for the analysis presented in this paper for declaring a QTL present or not in the MAPMAKER/QTL 1.1 program (Lincoln et al 1992b). LOD peaks for each significant QTL were used to position the QTL on the RI/RFLP map. The proportion of the total phenotypic variation explained

by each QTL was calculated as an iR^2 value (iR^2 = ratio of the sum of squares explained by the QTL to the total sum of squares). In cases where more than one peak was found on a chromosome for the same trait, multiple-QTL models were employed to determine whether the chromosome possessed single or multiple QTLs.

Epistasis analysis

Two-way interactions were performed between significant markers linked to QTLs and all other marker loci by the PROC GLM in SAS (SAS Institute 1988). For example, p_1, p_2, p_3, and p_4 were designated for the phenotypic effects of BC_1F_7 families with genotypes *II;II, II;IJ, IJ;II* and *IJ;IJ,* respectively, in the population backcrossed to I (hereafter referred as to BC/I), and *JJ;JJ, JJ;IJ, IJ;JJ.* and *IJ;IJ,* respectively, in the population backcrossed to J (hereafter referred to as BC/J). The null hypothesis (no epistasis) for this test is: $(p_1 + p_4) - (p_2 + p_3) = 0$, with a degree of freedom of 1 [(2-1) × (2-1) = 1].

Relationship between genome heterozygosity and trait expression

Hypergene (Young and Tanksley 1989) was used for calculating genome ratios (percentage of total genome originated from one parental genome) for each line in the RI population. The rules for genome calculation are as follows: if two consecutive markers delimiting the chromosome region in the line were from the same parent, the interval between them was considered to have the parental genome. If an interval was bounded by consecutive markers with alleles from the two parents, respectively, then one half of the interval was considered to be from one parent and one half from the other parent. The genome heterozygosity of a BC_1F_7 family in the BC/I is equal to the percentage of the J genome in the F_7 line, which was used to be backcrossed to generate the BC_1F_7 family. The genome heterozygosity of a BC_1F_7 family in the BC/J is equal to the percentage of the I genome in the F_7 line, which was backcrossed to generate the BC_1F_7 family. The relationships between genome heterozygosity and expression of traits were tested by regressing trait values on the genome heterozygosity in the 194 BC_1F_7 families for each of the two BC_1F_7 populations.

Results

F_1 heterosis

For heterobeltiosis (heterosis over the better parent), grain yield showed the strongest significant heterosis (20.6%), followed by 1000-grain weight (10.l%), plant height (9.9%), panicle length (5.0%), and days to maturity (3.2%). Number of grains plant^{-1}, percent seed set, and spikelets plant^{-1} also exhibited positive heterosis, but not significantly. No heterosis was observed for days to heading; number of spikelets panicle^{-1} (-16.3%) and number of grains panicle^{-1} (-12.1 %) showed significant negative heterosis. Panicles plant^{-1}, days to heading, and grains plant^{-1}, for which no heterobeltiosis was observed, showed significantly positive heterosis over the midparent. The grain yield is the function of three yield components: number of plants unit area^{-1}, number of grains plant $^{-1}$ and grain weight. In the field trial, the number of

plants unit area^{-1} was held constant, i.e., 300,000 plants ha^{-1}. For the other two components, increased grain weight (measured in 1000-grain weight) accounted for 73% of the increased grain yield in this heterotic F_1 hybrid (1.61 t ha^{-1} over the midparent or 1.35 t ha^{-1} over the better parent), which benefited from heterosis; the remaining 27% was due to the increased number of grains plant^{-1}.

It should be noted that the spikelet fertility of the parents (70-71%) and F_1 (74%) observed in this study is somewhat lower than normal (80-85%). This could be due to the high temperature in August in Changsha, Hunan, at heading and fertilization stages.

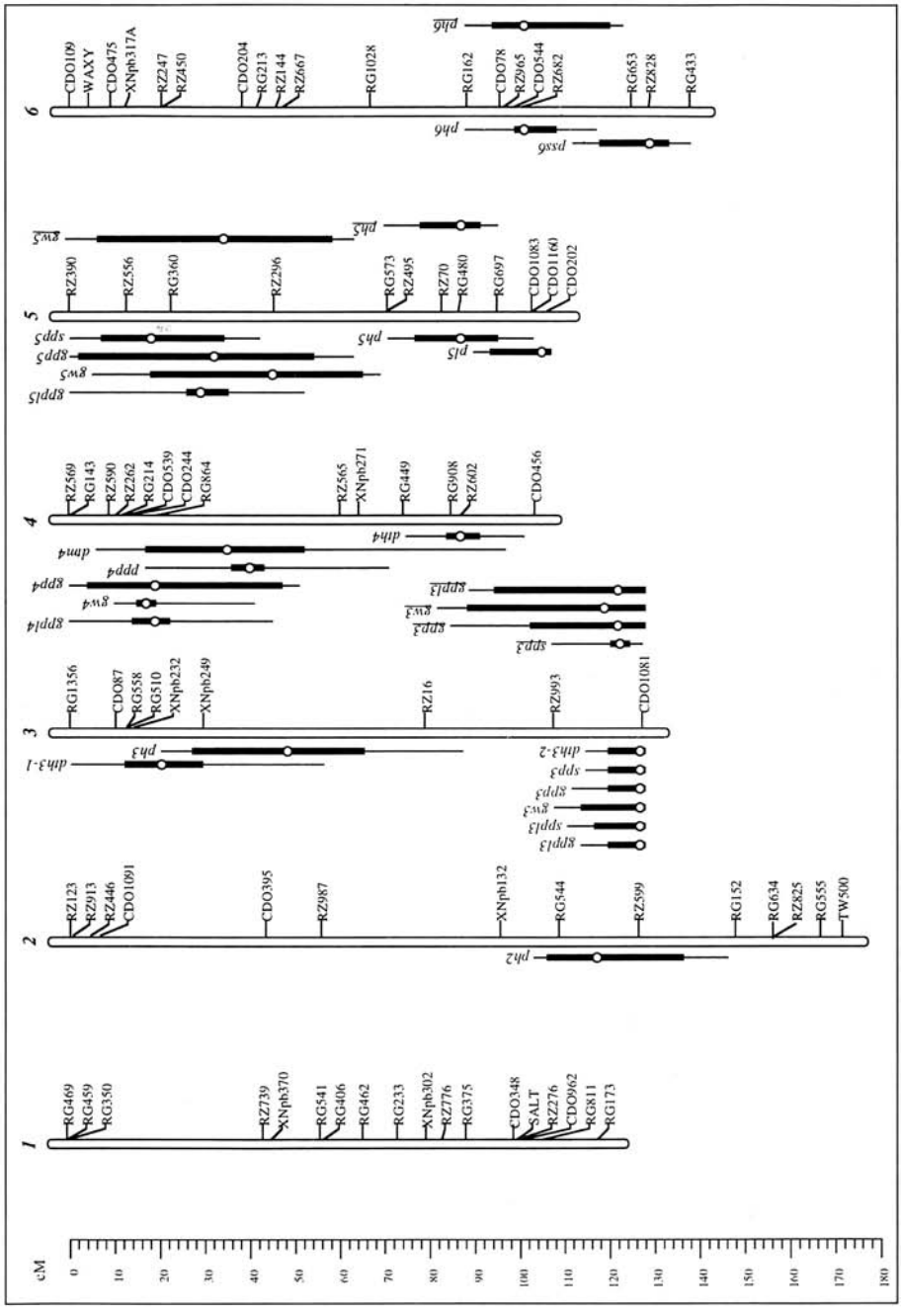
Genetic map

The genetic map shown in Figure 1 was based on 141 RFLP markers segregating in the RI population with 194 lines that served as the base population for generating the two backcross populations employed in this study. Those 141 markers are estimated to cover approximately 95% of the rice genome in comparison with the high-density molecular map of rice (Causse et al 1994). All RFLP markers had been previously placed on either the *Oryza sativa/O. longistaminata* (SL) map based on an interspecific backcross population generated at Cornell University, USA (Causse et al 1994), or the map derived from an intersubspecific (indica/japonica) F_2 population developed at National Institute of Agrobiological Resources, Japan (Saito et al 1991). The marker order on this RI map was in good agreement with that of the two maps mentioned above. A few exceptions were observed on this RI map as follows: 1) the single-copy clone, RZ262, which was mapped to a position close to the lower end of chromosome 4 on the SL map was assigned to the upper end of chromosome 4 on the RI map, 2) linked markers RG213 and RZ667 on chromosome 6, and RZ562 and RG333 on chromosome 8 on the SL map were reversed in order based on our mapping analysis, and 3) RZ825, CDO204, CDO109, RG634, and RG98, which were assigned to chromosomes 1, 2, 3, 7, and 11, respectively, on the SL map, were placed on chromosomes 2, 6, 6, 2, and 12, respectively, on the RI map. We checked the mapping films and found those five clones had two or more copies on both mapping populations. Presumably, different copies of the same clone were mapped on the two mapping populations.

Mapping QTLs' underlying traits

Each trait was subjected to QTL detection based on single-point analysis (one-way ANOVA) and interval mapping for each of the two BC_1F_7 populations. Single-point analysis and interval analysis gave basically the same result in detecting QTLs for each trait, but single-point analysis usually underestimated the phenotypic effect of a QTL, which did not lie exactly at the marker locus. This can be seen for the QTL for plant height on chromosome 3 and the QTLs for days to maturity and panicles plant^{-1} on chromosome 4 in the BC/I. Because the interval mapping more precisely estimates the phenotypic effects of the QTLs, all further analyses were based on the results from the interval mapping.

Plant height. Five QTLs were detected in the BC/I. For three of these QTLs (chromosomes 2, 3, and 8), the heterozygotes increased plant height compared with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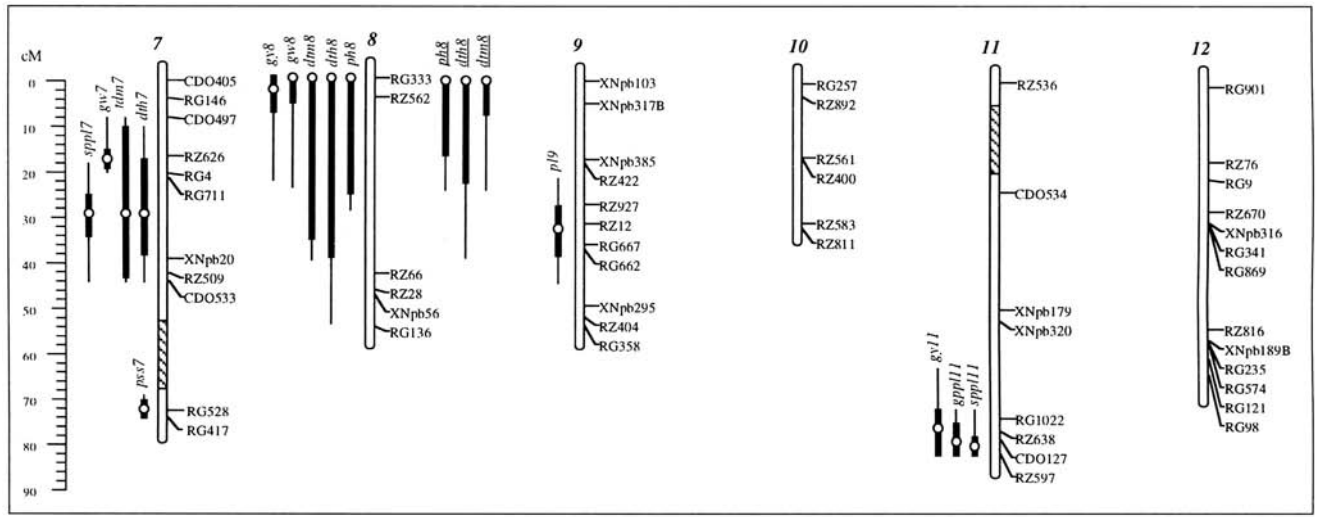

Fig. 1. Genetic linkage map of rice based on 194 RI lines derived from an indica (9024)/japonica (LH422) cross. Distances are given in Kosambi centiMorgans. RG = rice (IR36) genomic, RZ = rice cDNA, CDO = oat cDNA, XNpb = rice (Nipponbare) genomic; TW500 = rice cDNA; *WAXY* and *SALT* are known genes. Striped bars on chromosomes 7 and 11 indicate markers are linked with LOD scores < 2.0. Darkened bars show LOD scores ≥ 2.0 with the extensions representing LOD scores > 1.0 and < 2.0. The names of the QTLs are given above the respective extensions and are based on the origins of the chromosomes, for example, the QTL for plant height, bordered by RG544 and RZ599 on chromosome 2, is named *ph2.* The small circles (o) indicate the map positions of the peak LOD scores, which are the most likely positions for the putative QTLs. QTLs underlined are detected in both populations.

the respective homozygotes. The QTL bordered by markers XNpb249 and RZ16 on chromosome 3 accounted for 26% of the total phenotypic variation. For the other two QTLs (chromosomes 5 and 6), the heterozygotes caused a decrease in plant height.

Three QTLs in approximately the same map position (chromosomes 5, 6, and 8) were also detected in the BC/J. For the QTLs on chromosomes 5 and 6, the heterozygotes were superior to the homozygotes—a result compatible with additive gene action. The remaining QTL on chromosome 8 resulted in the heterozygote with reduced height, also suggesting additive gene action.

Days to heading. Three QTLs were revealed in the BC/I. For the two QTLs on chromosomes 3 and 4, the heterozygotes reduced days to heading. The heterozygote for the QTL on chromosome 8, which contributed to 36.6% of the total phenotypic variation, increased days to heading. In the BC/J, three QTLs were detected on chromosomes 3, 7, and 8. The two QTLs on chromosomes 3 and 7 increased days to heading in the heterozygotes; the QTL on chromosome 8 was found at the same map position as the QTL in the BC/I. However, in this case, the heterozygote decreased days to heading suggestive of additive gene action.

Days to maturity. Two QTLs were found in the BC/I. The heterozygote for the QTL on chromosome 4 shortened growth duration, while for the QTL on chromosome 8, to which 41.6% of the total phenotypic variation was attributable, the heterozygote lengthened growth duration. In the BC/J, two QTLs were identified. The heterozygote of the QTL on chromosome 7 increased growth duration, but the QTL, which was detected and mapped to the same chromosomal location on chromosome 8 in the BC/I, shortened growth duration.

Panicle length. Only two QTLs, on chromosomes 4 and 8, were detected in the BC/I and the BC/J, respectively; for both QTLs, the heterozygotes demonstrated increased panicle length compared with homozygotes.

Panicles plant^{-1}. Only one QTL (chromosome 4) was detected in the BC/I, and the heterozygote had fewer panicles plant^{-1}. No QTL was found over the threshold set for declaration in the BC/J.

Spikelets panicle^{-1}. One QTL was found on chromosome 3 in the BC/I for which the heterozygote had increased spikelets panicle^{-1}. Two QTLs were revealed in the BC/J. For the QTL on chromosome 3, which was found in the same map position as in BC/I, the heterozygote decreased spikelets panicle^{-1}. For the QTL on chromosome 5, the heterozygote had increased spikelets panicle^{-1}.

Grains panicle^{-1}. Two QTLs were detected on chromosomes 3 and 4 in the BC/I, and their heterozygotes had increased grains panicle^{-1}. Two QTLs were found in the BC/J. For the QTL at the same map position as in BC/I on chromosome 3, the heterozygote of the QTLs decreased grains panicle^{-1} in comparison with the homozygote. The heterozygote of the QTL on chromosome 5 enhanced grains panicle^{-1}.

Percent seed set. Two QTLs were mapped on chromosome 6 in the BC/I and on chromosome 7 in the BC/J, respectively. For the QTLs, the heterozygote of the QTL raised seed set rate compared with the homozygotes.

1000-grain weight. Three QTLs were detected in the BC/I. For the QTL on chromosome 3, the heterozygote lowered grain weight, while the heterozygotes of

QTLs on chromosomes 5 and 8 increased grain weight. Four QTLs were identified in the BC/J. For the QTLs on chromosomes 3, 4, and 7, the heterozygotes enhanced grain weight compared with their corresponding homozygotes. The heterozygote of the QTL on chromosome 5 decreased grain weight. The QTLs on chromosomes 3 and 5 were detected in the two backcross populations and had the same map positions.

Spikelets plant^{-1}. Three QTLs were identified in the BC/I on chromosomes 3, 5, and 11. In all cases, the heterozygotes of the QTLs had an increased number of spikelets plant^{-1}. No significant QTL for this trait was found in the BC/J.

Grains plant^{-1}. Three QTLs on chromosomes 3, 4, and 11 were detected in the BC/I, and all the heterozygotes increased grains plant^{-1}. Two QTLs were found for the BC/J. For the QTL on chromosome 3 (found at the same map position as in the BC/I), the heterozygote reduced grains plant^{-1}, when compared with the homozygote. The heterozygote of the QTL on chromosome 5 enhanced grains plant^{-1}.

Grain yield. Two significant QTLs were found—one on chromosome 11 in the BC/I and one on chromosome 8 in the BC/J, respectively. For the QTL on chromosome 8, the heterozygote decreased grain yield in comparison with the homozygote. The heterozygote of the QTL on chromosome 11 increased grain yield.

The fact that only two significant QTLs were detected for grain yield may be due to severe spikelet sterility (as measured in percent seed set), which was observed in both backcross populations. Percent seed set ranged from 47 to 77% in the BC/I, and from 42 to 83% in the BC/J. Greater than 95% of both BC/I and BC/J families showed lower percent seed set than the F_1. There was no correlation (r = 0.062 in the BC/I, and 0.090 in the BC/J) between grain yield and 1000-grain weight, which made more than 73% contribution to the F_1 heterosis of grain yield.

Epistasis

Markers associated with each of the 47 significant QTLs were tested for possible two-way interactions with all other markers in the genome. A total of 6,580 two-way tests were performed. Only 5.40, 1.18, and 0.17% of pairwise tests were significant at $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively, which were close to the frequencies that would be expected by chance. The markers with significant interactions were rarely found coinciding with other markers associated with other QTLs for the same trait.

Relationship between traits and genome heterozygosity

The effect of genome heterozygosity on the performance of each trait for the two backcross populations was evaluated by regressing the trait value of each BC_1F_7 family on its percentage of genome heterozygosity. The r value (correlation coefficient) obtained from such regression analysis should reflect the importance of heterozygosity per se to the expression of a particular trait. Only for plant height and panicle length were there significant positive relationships with genome heterozygosity in the BC/I. All other traits for both populations and plant height and panicle length for the BC/J showed no relationship between genome heterozygosity and trait performance. indicating that the overall genome heterozygosity alone had little effect on trait expression in both BC_1F_7 populations.

Trait expression in the RI lines

Figure 2 shows the distribution of phenotypic means over replications for each trait in the RI (F_8) population. For each of the traits evaluated, there were some recombinant lines having a higher phenotypic value than the F_1 hybrid (see Fig. 2). For grain yield, which showed the strongest significant heterosis in the F_1, there were two RI lines having grain yields (8.17 t ha^{-1} and 8.20 t ha^{-1}) that are significant (P £ 0.05), higher

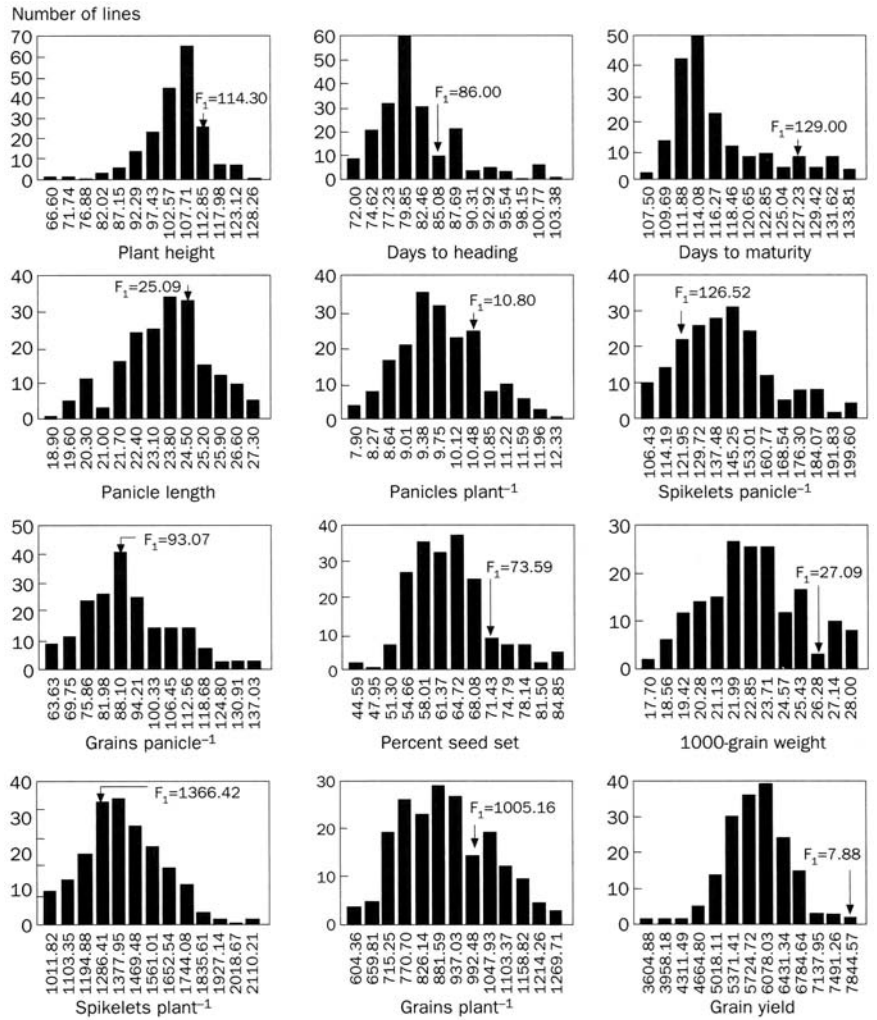

Fig. 2. Frequency distribution of phenotypes for each trait for the 194 RI lines (F_8) derived from the 9024/LH422 cross. Phenotypes for F_1 are shown by arrows. The values indicated in the x-axis are the lower limit of each group.

than the F_1s (7.88 t ha^{-1}). This is despite the fact that 91.10% of the recombinant lines experienced spikelet fertility problems (which is a common phenomenon observed in progeny of indica/japonica cross and caused by incompatibility between indica and japonica), i.e., seed set rates were lower than the F_1s. The occurrence of some recombinant lines having a higher phenotypic value than the F_1 hybrid was due to genetic reasons rather than environmental noise, as our experiment was designed to control environmental noise and experimental errors, and data used for the analyses were means obtained from a replicated trial rather than from single plants in the BC_1 or F_2 populations. This was clearly demonstrated by the fact that the proportions of phenotypic variance explained by genetic to the total phenotypic variance ranged from 81.13% for panicles $plant^{-1}$ to 99.88% for days to heading estimated by two-way ANOVA analyses from the PROC GLM in SAS (SAS Institute 1988) and that there was no significant difference ($P \leq 0.05$) between the two replications for all traits studied.

Discussion

Genetic basis of heterosis

The QTL mapping results revealed the following. Most of the significant QTLs (27/37 = 73%) were detected in only one of the two backcross populations. In 82% of these cases, the heterozygotes had higher phenotypic values than their respective homozygotes. Ten QTLs were detected with significance in both backcross populations and each pair was mapped to the same chromosomal location, and in each of all cases the phenotype of the heterozygote fell between those of the two homozygotes. This result suggests that the complementation of dominant (including partial dominant) alleles at different loci in the F_1 hybrid is the major contributor to F_1 heterosis.

This conclusion is supported by the general lack of significant correlations between genome heterozygosity and the phenotypic traits. Finally, one of the predictions of the dominance hypothesis is that true inbreeding individuals, like F_1 in vigor, can be obtained from its segregating populations. The prediction was met in this experiment for each of all traits including grain yield, since at least two RI lines were observed whose phenotypes exceeded that of the F_1. This result may be attributed to the segregation and recombination of genes at different loci in the seven meiosis experienced during population development in this study. These lines of evidence reinforce the conclusion that the genetic basis of heterosis in the F_1 hybrid examined is largely due to dominance.

The degree of correlation between genome heterozygosity and phenotypic traits reflects the importance of overall genome heterozygosity to the trait expression. For most of traits, such correlation is very low and not significant. This is consistent with the QTL mapping results. For most of the traits, not all QTLs detected for the trait had higher phenotypes in heterozygotes than in respective homozygotes, i.e., for some of the QTLs, heterozygotes showed higher phenotypes than the respective homozygotes; for the other QTLs, the heterozygotes exhibited lower phenotypes than the respective homozygotes. Therefore, overall genome heterozygosity would show no correlation with the trait.

Digenic interactions between markers associated with significant QTLs and all other markers were not found significant in this study. This suggests that strong epistasis is not likely to be involved in this study. However, as discussed by Tanksley (1993), marker-based QTL studies are inherently inefficient at detecting epistasis and one cannot exclude the possibility that some level of epistasis is occurring.

Genotype-by-environment interaction is interesting to geneticists and breeders. Since molecular markers were introduced in quantitative genetics, a number of QTL studies have been carried out to detect possible QTL by environment interaction. While QTL-by-environment interaction has been detected in some instances, it is usually of the type where QTLs found in one of the environments differ in the magnitude of their effects in different environments. To our knowledge, there are no instances where the gene action of a QTL has changed—e.g., change from dominance to recessiveness, partial dominance to overdominance, from one environment to another. Although the conclusion that heterosis in rice is largely due to dominance is drawn from studying QTLs in one environment, we believe that this conclusion is likely to extend to other environments.

Comparison with maize

The conclusion that heterosis in rice is largely due to dominance contrasts with QTL studies in maize, suggesting overdominance is implicated as the prominent factor conditioning heterosis (Stuber et al 1992). One possible explanation for this difference is that maize actually possesses a large number of genes for which alleles interact in a truly overdominant manner whereas rice does not. Rice and maize are both members of the Gramineae, evolved from a common ancestor and share many orthologous genes (Ahn and Tanksley 1993). For maize alone to harbor alleles that are truly overdominant would be remarkable. An alternative explanation is that maize does not contain a higher frequency of overdominant alleles and that the observed overdominant gene action detected in QTL studies is due to pseudo-overdominance or the occurrence of dominant and recessive alleles in coupling at closely linked loci (Crow 1952).

In this regard, it is important to note that the QTLs discovered from mapping studies are defined with only limited resolution. From a primary mapping study, it is normally not possible to localize a QTL to a region less than 10 cM. This leaves open the possibility that an overdominant QTL may actually be a deleterious recessive allele at one locus in cis with a beneficial dominant allele at a closely linked locus. This would be detected in a mapping study as a QTL with overdominant gene action. This phenomenon was termed pseudo-overdominance and has been acknowledged as a possible explanation for some of the overdominant gene action observed in maize (Stuber et al 1992). One might predict that pseudo-overdominance would be more likely to occur in plants in which deleterious recessive alleles are more abundant. Breeding and genetic studies would suggest that deleterious recessives are more frequent in maize and other outcrossing species than in self-pollinated species such as rice.

The difference in the reproductive biology of maize and rice could account for the greater accumulation of deleterious recessives in maize than in rice. In maize and

other outcrossing species, recessive alleles are usually masked by their corresponding dominant counterparts. In rice and other self-pollinated species, populations and individuals are more highly inbred, a condition in which deleterious recessive mutations are more likely to be eliminated by natural and artificial selections.

To definitively distinguish overdominance from pseudo-overdominance will require fine mapping of QTLs displaying overdominant gene action. In the case of pseudo-overdominance, it should be possible to break the tight linkage of the dominant and recessive alleles, which would result in loss of the observed overdominance behavior. In the case of true single-gene overdominance, fine mapping will more precisely define the position of the locus, but the overdominant gene action will persist. In the past, fine mapping of overdominant loci was impractical. Now, with the availability of high-density molecular linkage maps, fine mapping is a feasible proposition and the hypothesis that overdominant QTLs in maize are a result of tight linkage of dominant and recessive alleles can be tested empirically (Paterson et al 1990, Jansen and Stam 1994).

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Notes

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Genetic and molecular analysis of the anthocyanin pigmentation pathway in rice

A.R. Reddy

The anthocyanin pigmentation in rice is an example of many dispersed genes controlling a single trait, the purple/red color. We are interested in unraveling the genetic and molecular basis of expression and regulation of genes of the anthocyanin pathway for their eventual manipulation toward resistance of rice plants to various stresses, particularly diseases. Genetic analysis of phenotypic variation in tissue-specific accumulation of anthocyanins among different indica rice lines was performed. A dominant inhibitor gene, named *Ilb,* which inhibits pigmentation in the leaf blade, was uncovered. The major anthocyanidin pigment in rice was identified as cyanidin and the minor one as peonidin. The major proanthocyanidin in rice was tentatively identified as procyanidin. Molecular cloning of rice genes concerned with flavonoid and anthocyanin production has been achieved by using the corresponding cDNA sequences of maize as probes. A cDNA library, made from the leaf poly A + RNA preparation of young Purpleputtu (PP) seedlings, was screened with maize cDNA sequence probes. The gene-specific clones were isolated, restriction-mapped, and sequenced. The salient features of a rice cDNA clone named OsCHS (chalcone synthase), which hybridizes to the maize c2 cDNA, were described. Sequence comparison and Western analysis revealed an extensive homology between the rice and maize CHS cDNA and protein. The *CHS* locus of rice has been mapped to chromosome 11 using the restriction fragment length polymorphism approach. We have demonstrated that the anthocyanin biosynthesis in rice, particularly PP, is ultraviolet (UV) light-responsive. The abundance of the anthocyanin gene-specific transcripts in mRNA preps from UV-induced seedlings has been demonstrated. Molecular cloning and characterization of genes of the anthocyanin pathway, coupled with the availability of reliable and relatively easier genetic transformation methods in indica rice, offer unique advantages in genetic engineering of the pathway to unambiguously prove the role of flavonoids in resistance to disease and abiotic stresses as well.

Anthocyanin pigments, with their vivid display of colors ranging from bright red/ purple to blue, belong to the general class of plant pigments called flavonoids. Classified as secondary metabolites, they are ubiquitous among flowering plants and are synthesized by a multistep biosynthetic pathway, generally referred to as the anthocyanin pathway. Many functions are attributed to flavonoids: insect attractants for pollination, protection from ultraviolet (UV) light, defense against pathogens and insect attack, signal molecules in the nodulation process, male sterility and gametogenesis, activation of the *Ti* plasmid *Vir* region of *Agrobacterium tumefaciens,* modulation of hormone response, response to wounds. and resistance to a variety of biotic and abiotic stress conditions. Flavonoids, therefore, seem to emerge as the survival compounds of flowering plants.

In a few plant species, the anthocyanin pathway has been exploited to discover and elucidate a variety of cytogenetic, genetic, and molecular biological phenomena such as the breakage-fusion bridge cycle, the correlation between genetic and cytological crossing over, gene interaction, linkage and mapping studies, phenotypic marker lines, transposable elements, gene isolation, and regulation and evolutionary relationships. Recently, anthocyanin genes have been used extensively in transformation experiments in basic and applied studies (Meyer et al 1987, Vander Krol et al 1990, Lloyd et al 1992, Holton and Tanaka 1994, Marrs et al 1995). Anthocyanin pigmentation could also be used as a marker in nondestructive cell-specific assays both in transgenic and transient assay systems.

Anthocyanin pigmentation in rice plants has been extensively reported by earlier breeders and geneticists (e.g., Ramaiah and Rao 1953, Nagao et al 1962, Kadam 1974, Takahashi 1982, Maekawa and Kita 1987, Kinoshita and Takahashi 1991). Genetic analyses were mainly concentrated on phenotypic descriptions, identification of specific loci, and chromosomal mapping. Nonallelic gene interactions leading to modifications of the F_2 phenotypic ratios have been described. These studies revealed that the anthocyanin gene pigment system consists of mainly three basic genes: the *C* (chromogen), the *A* (activator), and the *P* (distributor). The tissue-specific distribution and accumulation of anthocyanin pigments are determined by additional loci. This CAP control system of the pathway is an overview and does not adequately explain critical aspects of pigment synthesis, distribution, and regulation. A collation of known genes of rice, their phenotypic effects, and interaction patterns is presented briefly in Table 1. However, nothing is known about specific gene products and enzymes controlling individual biosynthetic step reactions and the associated regulatory circuits of the pathway. In the absence of such information, any postulation about the gene action and regulation of the pathway is at best speculative.

A working hypothesis of the genetic basis of anthocyanin biosynthesis in rice plant is as follows. The known genes can be grouped tentatively into structural and regulatory genes. Among the structural genes, the *C, A, Rc,* and *Rd* genes may encode enzymes of the pathway whereas the regulatory genes, *P* and *Pl,* with their diverse alleles, determine temporal and spatial regulation of pigmentation. In addition, nonallelic dominant inhibitor loci were reported. which either down-regulate or eliminate the pigment synthesis in different tissues. The pattern of anthocyanin

Table 1. The anthocyanin gene-pigment system and their phenotypic effects on rice.

Gene	Phenotypic effect
Structural	
C (chromogen)	Responsible for anthocyanin production: with an allelic series of C^B, C^{Br}, C^+ (null), etc.
A (activator)	Activation of C gene; essential for anthocyanin production: with an allelic series of A^S, A^E, *A*, A^+ (null), etc.
Rc (brown pericarp)	Synthesis of brown pigments in pericarp
Rd (brown pericarp)	Synthesis of brown pigments in pericarp
Regulatory	
P (purple)	Distributor of anthocyanin pigments in the apiculus: alleles *P*, P^K, P^+ (null), etc.
Pl (purple leaf)	Localizer of anthocyanin in leaf: alleles Pl^W (leaf blade, leaf sheath, auricles, ligule, and pericarp); *Pl* (leaf blade, leaf sheath, collar, auricles, ligule, node, and internode); Pl^i (leaf blade, leaf sheath, ligule, and internode). Pl^+ (null allele resulting into colorless phenotype of tissues).
Pn (purple node)	Localizer of anthocyanins in the node
Prp (purple pericarp)	Localizer of anthocyanins in the pericarp
Inhibitory	
I-Pl (inhibitor of purple leaf)	Dominant inhibitor to purple anthocyanin
I-Pl1,I-Pl2, I-Pl3	Inhibit action of both Pl^W and Pl^i alleles
I-Pl4, I-Pl5	Inhibit action of the *Prp* locus
I-Pl6	Inhibits action of Pl^i allele
Ilb (inhibitor of leaf blade)	Inhibits leaf blade pigmentation

Sources: Chang and Jordan (1963), Takahashi (1982), Kinoshita and Takahashi (1991); adapted from Reddy et al (1995).

pigmentation in the rice plant is thus determined mainly by the allelic status of individual genes and complex interactions between them. Intensity and shades of color are marginally influenced by nongenetic factors such as soil condition, mineral nutrition, temperature, and light.

Variation in tissue-specific distribution of anthocyanins among rice lines

There is significant variation, both qualitative and quantitative, among rice varieties in the display of red/purple color phenotypes in various plant organs such as leaf, stem, node, internode, auricle, ligule, leaf sheath, stigma, apiculus, and pericarp. The

distribution of red/purple color in different plant parts among indica lines showed considerable variation (Reddy et al 1995). Some lines show color in almost all aerial tissues, whereas some show no red/purple color in any tissues. A few others exhibit an intermediate phenotype with color in various parts and finally, certain lines show no anthocyanin color in aerial parts but exhibit brown color in pericarp tissue (Reddy et al 1995). The genotype of the above selected lines is predicted and then classified, based on the pigment variation among well-defined japonica lines. These lines served as a base material for isolation of mutants and subsequently, the genes of the pathway.

Dominant inhibitory mutants

Among the members of the abovementioned classes, three indica lines—Purpleputtu (PP), N22B, and N22W—are of particular interest. The PP plants display purple color in all aerial parts except the node and pollen, N22B plants totally lack purple pigments and thus display green color in vegetative parts and brown color in the pericarp, and the N22W plants display green color in vegetative parts and no color in the pericarp. Genetic analysis revealed that the PP plants carry a full complement of structural and regulatory genes required for pigmentation of vegetative tissue and pericarp, whereas the N22B and N22W plants carry a dominant inhibitor allele, named *Ilb* (inhibitor of leaf blade color), which inhibits pigmentation of the leaf blade even in the heterozygous condition (Reddy et al 1995). In rice, it is a general observation that crosses between colored and colorless lines produce colorless F_1 plants, suggesting a wide distribution of dominant inhibitor alleles among rice cultivars.

The *Ilb* allele is unique in that its inhibitory activity is strictly leaf blade-specific. In contrast, many dominant inhibitors of anthocyanin pigmentation reported earlier in rice show inhibition in more than one tissue. This is an interesting observation because rice, unlike other cereals, seems to have several dominant inhibitor loci dispersed in the genome (Table 1). Such a diverse array of inhibitors in the rice genome is presumably a result of a conscious effort of breeders to suppress completely anthocyanin production throughout the plant. Alternatively, for some reasons, the rice plant had acquired, over time, a fail-proof genetic system of regulation to suppress anthocyanin coloration in a variety of plant parts through distinct tissue-specific inhibition mechanisms. However, such a burst of inhibitors during evolution does not seem to be common among grasses. Although one such inhibitory gene, *C1-I,* which inhibits seed color in maize, is well known, no such preponderance of inhibitors among cultivars and land races of maize has been reported. Molecular cloning and analysis of *C1-I* and its allele, *C1,* revealed that the *C* locus encodes a DNA-binding protein that is associated with regulation of the anthocyanin pathway (Cone et al 1986; Paz Ares et al 1986, 1987, 1990; Franken et al 1994; Scheffler et al 1994). The mode of these inhibitory genes in rice needs to be clarified. In any event, the inhibitor alleles, as a distinct group, are a handy tool to genetically block anthocyanin production, which eliminates the purple color in the plant and seed. Clearly, the homozygous/heterozygous mutants at the inhibitor loci mimic the phenotypic effects of antisense mutants.

Biochemical characterization of pigments

Rice plants accumulate mainly two anthocyanin pigments, cyanidin and peonidin, an o-methyl derivative of cyanidin. This was confirmed by a variety of standard techniques: thin layer chromatography, proton-NMR spectroscopy, UV-VIS spectroscopy, and standard organic methods and cochromatography with authentic compounds (Reddy et al 1995). Incidentally, this combination of anthocyanin pigments appears to be exclusive to indica rice. In certain japonica plants, the presence of malvidin was reported (Takahashi 1957). In addition, rice plants seem to accumulate a unique class of pigments called proanthocyanidins, imparting brown color, particularly in the pericarp of N22B. These pigments yielded cyanidin and peonidin on hydrolysis (Reddy et al 1995).

Qualitative and quantitative analyses of pigment extracts revealed that floral-derived tissue accumulates as much as five times more pigments than vegetative tissue with about a tenfold increase in peonidin content in the apiculus, hull, and pericarp. On the other hand, vegetative tissues have cyanidin as the major pigment with very little of peonidin. Thus, the observed pigmentation pattern in a given tissue in rice reflects complex nonallelic gene interactions and the role of tissue-specific regulatory mechanisms. The assorted color shades from brown to red to deep purple could be due to different chemical modifications such as hydroxylation, glycosylation, methylation, acylation, and polymerization of the basic anthocyanidin molecule, apart from the effects of pH and the presence of copigments (Holton et al 1993). For instance, methylation appears to be predominant in floral-derived tissue, being at its maximum in the pericarp of PP. Inasmuch as all our pigment analyses were with anthocyanidin aglycones, it was not possible to assess the native glycosidic nature of these pigments. Also, the composition of flavonoids in rice has yet to be established.

Genetic blocks of the anthocyanin pathway

The identification of the proanthocyanidin-accumulating line, N22B, and the leucoanthocyanidin-accumulating (transient) line, G962, is a major step toward characterizing rice lines with genetic blocks in the pathway (Fig. 1). The anthocyanin pathway in the N22B pericarp is blocked and diverted to form proanthocyanidins instead of normal anthocyanins. This represents a block in the conversion of leucocyanidin into cyanidin leading to the condensation product of leucocyanidin and catechin (Stafford et al 1985; Fig. 1). By contrast, the N22W pericarp tissue does not accumulate leucoanthocyanidins or any known flavonoids, suggesting a very early block in the pathway. The genes involved are tentatively identified as *Rc* and *Rd*, as they were earlier implicated in the accumulation of reddish-brown pigments in the pericarp tissue of certain japonica rice lines (Nagao 1962, Takahashi 1963 as reviewed in Takahashi 1982). The availability of proanthocyanidin-accumulating mutants in rice opens an interesting area of research since these compounds are thought to be plant defense chemicals.

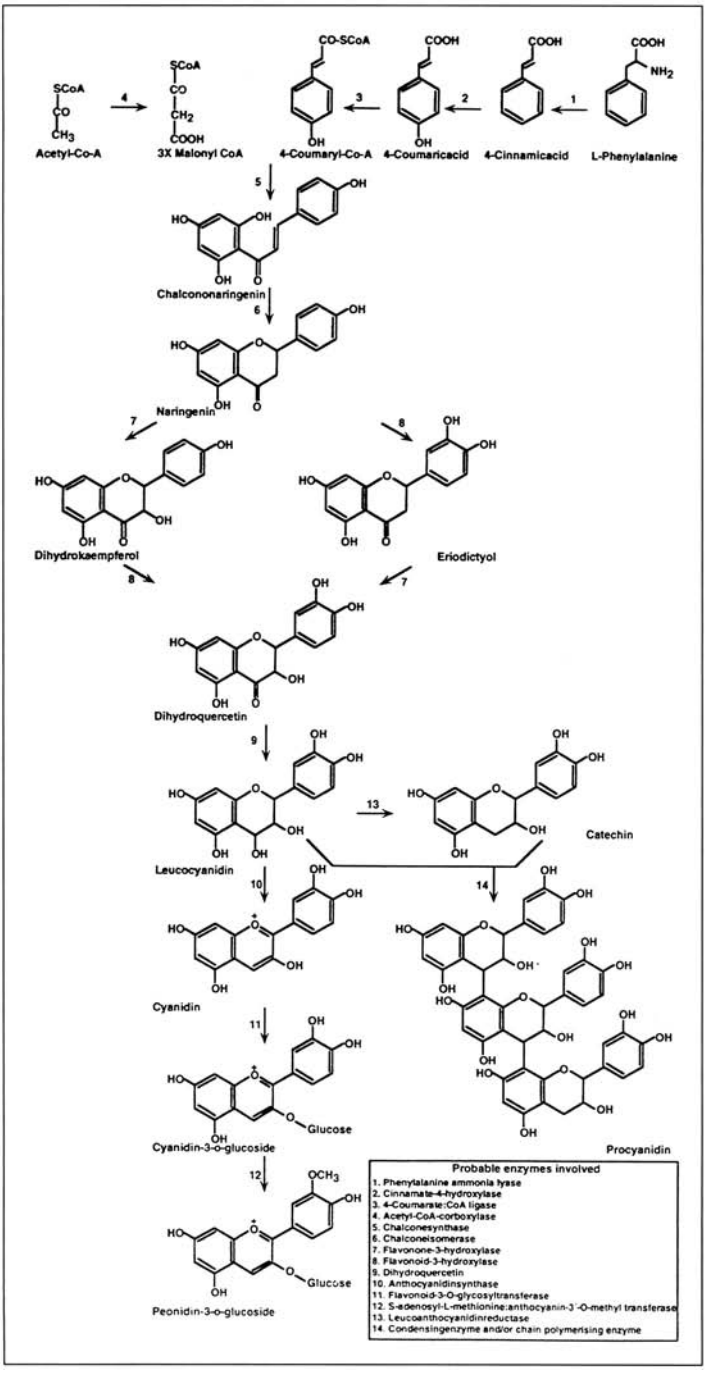

Fig. 1. Anthocyanin biosynthetic pathway.

Molecular biology of the anthocyanin pathway

The molecular basis of anthocyanin gene expression and regulation in rice could not be clarified since none of the structural or regulatory genes of the pathway have been cloned. With the available anthocyanin mutants and variants, we have attempted the molecular isolation of anthocyanin genes. The corresponding genes of maize were used as cDNA probes because anthocyanin genes of maize are structurally and functionally well defined. In addition, because rice and maize are closely related members of the Graminae, significant sequence similarities between anthocyanin genes are expected. In fact, a number of anthocyanin genes from plant species belonging to different genera such as *Antirrhinum,* petunia, maize, and parsley were identified and isolated depending on their sequence homology at the DNA level. Such homologies indicated their functional relatedness, since many of the anthocyanin regulatory and structural genes are used to complement the biochemical function of the corresponding genes in transgenic plants. Maize regulatory genes *R* and *C1* were used to activate anthocyanin biosynthesis in transgenic *Arabidopsis* (Lloyd et al 1992) and *Antirrhinum* (Goodrich et al 1992). The *A1* (anthocyanin-1) gene of maize was demonstrated to be active, both transcriptionally and translationally, in transgenic petunia plants (Meyer et al 1987). Several rice cDNA clones from the PP cDNA library, hybridizable to at least five extensively characterized maize genes, have been isolated. Each of these genes encodes an enzyme of the anthocyanin pathway: *C2*-chalcone synthase (CHS), *A1*-dihydroflavono 14-reductase (DFR), *A2*-NADPH-dependent oxidoreductase (ANS), *Bzl*-flavonol-3-O-glucosy1 transferase (F3GT), and *Bz2*-glutathione-S-transferase (GST).

Molecular isolation and characterization of rice cDNA clones

Chalcone synthase (CHS)

A cDNA library was made from poly A^+ mRNA from young developing leaves of PP in l gt NM 1149-Pop13 cells using standard protocols. This library was screened and several cDNA clones were isolated. A summary of isolated cDNA clones, the gene-specific transcripts and polypeptides, and enzyme activities in rice is presented in Table 2. Using maize *Zm:CHS:C2,* the maize *C2* cDNA encoding chalcone synthase as a probe, a cDNA clone, *OS-CHS,* was isolated. This cDNA clone was further characterized and sequenced (Scheffler et al 1995). The sequence comparison of *OS-CHS* with *Zm:C2:CHS* revealed 86.3% homology, and with *Zm:Whp:CHS,* 85.4% homology within the translated region. Western analysis using *Zm:CHS* antisera also leads to the same conclusion. Further, these proteins are of comparable size. The *OS-CHS* sequence was mapped to chromosome 11 of rice using a restriction fragment length polymorphism mapping strategy (Reddy et al 1996).

CHS and conditional genetic male sterility

Os:CHS cDNA also shows extensive sequence homology to that of the white pollen *(Whp)* gene of maize at the DNA and protein levels. *Whp,* discovered by Coe et al

Table 2. Status of genes/enzymes involved in anthocyanin biosynthetic pathway in rice.

Gene	Enzyme	Protein	Transcript	cDNA
Chs	Chalcone synthase	+[b]	+	+
Dfr	Dihydroflavonol reductase	-[c]	+	+
Ans	Anthocyanidin synthase	-	+	+
Fgt[a]	Flavonol-3-O-glucosyl transferase	+	+	-
Bz2[a] *(Gst)*	Glutathione-S-transferase	+	+	-

[a]Not yet clearly established in rice. [b]+ = detected in indica rice (Purpleputtu). [c]-= not detected.

(1981), encodes CHS and is a duplicate gene of *C2.* The *whp whp* plants produce pollen devoid of flavonoids and are found to be conditionally male sterile. Similarly, flavonoids are implicated in pollen viability, pollen germination, and the male reproductive pathway in petunia (Mo et al 1992, Taylor and Jorgensen 1992). These observations suggest that the pathway could effectively be used to control male fertility in crop plants. The extensive sequence homology between *OS-CHS* and *Zm2:Whp: CHS* allows speculation that rice plants deficient in *CHS* gene expression would eventually lead to conditional genetic male sterility.

Anthocyanidin synthase (Ans)

A cDNA clone hybridizable to the *Zm:A2* probe was isolated and partially sequenced. The sequence comparison between *Os:Ans* and *Zm:A2* at the DNA and protein (deduced from cDNA) levels revealed an extensive homology (data not presented). The *a2* mutant of maize is known to cause a genetic block in the anthocyanin pathway leading to accumulation of colorless leucoanthocyanidin (Coe et al 1988). The *ans* mutant, therefore, in principle, should either accumulate leucoanthocyanidin or proanthocyanidin. In fact, the mutant lines N22B and G962 accumulate detectable amounts of proanthocyanidins and leucoanthocyanidins, respectively (Reddy et al 1995), and therefore are potential *ans* mutants. Further tests are required to define the genetic identity of these mutants. It is interesting to note that N22B and N22W are homozygous to the *Ilb* allele.

The anthocyanin pathway in rice is ultraviolet light-responsive

The fact that the anthocyanin pathway in rice is ultraviolet (UV) light-responsive (Reddy et al 1994) is significant in view of the continuous depletion of the ozone layer in the atmosphere and the resulting increase in the incidence of UV light causing damage to plant life. Biochemical analysis revealed that this UV light-responsive anthocyanin synthesis appears to be mediated by a specific phase of phenylalanine ammonia lyase activity (Reddy et al 1994). Preliminary analysis suggests that rice

seedlings seem to have a specific UV-B receptor in addition to general photoreceptor, the phytochrome. The UV-B-induced anthocyanin biosynthesis precedes the activation of genes of the anthocyanin pathway. Enzyme analyses revealed that phenylalanine ammonia lyase (Reddy et a1 1994), CHS, and F3GT (unpubl. data) showed enhanced activities under UV-B light. Northern analysis also substantiated these results. In addition, expression of the putative *Gst* gene (encoding glutathione-S-transferase) has also been shown to be inducible by UV-B in rice seedlings (Madhuri et a1 1994).

A cDNA library from poly A^+ mRNA of UV-B light-induced leaves was constructed and screened for genes of the anthocyanin pathway. This library would be a source of not only the anthocyanin genes, but also of UV-B responsive elements. Thus, the anthocyanin pathway could serve as a model system to study the genetic basis of response to light, particularly UV-B, and molecular mechanisms of signal transduction.

Molecular manipulation of the anthocyanin pathway to improve disease resistance in rice

Flavonoids as plant defense molecules

The antibacterial and antifungal properties of flavonoids are well documented. Lamb et al (1989) reported that flavonoids play a role in conferring disease resistance in many plants. Proanthocyanidins (Scalbert 1991) and 3-deoxyanthocyanidins (Snyder et al 1991) have been shown to accumulate when plants are infected and are believed to be defense compounds. However, to date, direct evidence for such protective roles of these compounds is still lacking. Hence, we are looking at the toxic effects of flavonoids against major rice pathogens such as *Xanthomonas oryzae* pv. *oryzae (Xoo;* bacterial leaf blight), *Pyricularia oryzae* (blast), and *Rhizoctonia solani* (sheath blight).

Some purified flavanones, flavonols, and phenylpropanoids were screened against the rice pathogens in an in vitro toxicity assay. The reaction response was highly varied where the tested compounds caused significant growth inhibition of the pathogens at micromolar concentrations. Naringenin (the first flavonoid intermediate committed to the anthocyanin pathway) showed a broad spectrum inhibition to six strains of *Xoo* tested. Liquid culture assays with naringenin also showed a tenfold reduction in the growth of *Xoo* after a 12-h shaker incubation at 28 ºC. However, none of the compounds had any significant effect on the mycelial inhibition of *P. oryzae* or *R. solani* (Padmavathi et a1 1996). Attempts are under way to study the response of pigmented and nonpigmented rice cultivars, under blast and blight infection, by determining the flavonoid profiles, levels of phenylpropanoid and flavonoid pathway enzymes, and specific transcripts.

Role of flavonoids in disease resistance

The present trend in developing defense strategies in many plants is to manipulate the response of defense genes to pathogen attack or to develop transgenic plants with engineered genes that inhibit the pathogen. A possible alternative strategy is to enhance the endogenous levels of selected phenylpropanoids and flavonoids by transferring

the appropriate regulatory genes into rice plants. Enhanced accumulation of specific intermediates can also be achieved by using a combination of overexpression and antisense strategies. Such "smart plants" should hyper-accumulate flavonoid intermediates of interest and thereby should have their resistance to pathogen attack enhanced. Efforts are under way to make a complete repertoire of transcriptional fusion constructs of specific anthocyanin genes for overexpression and also inhibition, by antisense sequences, of the anthocyanin pathway. Such constructs are made for almost all the required structural and regulatory genes of the pathway.

These constructs will enable us, through a transgenic approach, to test the premise that flavonoids play a role in disease resistance. Thus. the manipulation of the pathway and its eventual use to generate transgenics with improved disease resistance would ultimately prove to be a powerful technique. In addition. these gene constructs can be used possibly to alter the shades of color and intensity, tissue-specific distribution, and the composition of the responsible pigments in the variety of plant species, including ornamentals.

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Sorting of storage protein mRNAs on distinct endoplasmic reticulum membranes in developing rice endosperms

D.G. Muench, Y. Zhang, Y. Wu, and T.W. Okita

Electron microscopic studies reveal that rice endosperm cells contain two types of endoplasmic reticulum (ER), the cisternal ER (C-ER) and protein body ER (PB-ER). As demonstrated by biochemical techniques and in vitro and in situ hybridization, mRNAs that code for the prolamine storage proteins are enriched up to 10-fold over the mRNAs that code for the glutelin storage proteins on the PB-ER, whereas glutelin mRNAs are about 2-fold more abundant than prolamine mRNAs on the C-ER membranes. Therefore, the initial targeting of glutelins and prolamines into separate protein bodies is facilitated by the segregation of their transcripts on the two distinct ER types. We have obtained evidence suggesting that at least one process that is responsible for targeting of the prolamine mRNA to the PB-ER is the interaction of the prolamine transcript with a receptor on the PB surface. In addition to these studies, we discuss our current efforts to obtain more direct evidence for the mechanism of sorting by generating transgenic plants containing hybrid gene constructs.

The seed storage proteins of plants are a group of evolutionary conserved proteins that serve as a source of carbon, nitrogen, and sulfur for the germinating seedling (Shotwell and Larkins 1989). They are synthesized during seed development and are packaged in protein bodies (PBs) via one or more steps of the secretory pathway. Recent elucidation of primary sequences of plant storage proteins indicates that two broad classes of storage proteins exist (Shotwell and Larkins 1989). Although the prolamines are the major storage proteins of cereals, rice and oat are unusual in that they accumulate both classes, prolamines and globulins, in their seeds (Juliano 1972, Shotwell and Larkins 1989, Muench and Okita 1996). The major storage protein of rice is an insoluble glutelin fraction that displays protein sequence, subunit composition, and charge distribution similarities to the 11S globulins of the legumes (Zhao et al 1983, Robert et al 1985, Wen and Luthe 1985, Takaiwa et al 1986). The glutelins

constitute about 60% of the total protein fraction in rice seed whereas the prolamines comprise 18-20% of the total protein (Ogawa et al 1987, Li and Okita 1993). Here, we discuss the cellular processes by which the rice storage proteins are packaged into PBs and recent results on the biochemical and cellular mechanisms that are responsible for sorting these proteins into separate PBs.

Segregation of rice storage protein mRNA on the endoplasmic reticulum

Rice is unique among the cereals in that it packages glutelin and prolamine polypeptides into separate PBs. The prolamine PBs are derived directly from the endoplasmic reticulum (ER) whereas the glutelin PBs are deposited in the vacuole via the Golgi apparatus (Tanaka et al 1980, Krishnan et al 1986, Yamagata and Tanaka 1986).

Electron microscope studies reveal that rice endosperm cells contain two types of ER, the cisternal ER (C-ER), consisting of single-lamellar stacks distributed throughout the cell, and the protein body ER (PB-ER), which delimits the prolamine PBs (Li et al 1993a). Biochemical evidence shows that these two types of ER membranes differentially recruit prolamine and glutelin mRNA. When polyadenylated mRNA was isolated from purified PBs and translated in vitro, only prolamine was synthesized (Yamagata et al 1986). On the other hand, C-ER membrane-bound polysomes contained about 2-fold more glutelin mRNA than prolamine mRNA (Kim et al 1993). These results suggest that the storage proteins of rice are not randomly distributed on the ER membrane, but rather are localized on different ER types.

Subcellular fractions enriched for both types of ER membranes were purified and analyzed for their prolamine and glutelin mRNA contents by blot hybridization. These results were supported by Li et al (1993a), who showed that prolamine transcripts are present at a 10-fold molar excess over the glutelin transcripts in the PB-ER fraction. Conversely, the glutelin mRNAs are enriched 2-fold over prolamine mRNA on the C-ER.

More direct evidence of the sorting of prolamine and glutelin mRNAs to different parts of the ER was obtained using single- and double-probe in situ hybridization of ultrathin rice endosperm sections (Li et al 1993a). Taking into consideration the probe and gold particle sizes, the PB-ER was shown to have about 7-fold more prolamine mRNA than glutelin mRNA and the C-ER had about 2-fold more glutelin transcript over prolamine transcript. These results are consistent with the previous biochemical and in vitro hybridization estimations, further demonstrating the preferential sorting of these transcripts to different parts of the ER. Thus, it appears that the segregation of glutelin and prolamine mRNA on the C-ER and PB-ER, respectively, facilitates the initial targeting of glutelin and prolamine to distinct PBs. The biochemical and molecular bases for the segregation of prolamine and glutelin mRNAs are not known. However, examples of nonrandom mRNA localization in certain animal cell types are well established (reviewed by St. Johnston 1995).

Mechanism of mRNA sorting in rice endosperm cells

Our current efforts are directed toward determining the mechanism responsible for the sorting of glutelin and prolamine mRNAs to distinct domains of the ER in rice endosperm cells. One possible mechanism may involve the directed sorting of prolamine transcripts to the PB-ER in a stepwise localization pathway where cis-sequences of the prolamine transcripts are recognized by proteins that are responsible for transport and anchoring to the PB periphery. A second mechanism proposed by Masumura et al (1990) may involve a cotranslational process whereby the prolamine and glutelin signal peptides are recognized by different signal recognition particles (SRPs) which, in turn, would interact with their corresponding SRP receptors that are asymmetrically distributed on the PB-ER and C-ER.

Alternatively, the sorting of glutelin and prolamine mRNAs may be a result of indirect processes instead of an active sorting mechanism. For example, the prolamine and glutelin polysome complexes may be randomly distributed on the PB-ER but then an enrichment of prolamine mRNAs may occur due to an increased residence time of the prolamine translation complex on the PB-ER. A plausible explanation for the stability of the prolamine translational complexes on the PB-ER is the direct assembly of the nascent polypeptide onto the protein granule during the synthesis and translocation process. Such a self-assembly model for prolamine PB formation has been proposed for the maize zeins based on modeling studies of this hydrophobic polypeptide (Argos et al 1982). More recent studies, however, suggest the possible role of the lumenal chaperone, binding protein, or BiP in maize PB formation (Zhang and Boston 1992, Li et al 1993b).

Role of a receptor in prolamine mRNA targeting

The mRNA localization pathway proposes that mRNA sorting occurs by a two-step process where the mRNA is selectively transported to a specific intracellular location and then anchored to a receptor at the destination site (Yisraeli et al 1990). We have obtained evidence that supports the existence of a salt-sensitive, detergent-resistant receptor that binds prolamine mRNA to the prolamine PB. This receptor would be present in addition to the SRP and ribosome receptors of the ER polypeptide translocation complex (Rapoport 1991, 1992). When an enriched rice endosperm PB fraction is prepared in a low ionic strength buffer, the bulk of the prolamine polysomes is observed in this fraction. Treatment of this PB fraction with 1% Triton X-100 for 10 min released less than 5% of the total polysomes into the supernatant. The retention of the bulk of the prolamine polysomes with membrane-free PBs suggests that the polysomes are complexed to the PB surface by a second receptor in addition to the SRP and ribosome receptors. Incubation of the Triton X-100-washed PB fraction with puromycin, a drug that mediates premature release of nascent polypeptide chains from ribosomes, had little effect on the release of RNA from detergent-treated PBs. Since we have demonstrated the release of the nascent chain/BiP complex under these same incubation of conditions (Li et al 1993b), these results indicate that the PB polysomes were not bound by the nascent polypeptide chain, but most likely by the mRNA.

To obtain additional evidence demonstrating that the interactions between the prolamine PBs and prolamine mRNA are not mediated by a ribosome or nascent polypeptide interaction, we conducted experiments that investigated the interaction of naked prolamine mRNA with the PBs (Y. Wu and T.W. Okita, Washington State University, unpubl. data). Half-sectioned, mid-developing seeds were incubated overnight in MS media supplemented with asparagine and 2% sucrose and containing either 0 or 25 mM NaF. NaF decreases the ATP content of the cell. Under these conditions, the initiation step of translation is prevented while still allowing the loaded ribosomes to "run-off' the transcript. Crude extracts of these seeds were prepared and then detergent-washed PBs were isolated from this crude extract and treated with 300 mM NaCl. The PBs were removed by centrifugation and the supernatant was loaded onto a 15-60% sucrose gradient and centrifugated. Supernatant, monosome, and polysome fractions were isolated from the sucrose gradient, and the amounts of prolamine mRNA within each fraction quantified by dot blot hybridization. The

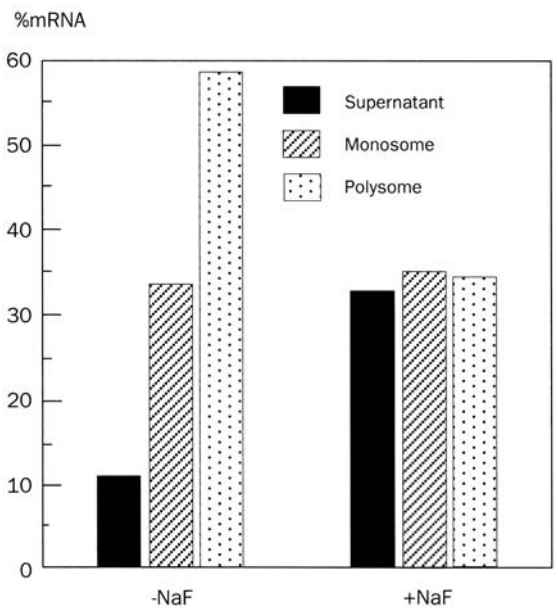

Fig. 1. Distribution of prolamine mRNA in polysomes from NaF-treated seeds. Seeds were treated with or without 25 mM NaF as described in the text, and detergent-washed PBs were then centrifuged. The supernatant was collected and loaded on a sucrose gradient to separate polysomes and monosomes from free RNA. Fractions corresponding to the soluble phase, monosomes, and polysomes were collected and RNA samples from each of these fractions obtained and analyzed for their prolamine RNA content.

prolamine mRNA concentration in these fractions differed in the NaF-treated vs the nontreated seeds (Fig. 1). Although the prolamine mRNAs in the form of monosomes did not vary substantially between the two treatments, there was a large increase in free prolamine mRNA found in the supernatant fraction of the NaF-treated seeds. This increase in free mRNA was proportional to the decrease in polysomal prolamine mRNA in NaF-treated seeds (Fig. 1).

Overall, these results support the existence of a salt-sensitive, detergent-resistant receptor that binds prolamine mRNA to a nonmembrane component on the surface of the prolamine PB by an unknown mechanism. This receptor may be a component of the mRNA localization pathway involving transport and anchoring of prolamine mRNA to the PB surface via a cis-acting RNA sequence. As seen in other cell types that demonstrate mRNA localization, the cytoskeleton may have a role in the transport and anchoring of prolamine mRNA to the PB via an RNA signal as discussed in more detail below.

Association of prolamine PBs and mRNA with the cytoskeleton

Extracts from mid-developing rice seed were separated on a sucrose gradient in order to resolve the C-ER/polysomes and the PBs. Fractions enriched for these membranes were pooled, subject to treatment with detergents and/or high ionic strength buffer and then refractionated by centrifugation on a 20-80% sucrose density gradient. Panels A and B of Figure 2 depict the sedimentation of the enriched C-ER and PBs fractions, respectively, on the sucrose density gradients. Other than the peaks corresponding to the C-ER/polysomes and PBs, the only other major peak evident in these gradients corresponds to the pellet that is displaced during the fractionation of the gradient with dense CsCl solution. Fractions corresponding to the soluble fraction, the C-ER/polysome fraction, the PB fraction, and the pellet fraction were pooled and analyzed for their content of prolamine RNAs (Fig. 3) and the cytoskeleton component tubulin (Fig. 4). From the C-ER gradient (Fig. 2a, top panel), the C-ER/polysome fraction contained the majority of prolamine transcript (Fig. 3), whereas in the PB gradient (Fig. 2b, top panel), the PB peak contained most of the prolamine transcript (Fig. 3). Associated with the C-ER/polysome peak in the C-ER gradient were significant amounts of tubulin and prolamine (Fig. 4a) as well as actin (not shown). The presence of prolamine mRNA in the C-ER fraction likely represents mRNA involved in the synthesis of prolamine into newly developing PBs at localized areas of the C-ER. The PB gradient shows a similar association of prolamine PBs with the tubulin (Fig. 4b) and actin (not shown).

Detergent treatment of the PB fraction with 1% polyoxyethylene 10-tridecyl (PTE) in the low-salt buffer resulted in a sucrose gradient profile similar to that obtained for the untreated detergent sample (Fig. 2b, middle panel) with no apparent change in the distribution of prolamine RNAs and polypeptides and of tubulin within the gradient (Figs. 3 and 4b). In contrast, detergent treatment of the C-ER/polysome fraction resulted in the disappearance of the C-ER/polysome peak and the appearance of a new peak sedimenting with the PBs (Fig. 2a, middle panel). This shift in sedimentation pattern is also evident for prolamine mRNAs and polypeptides and cytoskeletal ele-

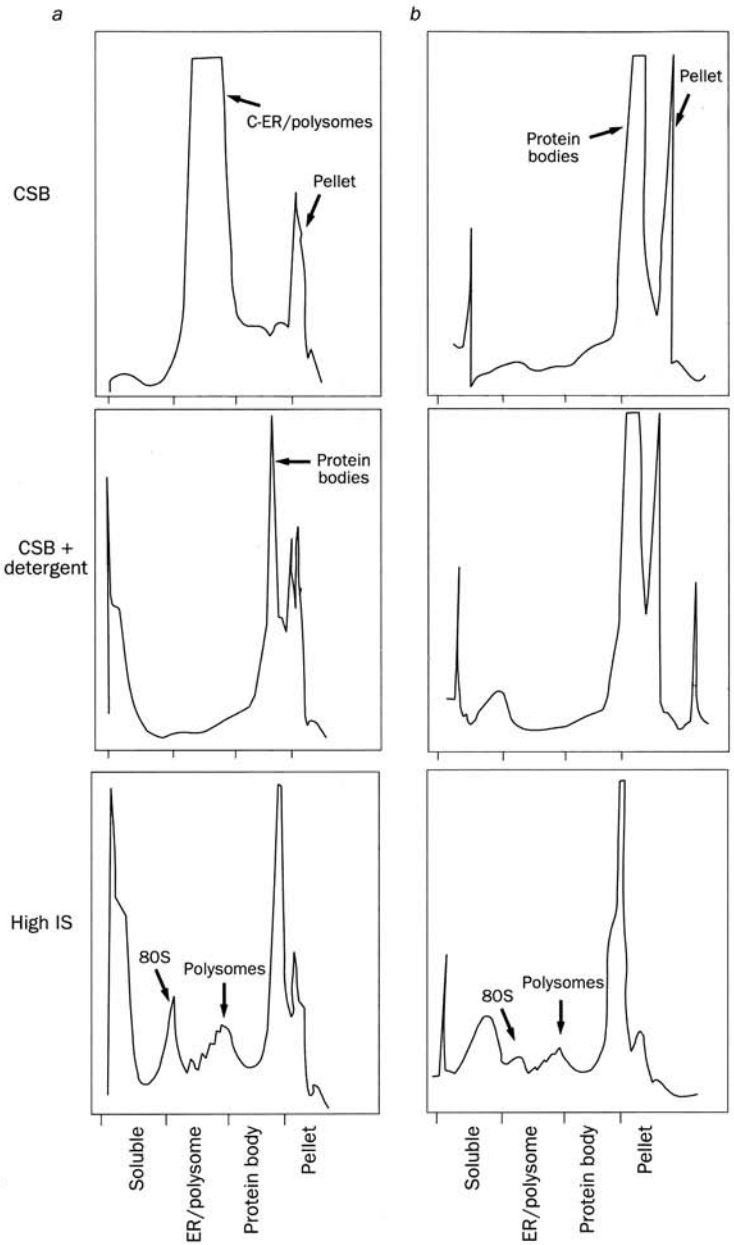

Fig. 2. Sucrose density gradient (20-80%) absorbance profiles of a) C-ER and b) PB fractions extracted in cytoskeleton stabilizing buffer (CSB), CSB + detergent or CSB + detergent + 200 mM Tris-HCl pH 7.5 (High IS). The gradients were separated into four fractions as shown on the horizontal axis and the RNA and protein isolated for further analysis. The components of each peak are identified by the arrows. 80S refers to the monosome peak.

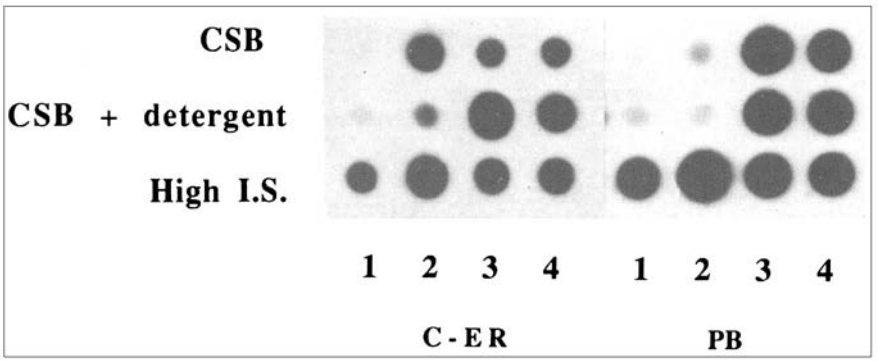

Fig 3. Effects of salt and detergent pretreatment on the sedimentation of prolamine mRNA. RNA was purified from the fractions obtained from the C-ER and PB sucrose gradients. The RNA was blotted to nitrocellulose and probed with labeled prolamine DNA. The numbers at the bottom of each panel correspond to 1, soluble fraction; 2, C-ER/polysome fraction; 3, protein body fraction; and 4, pellet fraction. The different treatments shown left of the panels are as described in Figure 2.

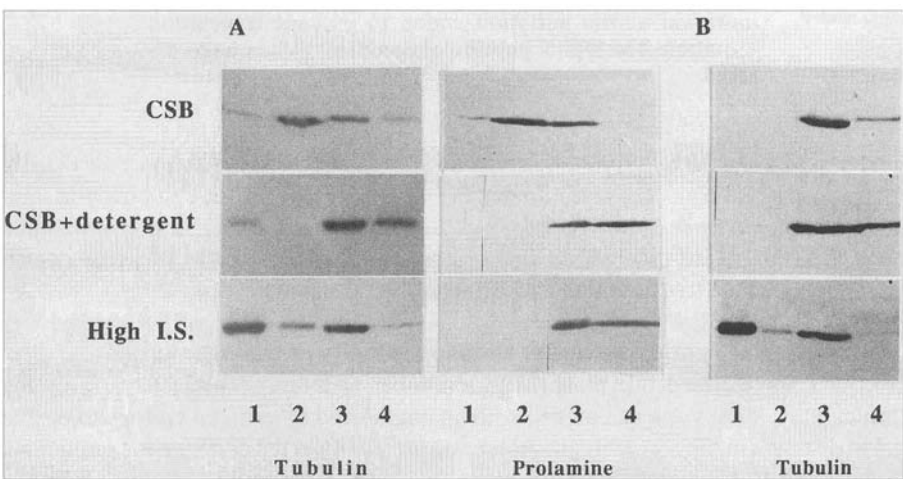

Fig. 4. Effects of salt and detergent pretreatment on the sedimentation of tubulin and prolamine in the a), C-ER and b), PB sucrose gradient profiles shown in Figure 3. Protein from the four sucrose gradient fractions was electrophoresed on either 7% (tubulin) or 15% (prolamine) acrylamide gels, electroblotted to nitrocellulose and probed with either anti-tubulin or anti-prolamine antiserum. Figure labels are as in Fig. 2.

ments (Figs. 3 and 3a). Treatment of the C-ER and PB fractions with 200-mM Tris (pH 7.5) and PTE released most of the polysomes from these fractions, as shown by the polysome profile in the C-ER/polysine fraction (Fig. 2a,b, bottom panels) and the mRNA analysis (Fig. 3). A majority of the tubulin (Fig. 4) and actin was solubilized

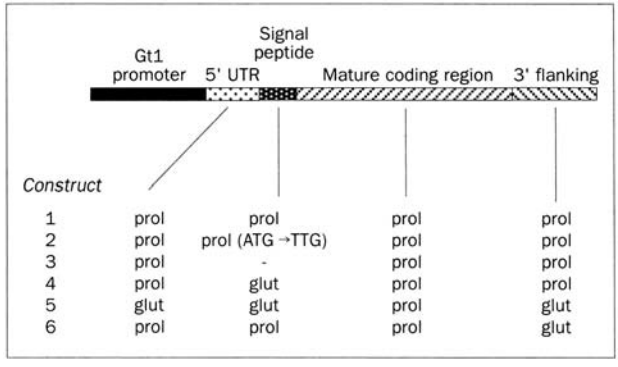

Fig. 5. Hybrid prolamine gene constructs. The map shows the hybrid DNA construct that contains the corresponding regions of either the prolamine or glutelin DNA. All constructs are under the transcriptional control of the Gt1 promoter. The six constructs differ by swapping the regions of prolamine (*prol*) or glutelin (*glut*) genes. The prolamine signal peptide of construct 2 contains a point mutation at the initiation codon to prevent translation initiation. The signal peptide of construct 3 has been deleted.

under these conditions and was observed in the soluble fraction of the sucrose density gradient. These results demonstrate that the prolamine mRNA/PB complex is associated with the cytoskeleton. It is possible, therefore, that the targeting of prolamine mRNA to the PB-ER involves binding of a receptor molecule to the transcript, which may then mediate transport and anchoring to the PB via the cytoskeleton.

Distribution of hybrid mRNAs in endosperm cells of transgenic rice

We have transformed rice plants with a number of hybrid constructs carrying the prolamine coding sequence tagged with an engineered C-terminal epitope to enable us to distinguish the recombinant prolamine protein from the endogenous prolamine. Subcellular fractionation of PB-ER and C-ER membranes will be obtained from the endosperm tissue of the transgenic plants, and the distribution of the hybrid mRNA and its corresponding protein identified. Six constructs, each driven by the endosperm-specific glutelin Gt1 promoter, were assembled for transformation (Fig. 5). Construct 1 encodes the wild-type prolamine coding and flanking sequences as a control to ensure proper distribution of hybrid mRNA and protein in the developing endosperm cell. Construct 2 contains a mutated initiation codon, which would prevent translation of the hybrid transcript, and should reveal if the targeting of prolamine transcript to the PB-ER is dependent on translation. If the hybrid mRNAs of the transformed plants expressing this construct are targeted to the prolamine PBs, then these data would support the existence of an mRNA targeting signal. In construct 3, the prolamine signal peptide was deleted. and in construct 4, it was replaced with the glutelin signal

peptide sequence. These two constructs will determine if cotranslational events are necessary for targeting. If the prolamine signal peptide is required for targeting of prolamine mRNAs to the PB-ER, then the hybrid prolamine mRNA from construct 3 will not be associated with the PB-ER. If there exists a population of SRPs that can differentiate between the prolamine and glutelin signal peptides, then hybrid mRNA, which contains the glutelin signal peptide sequence from construct 4, will primarily target to the C-ER. Construct 5 contains the glutelin 5' UTR, 3' flanking region and signal peptide, and the mature prolamine coding region. If the prolamine polypeptide alone is required for the targeting of prolamine mRNA to the prolamine PBs independent of any other flanking sequences or the signal peptide, plants transformed with construct 5 should show sorting of the transgenic prolamine mRNA to the same location as the wild-type plants. Since BiP is known to interact with the prolamine polypeptide and has a role in folding and assembly. perhaps this interaction stabilizes the prolamine translation complex on the PB-ER. Construct 6 will determine if the glutelin 3' UTR has any role in the translocation of glutelin mRNA to the C-ER since a glutelin mRNA signal has not been ruled out as a signal for C-ER membrane targeting. These constructs, together, should provide us with good evidence as to the signal responsible for mRNA targeting in rice.

Conclusions

Since the glutelins and the prolamines are both synthesized on the ER, an effective mechanism is required for the deposition of these proteins into two distinct PB types within the endosperm of rice cells. It appears that rice specifically targets prolamine mRNA to the PB-ER to prevent the large intracisternal prolamine aggregates from impeding the transport of glutelin from the ER into the secretory pathway. This targeting may also be an effective mechanism for the cell to concentrate newly synthesized prolamine polypeptides. Evidence suggests that the intracisternal deposition of prolamines in barley endosperm (Rechinger et al 1993) and granule formation in hamster pancreatic cells (Tooze et al 1989) require a critical concentration of intracisternal protein, above which protein aggregation will occur (Okita and Rogers 1996). Below this critical concentration, the newly synthesized proteins are transported from the ER via the secretory pathway. In rice, prolamine mRNA sorting and the interaction of prolamine with BiP and other chaperones and lumenal proteins may concentrate the newly synthesized prolamine within a localized region of the ER. Together, localized concentrations of prolamine may initiate intracisternal aggregation of prolamine and subsequent PB formation.

Our observation that prolamine mRNA is associated with membrane-free PBs as well as the cytoskeleton is consistent with a translation-independent sorting of prolamine mRNAs to the PB. Many examples of nonrandom localization of mRNA have been observed in animal systems and may provide models of how mRNA sorting occurs in rice. The results we will obtain from the transgenic plants and from the microinjection experiments will more clearly explain the mechanisms responsible for mRNA sorting in rice endosperm cells.

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Concurrent session papers

Phylogenetic relationships between cultivated and wild species of rice as revealed by DNA polymorphisms

T. Ishii, T. Nakano, H. Maeda, O. Kamijima, and G.S. Khush

To clarify the phylogenetic relationships among A genome species, restriction fragment length polymorphism (RFLP) analyses on chloroplast, mitochondrial, and nuclear genomes and random amplified polymorphic DNA (RAPD) analysis were carried out using 30 accessions (8 *Oryza sativa,* 6 *O. glaberrima,* 13 *O. perennis,* and 3 *O. breviligulata).* Chloroplast DNA was isolated and digested with two restriction endonucleases. To examine mitochondrial and nuclear DNA, total DNA was digested with two endonucleases and subjected to Southern hybridization using 8 mitochondrial and 12 nuclear DNA specific probes, respectively. From the proportion of common fragments, the genetic distances between 30 accessions were calculated, and dendrograms were constructed. RAPD analysis using 14 primers was also carried out, and a phylogenetic tree was made based on the proportion of common fragments. The differentiations of three genomes are mostly harmonious; however, the magnitude of nuclear genome differentiation is much bigger than the other two. The Oceanian, American, and African forms of *O. perennis* differentiated greatly from others, whereas the *O. perennis* Asian form and O. *breviligulata* formed clusters with O. *sativa* and *O. glaberrima, respectively. This indicates that O. sativa* and *O. glaberrima* had originated from the *O. perennis* Asian form and *O. breviligulata,* respectively.

The genus *Oryza* includes two cultivated species and more than 20 wild species. Genome analysis revealed that the cultivated species, *O. sativa* and *O. glaberrima,* and the wild species, *O. perennis* and *O. breviligulata,* share the same genome, A. *O. sativa* and *O. perennis* are further subdivided because of their broad genetic differentiation. *O. sativa* has three groups, japonica, javanica, and indica, while *O. perennis* consists of four geographical forms, Asian, American, African, and Oceanian (Morishima 1969).

There are three organelles in a plant cell, i.e., nucleus, chloroplast, and mitochondrion. Each organelle has its own genetic information. Nuclear genetic information is inherited from both parents, while that of chloroplast and mitochondrion is maternally inherited. Although these genomes are autonomous and independent in inheritance, they interact with each other in terms of gene expression.

Recently, analysis of DNA variation has become useful in elucidating genetic differentiation. Therefore, in this study, we analyzed chloroplast (ct), mitochondrial (mt), and nuclear DNA using 30 accessions in the A genome species to clarify the phylogenetic relationships between cultivated and wild species and to compare differentiation of the three genomes at the DNA level. In addition, we carried out randomly amplified polymorphic DNA (RAPD) analysis using the same accession.

Materials and methods

Plant materials

Thirty accessions of the A genome species were used in the study. Their classification and names are listed in Table 1.

Table 1. Plant materials used in this study.

Taxon		Code	Name	Source[a]
O. sativa	Japonica	J1	Nipponbare	K
O. sativa	Japonica	J2	Taichung 65	K
O. sativa	Javanica	Jv1	532	S
O. sativa	Javanica	Jv2	647	S
O. sativa	Indica	I1	IR36	K
O. sativa	Indica	I2	108	S
O. sativa	Indica	I3	419	S
O. sativa	Indica	I4	C8005	S
O. glaberrima		G1	W401	S
O. glaberrima		G2	W438	S
O. glaberrima		G3	W440	S
O. glaberrima		G4	W446	S
O. glaberrima		G5	W492	S
O. glaberrima		G6	W528	S
O. perennis	Asian	As1	W108	S
O. perennis	Asian	As2	W120	S
O. perennis	Asian	As3	W149	S
O. perennis	Asian	As4	W593	S
O. perennis	Asian	As5	W630	S
O. perennis	American	Am1	W1167	S
O. perennis	American	Am2	W1169	S
O. perennis	American	Am3	W1192	S
O. perennis	African	Af1	W1540	S
O. perennis	Oceanian	01	W1300	S
O. perennis	Oceanian	02	W1627	S
O. perennis	Ocenian	03	W1629	S
O. perennis	Oceanian	04	W1633	S
O. breviligulata		B1	W607	S
O. breviligulata		B2	W653	S
O. breviligulata		B3	W1152	S

[a]K = Kobe University, Japan; S = Dr. Y. Sano, National Institute of Genetics, Japan.

Restriction fragment length polymorphism (RFLP) analysis

Purified ctDNA was digested with two restriction endonucleases, *Eco*RI and *Hin*dIII. The digests were electrophoresed in agarose gel, and the fragment patterns were observed by ethidium bromide staining.

Total DNA was digested with *Eco*RI and *Hin*dIII, electrophoresed in agarose gel, and transferred to nylon membrane. To detect mitochondrial and nuclear DNA fragments, 8 and 12 specific probes were used for Southern hybridization (Table 2).

RAPD analysis

In polymerase chain reaction, total DNA from 29 accessions and 14 kinds of decamer oligonucleotide (Operon Inc., USA) were used as templates and primers, respectively. The amplified products were observed after agarose gel electrophoresis.

Construction of dendrogram

Based on the band patterns, the proportion of common fragments between accessions was calculated. In RFLP analyses, genetic distances were further estimated according to Nei (1987). The phylogenetic trees were constructed by the unweighted pair-group method with arithmetic mean (Sneath and Sokal 1973).

Table 2. Probes used for RFLP analysis of mitochondrial (A) and nuclear DNA (B).

A			B		
MtDNA specific probe[a]	Size (kbp)	Plant source	NucDNA specific probe[b]	Size (kbp)	Chromo-some no.
COXI[1]	2.6	Oenothera	RG236	1.4	1
COXII[2]	1.9	Pea	RG144	0.8	2
COXIII[1]	1.1	Oenothera	RG69	?	3
COB[3]	1.8	Wheat	RG214	1.4	4
ATPA[1]	1.5	Pea	RG182	3.4	5
ATP6[1]	4.2	Oenothera	RG172	1.8	6
RRN26[4]	5.1	Wheat	RG351	0.8	7
RRN18+5[4]	3.2	Wheat	RG20	1.5	8
			RG358	1.0	9
			RG241	2.5	10
			RG118	2.0	11
			RG190	1.4	12

[a]Probes include whole or part of the region of the genes. They were provided by Dr. A. Brennicke, Institut für Genbiologische Forschung, Germany (1); Dr. A. Morikami, Nagoya University, Japan (2); Dr. W. Gray, Dalhousie University, Canada (3); and Dr. F. Quetier, Untversite Paris, France (4). [b]All probes were cloned from rice genomic DNA and mapped on the chromosomes (McCouch et al 1988). They were provided by Dr. S. McCouch, Cornell University, USA.

Results and discussion

Dendrograms showing genetic relationships in the A genome species of rice were drawn (Figs. 1-4). Since genetic distances were calculated from the results of RFLP analyses on ct, mt, and nuclear DNA, the extent of differentiation among the three genomes was compared. The magnitude of nuclear genome differentiation is much bigger than the other two, and that of the chloroplast is the smallest. These might be due to the different way of inheritance and the proportion of the coding region in each genome.

In all analyses, *O. perennis* showed wider differentiation than the other A genome species. The Oceanian, American, and African forms of *O. perennis* differentiated greatly. This might be caused by geographical isolation. On the other hand, the *O. perennis* Asian form and *O. breviligulata* formed clusters with *O. sativa* and *O. glaberrima,* respectively. This strongly indicates that *O. sativa* and *O. glaberrima* had originated from the *O. perennis* Asian form and *O. breviligulata,* respectively.

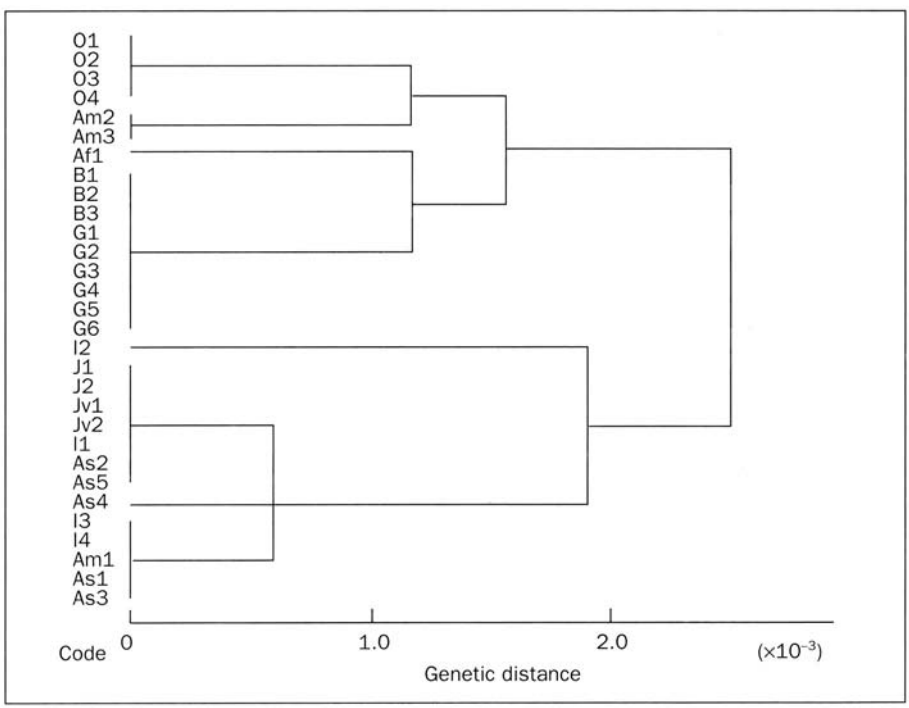

Fig. 1. A dendrogram showing genetic relationships between 30 accessions of A genome species of rice based on RFLP analysis of chloroplast DNA.

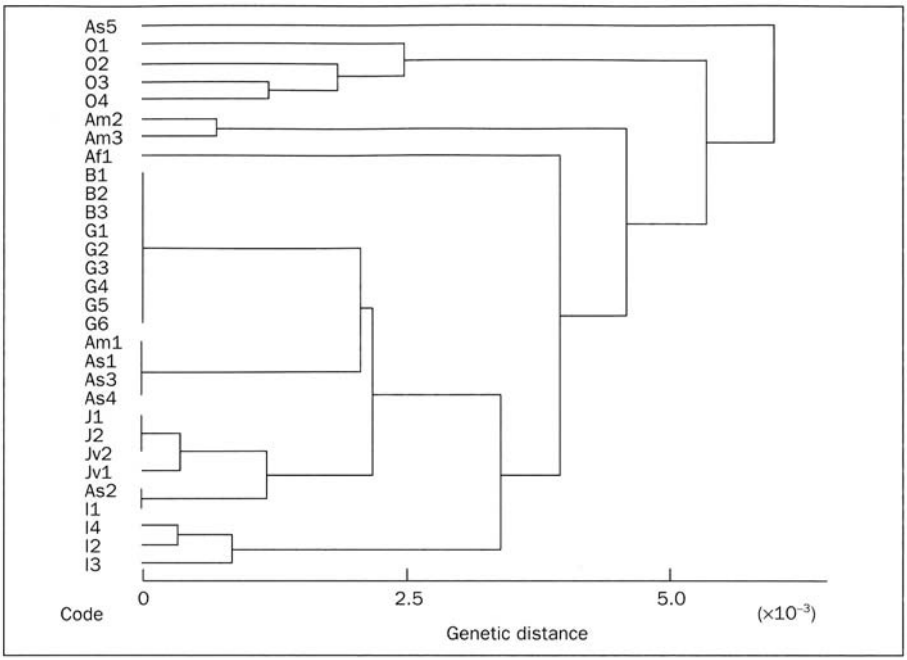

Fig. 2. A dendrogram showing genetic relationships between 30 accessions of A genome species of rice based on RFLP analysis of mitochondrial DNA.

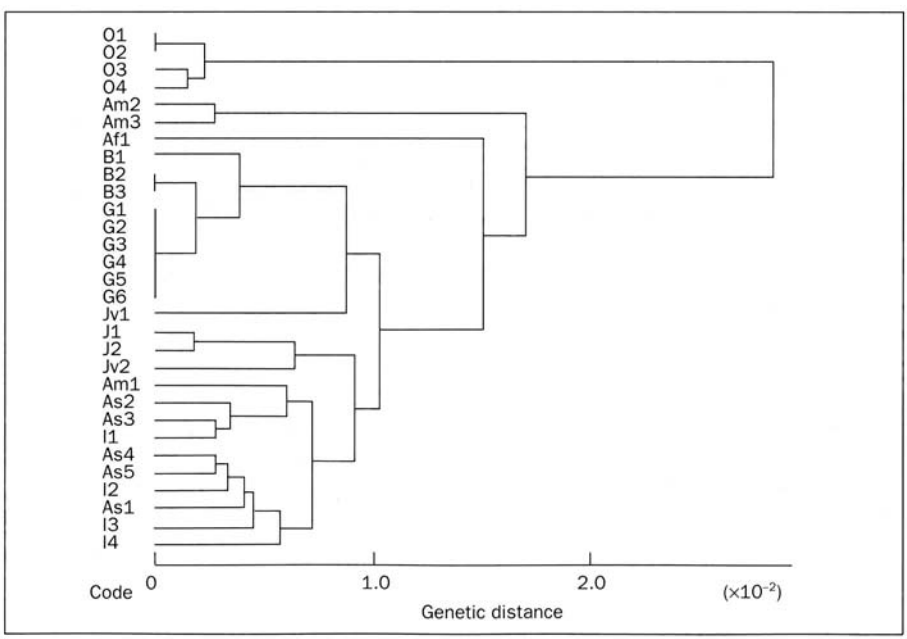

Fig. 3. A dendrogram showing genetic relationships between 30 accessions of A genome species of rice based on RFLP analysis of nuclear DNA.

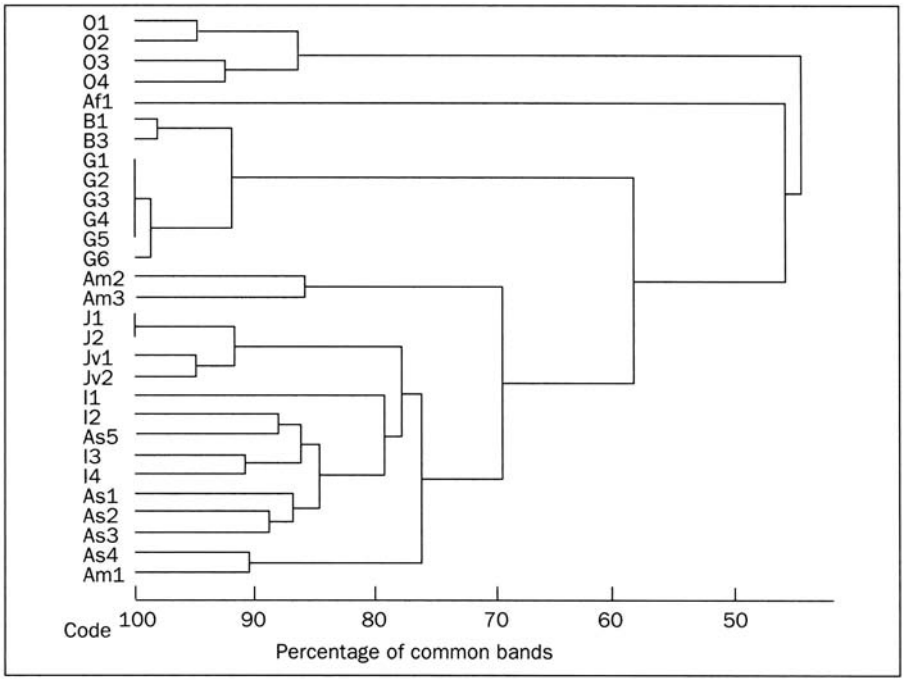

Fig. 4. A dendrogram showing genetic relationships between 29 accessions of A genome species of rice based on RAPD analysis.

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pRRD9, a repetitive DNA sequence that transfers from the chloroplast genome to the nuclear genome with diverse amplification across species

A.D. Han, C.X. Wang, Z. Xiao, H. Yu, W.H. Liu, and L.S. Liu

A repetitive DNA sequence of rice, pRRD9, has been characterized and sequenced and shows a wide distribution of its homologs in closely related species and also in distant families. Copy number analysis shows that pRRD9 amplifies gradually along evolutionary lines from wild rice to cultivated rice. DNA analysis shows that pRRD9 has an 89.3% homogeneity with a related sequence of the photosynthesis system II *QB* gene of rice. Further study indicated that pRRD9 comprises two regions: the right region (117-60), mostly derived from a chloroplast genome, and the left region (1-116), derived from a nuclear genome. The right region shares 99.7% identity with the related sequence of the *QB* gene while the left region shares only 45.7%. We found that pRRD9 homologs existed even in nuclear genomes of several distant plants, suggesting there is a complex mechanism in the evolution of repetitive DNA sequences.

Repetitive DNA sequences distribute widely in fungi, animals, and plants. They have been called "selfish" DNAs by Orgel and Crick (1980), but there is growing evidence that repetitive DNA sequences have many functions including the regulation of gene expression and speciation of organisms (Hohn and Dennis 1985, Leeton and Smyth 1993, Flam 1994, Nowak 1994), which we believe are "in hiding." We obtained some repetitive DNA clones from the rice genome via the renaturation kinetics method. This paper reports on one of the repetitive DNA clones.

Copy numbers of pRRD9 and its homologs in different plants

We used Ultrascan XL (Pharmacia LKB) to measure slot-blot signal intensity from total DNAs and calculated the copy numbers according to Cullis et al (1984). The average copy numbers estimated for japonica and indica rices are 4,740 and 3,310, respectively. from each of the eight subspecies. In wild rice, the copy numbers vary

Table 1. The relative copy numbers of pRRD9 or its homologs in different species.

Species	*Family*	*Relative copy no.*[a]
Oryza sativa	Poaceae	100.0
Michelia alba	Magnoliaceae	2.8
Acacla confusa	Fabaceae	9.0
Broussonetia papyrifera	Moraceae	52.0
Polygonum chinensis	Polygonaceae	10.4
Ophlopogon japonicus	Liliaceae	2.0
Cyperus rotundus	Lyperaceae	9.4
Gnetum parvifolium	Gnetaceae	2.0
Cordyline fruticosa	Liliaceae	45.8
Hygroryza aristata	Poaceae	22.0

[a] Calculated as relatlve percentages of that of rice, which is set at 100%.

with genome types: e.g., 1,320 for BBCC and 650 for CC, EE, and FF. The copy numbers of pRRD9 homologs of 42 additional species from approximately 20 families were calculated as relative percentages of that of rice, which is set at 100%. Table 1 shows that pRRD9 or its homologs appears to amplify gradually along evolutionary lines.

pRRD9 and its homologs are distributed widely in the plant kingdom

Based on the copy number test, we altered the DNA concentrations of different species in Southern hybridization to obtain strong signals. Previous work had shown fingerprintlike hybridization patterns in japonica and indica rices (Liu et al 1994). We were able to demonstrate that similar patterns also exist in wild rice species when *Hae*III-digested total DNAs were hybridized with pRRD9. Since no two species had identical patterns, it is very likely that pRRD9 has unique fingerprints in individual species or subspecies. In the Poaceae, obvious polymorphism was observed in the hybridization patterns. *Lingnania chungii, Bambusa texilis, Lingnania fimbriliguluka,* and *Bambusa ventricosa* produced single 3.2-kb bands similar to that in rice, which also exist in wheat, while *Bambusa vulgaris* had only a 1.1-kb band. We also obtained polymorphic signals in the Chlorideae, Eragrostideae, Zoysieae, Paniceae, Andropogoneae, Thuareinae, and Paspalinae.

Homogeneity scanning of pRRD9

The wide distribution of pRRD9 in the Plant Kingdom inspired us to conduct homogeneity scanning of pRRD9 in the data base. The maximum homogeneity (89.3%) found was in the chloroplast *psbA* gene of rice. which encodes a 32-kDa QB protein. Further analysis revealed pRRD9 as two different regions. The left region (1-116)

shares only 45.7% identity with the *psbA* gene region (72-193), while the right region (117-560) is nearly identical to the related sequence of *psbA* with only one different nucleotide. There seems to have been two major leaps during the evolutionary process of pRRD9 since the homogeneity comparison analysis extends to all 44 published *psbA* genes. Some factors might have triggered three dramatic mutations in the chloroplast gene at the nucleotide level in different eras.

Transfer of pRRD9 and its diverse amplification

The high homogeneity between the right region of pRRD9 and the related sequence of *psbA* suggests the formation of bipartite pRRD9 from two unrelated sequences. These may have been linked together by chance in the cloning process by the renaturation kinetics method. However, it leads us to believe that a homologous sequence of *psbA* may exist in the nuclear genome. So, we again performed Southern hybridization with nuclear DNAs from purified nuclei of several different species that probe either to pRRD9 or to the subclone of the right region. Contamination of plastid DNA was reduced to the lowest possible through selected lysis of plastid membranes by TritonX-100 with proper concentration and subsequent sucrose gradient centrifugation (Wang et al 1995). Indirect support was conferred by the molecular size of nuclear DNA released from agarose gel, which ranges from 200 to 2200 kb, generally larger than the size of plant chloroplast DNA.

As expected, there was no significant difference between hybridization patterns with total DNAs and nuclear DNAs. In hybridization with the subclone of the right region as a probe, ladderlike bands disappeared completely in rice, which could be characterized with pRRD9 as a probe. However, in *Cycas taiwaniana, Cyperus rotundus, Polygonum chinensis,* etc., the single hybridization bands were the same with probes prepared from either pRRD9 or subclone of the right region. In *Cordyline fruticosa, Gnetum parvifolium,* and *Ophiogon japonicus,* no hybridization signal was observed even under prolonged exposure. It should be noted that in *Polygonum chinensis,* the single hybridization band disappeared in hybridization with the subclone of the right region but existed in hybridization with pRRD9. Therefore, we conclude that the left region is relatively constant and exists only in the rice nuclei, and the right region originates from the chloroplast genome and had transferred into the nuclear genome during a previous era.

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Monitoring wild rice populations in permanent study sites in Thailand

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Since 1983, we have been monitoring seven natural populations of Asian common wild rice (*Oryza rufipogon*) in Thailand, including four annual and three perennial populations located in a suburb of Bangkok. The objective of this project is to obtain demographic-genetic data for understanding the population dynamics of this wild rice. We regularly visit the sites to make field observations and to take samples for genetic studies. By 1990, all annual populations in the study had become extinct. The perennial populations have survived a little longer, but have become nearly extinct. The decline of these populations was mainly due to catastrophic habitat destruction caused by development. The extinction rate has been higher in the annuals, which suggests that the annual population is maintained as a whole by repeated extinction/colonization events. Analyses of genetic diversity and the propagating system suggested that the perennial and annual populations have different genetic structures and survive under different dynamics. To cope with the threat of genetic erosion occurring in wild rice, the design and implementation of an in situ conservation project is an urgent matter.

Asian common wild rice (*Oryza rufipogon*) is widely distributed in monsoon Asia and is differentiated into the perennial and annual (often referred to as *O. nivara*) ecotypes. We are monitoring several wild rice populations at permanent study sites in Thailand. The original objective of this long-term project was to collect demographic-genetic data to gain a better understanding of population dynamics. But we soon witnessed a drastic change of natural environments and the subsequent extinction of these wild rice populations.

Materials and methods

In 1983, we selected eight study sites inhabited by *O. rufipogon* located in a Bangkok suburb, including some, which had been monitored since the 1970s. Among these, four annual and three perennial populations were visited regularly. As generally observed in this species, the annuals grow in shallow water and the perennials grow mainly in deep water. During every visit, population size, percentage of cover, plant height, water depth, major companion species, and other data were recorded. At the start of the rainy season, the number of seedlings and ratoons per unit area was counted to determine the relative proportion of sexual and asexual propagation. During the 1983-90 period, several 1- × 1-m quadrats were set up at each site and the number of wild rice plants was counted during every visit. Seed samples were taken regularly from the same populations. The first-generation plants derived from original seeds were grown in short-day plots at the National Institute of Genetics, Japan. Various characters and allozyme variations were examined.

Results

Population flux

At the beginning of this project, wild rice plants mostly covered all sites, except on one site where the annual population (NE2) was already declining. By 1990, all annual populations had become extinct at the original sites. During 1982-83 at the NE1 population site, annual plants invaded an abandoned ricefield and rapidly established a huge population (NE1-C), but it was destroyed by the construction of an army camp. Populations NE3 and NE4 disappeared due to a road-widening project. Population NE2 was the only one to become extinct due to natural causes. When found in 1973, this population fully occupied a roadside ditch, but it was replaced gradually by perennial competitors (*Mimosa* sp., *Aeschynomene* sp., etc.).

The perennial populations persisted longer and only recently did populations CP20 and NE88 become extinct due to the construction of a gasoline station and the destruction of a roadside ditch, respectively. CP24, a unique population which most probably absorbed genes of a different genome, is also declining probably due to environmental pollution.

Local extinction rates within a population

It is known that seed productivity and seed-dispersing ability are more advanced in annuals than in perennials (Oka and Morishima 1967). This was confirmed by checking whether young plants emerged from seedlings or ratoons (Table 1). We tried to estimate local extinction rates within a population. As shown in Table 1, the extinction rate, on a quadrat basis, was higher in the annuals than in the perennials. A weedy type, CP20-X growing in a deepwater ricefield, which potentially has a perennial habit, but actually propagates by both seeds and ratoons, showed a high local extinction rate. During the observation period, population size, as a whole, did not vary that much at each site. This implies that the annual and perennial populations are main-

Table 1. Propagating system and extinction rate within populations of wild rice estimated at permanent study sites.

Population	Quadrats studied (no.)	% of seedlings[a]	Ecotype	Local extinction rate, e_0[b]
NE3	6	100 ± 0	Annual	0.040
NE4	7	99.6 ± 0.5	Annual	0.055
CP20-X	3	72.0 ± 2.2	Weedy	0.078
CP20-Y	4	19.8 ± 8.7	Perennial	0.000
NE88	4	6.3 ± 8.2	Perennial	0.004

[a]Proportion of seedlings to total plant number (seedling + ratoon) observed in the early rainy season, averaged over 2-9 times. [b]Estimated from $X = (1 - e_0)^T$ where T = no. of years observed, X = proportion of extant quadrats after T years.

Table 2. Genetic parameter of the annual and perennial populations estimated based on allozyme data (Barbier 1989, Morishima and Barbier 1990).

Parameter	Annual				Perennial		
	NE1-C	NE2	NE3	NE4	CP18	CP20	NE88
Average gene diversity (H)	0.085	0.165	0.208	0.147	0.366	0.327	0.350
Observed inbreeding coefficient (F)	1.000	0.805	0.955	0.721	0.523	0.251	0.750
Outcrossing rate (t)		0.04-0.10				0.50-0.55	
Partitioning of gene Diversity by F-statistics	F_{IS}	0.868				0.400	
	F_{ST}	0.114				0.033	

tained by repeated local extinction/colonization events. In fact, we frequently observed a number of small patches of wild rice on the fringe of the original extinct populations. They were mostly annual types.

Genetic structure of natural populations

Table 2 shows various parameters obtained from allozyme variations. The annual populations had less gene diversity and an observed higher inbreeding coefficient than the perennials. The annuals proved to be predominantly self-pollinating and the perennials outbred, at least partially. Partitioning of gene diversity into inter- and intra-population components using F-statistics indicated that F_{IS} (correlation between uniting gametes within a population) and F_{ST} (a measure of population differentiation) were higher in the annuals than in the perennials. In the declining populations, gene diversity had clearly decreased. It is difficult, however, to conclude that a lack of genetic diversity caused the population extinctions. In NE2, average gene diversity H decreased from 0.24 to 0.02 during the 1974-80 period. Most probably, this is not the cause of the population's decline, but an effect.

Discussion

Perennial and annual types of *O. rufipogon* differ in both mating and propagating systems. Occupying different niches, they survive under different population dynamics. Annuals are adapted to disturbed habitats. Perennials seem vulnerable to disturbance, however, they do seem to contain enough genetic variability to derive new weedy genotypes. Catastrophic habitat destruction expels both annuals and perennials equally. This is what is happening in most wild rice-growing areas.

Because of the outbreeding nature of wild rice, natural hybridization between the two ecotypes, as well as between wild and cultivated rices. occurs frequently. Weedy derivatives of such introgression dominate after extinction of the "true" wild rices. The design and implementation of a feasible plan of in situ conservation of wild rices, as an alternative of ex situ conservation of germplasm, are urgently needed (Vaughan and Chang 1992). Judging from the results of our study. different strategies must be put forth for the conservation of natural populations of the perennial and annual ecotypes.

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Genetic dissection of crossability in rice

Y. Sano and S. Kobayashi

Unidirectional cross incompatibility was detected when a segment of chromosome 6 was introduced from a common wild rice into a japonica-type (T65*wx*) rice. When pollinated by T65*wx,* the plant carrying the segment produced aborted and ungerminable seeds showing reduced seed setting while the reciprocal cross between the same parents showed normal seed setting. Cytohistological observations revealed that retardation of the endosperm took place 4-5 d after fertilization and it seemed to cause defective seeds with disturbed tissue differentiation. Genetic experiments showed that a gene (*Lcr*) controlling incompatibility in the female parent was located near *Wx* (nonwaxy endosperm) on the segment and that a gene controlling compatibility in the male parent was also located near *S6* (gamete eliminator) on the segment. In addition, the segments from the indica type and *O. glaberrima* were compared. They carried no *Lcr* but genes controlling compatibility with the *Lcr* female. Furthermore, the japonica type tended to show reduced seed setting in crosses to the *Lcr* female while the indica type and common wild rice tended to show normal seed setting. Implications of the results on wide hybridization also were discussed.

Crossing barriers are often recognized in rice when distantly related taxa are intercrossed. A range of variation in the rate of success is also found even in crosses between forms sharing the same genepool (Chu et al 1969, Sitch 1990). Recently, Okuno and Nakayama (1993) reported that Japanese varieties Akihikari and Nipponbare showed reduced seed setting when crossed with IR varieties (IR28, IR36, and IR58) as material parents. The reciprocal crosses, however, showed normal seed setting, indicating unidirectional cross incompatibility. They suspected that the cross incompatibility might be the result of wide hybridization during breeding procedures. If so, this implies that using wild rice creates a new reproductive barrier among rice cultivars. We also found a similar type of cross incompatibility in hybrid derivatives

between a common wild rice (*Oryza rufipogon,* W593 from Malaysia) and a japonica-type rice (Sano 1992). We report here the genetic mechanisms and discuss its implications in breeding.

The segment responsible for cross incompatibility

Cross incompatibility was noticed while introducing a segment of chromosome 6 from W593 into T65 *wx* (a near-isogenic line [NIL] of Taichung 65 with *wx*) by successive backcrosses. The alien segment contained Wx^a and *S6* (gamete eliminator). The heterozygote of Wx^a *S6/wx* $S6^a$ was reciprocally backcrossed with T65*wx* (*wx* $S6^a$). When the heterozygote was pollinated by T65*wx*, the heterozygote showed reduced seed setting (15/186, 8.1%) while the reciprocal crosses showed higher seed setting (141/258, 54.7%). The factors causing the incompatibility reaction were not cytoplasmic since it transmitted through both micro- and megaspores.

To confirm the incompatibility reaction, four NILs carrying various lengths of the alien segment were obtained from BC_8F_4 and they were cross-pollinated (Table 1). The homozygote of Wx^a *S6/*wx^a *S6* showed unidirectional cross incompatibility with T65*wx*. The incompatible cross-pollination gave 153 aborted seeds in addition to 28 normal seeds (Fig. 1), suggesting abnormalities after fertilization.

Incompatibility reaction in the female and male parents

The incompatibility occurred only as T65*wx* pollinated lines carrying the segment from Wx^a to *Se1* as shown in Table 1, suggesting that different factors control the incompatibility reaction in the female and male parents. To analyze the gene(s) controlling the incompatibility reaction in the female parent, each F_2 plant of T65*wx Se1 S6*/T65 Wx^a *S6* was pollinated with T65*wx* and their seed settings were examined. In total, 53 F_2 plants were tested and they segregated into two classes with low or high seed setting (3:1 ratio). This indicates that the incompatibility reaction in the T65 Wx^a *S6* female is controlled by a single dominant gene. *Lcr,* which was linked to *wx* with a recombination value of 0.19 ± 0.06 (Sano 1992). Both alleles of *Lcr/*Lcr^+ transmitted

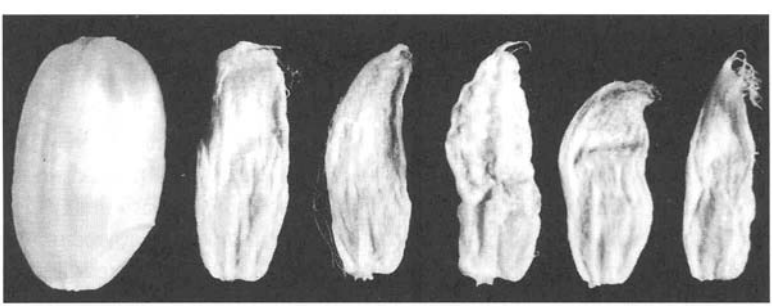

Fig. 1. Aborted and ungerminable seeds observed when plants carrying *Lcr* were pollinated with T65*wx*. Note the reduced seed setting.

Table 1. Percentages of seed sets in cross pollination among lines carrying different segments of chromosome 6 from W593 (Sano 1992).

Female parent	Male parent[a]				
	T65*wx*	T65*wx S6*	T65*wx Se1 S6*	T65*wx S6 En-Se1*	Tw65 *Wx*[a] *S6*
T65wx	73(268)	83(154)	59(111)	63(71)	82(107)
T65wx *S6*	54(191)	71(58)	79(47)	68(83)	71(52)
T65wx *Se1 S6*	61(57)	68(102)	71(52)	67(48)	67(63)
T65*wx En-Se1 S6*	11(89)	66(78)	72(66)	77(103)	73(88)
T65 *Wx*[a] *S6*	12(232)	76(96)	64(50)	76(49)	79(68)

[a]T65*wx Se1 S6*, T65*wx En-Se1 S6*, and T65 *Wx*[a] *S6* carry the segments of W593 from *S6* to *Se1*, *En-Se1* and *Wx*[a], respectively. T65*wx S6* carries only *S6* from W593. The gene order is *Wx*[a] - *En-Se1* - *Se1* - *S6*. Numbers of florets pollinated are shown in parentheses.

evenly through megaspores when pollinated with T65*wx*, suggesting that the genic effect of *Lcr* is sporophytic.

The unidirectional incompatibility was observed between a specific female and a specific male (Table 1). All the NILs with different segment lengths showed normal seed setting when pollinated by the *Lcr* female, showing the presence of a gene(s) controlling the compatibility reaction in the male parent. The results indicate that it may be located near *S6* and that W593 carries both genes controlling cross-incompatible female and cross-compatible male on chromosome 6.

Histological observations

Crossing barriers could be due to abnormalities in pollen-style interaction or due to abortion of embryo and/or endosperm at an early stage of seed development. Growth of pollen tubes was examined using a fluorescence microscope after staining with aniline blue (Sitch 1990). Pollen tubes of T65*wx* grew quickly on styles of *Lcr/Lcr* and reached around the micropyle for 4 h after pollination. To observe seed development, spikelets were fixed in FAA, paraffin-sectioned, and stained by safranin and fast green. Although fertilization took place in the incompatible cross, most of the F_1 endosperm began to deteriorate about 4-5 d after fertilization. However, the hybrid embryos continued to grow with disturbed tissue differentiation (Fig. 2), suggesting that embryo lethality may be caused by abortion of endosperm cells.

The genetic experiments revealed that the incompatibility reaction is accomplished by both factors from the female and the male parents. Regarding the possible mechanism, gene dosage effects in the triploid endosperm were ruled out because neither “escape” from the incompatibility system nor the reciprocal cross produced aborted seeds on selfing. The histological observations revealed that the incompatible reaction resulted from zygotic lethality. These questions were raised: why does *Lcr* have adverse effect when transmitted through the megaspores? Why is a factor(s) controlling compatibility in the male parent effective when transmitted through the

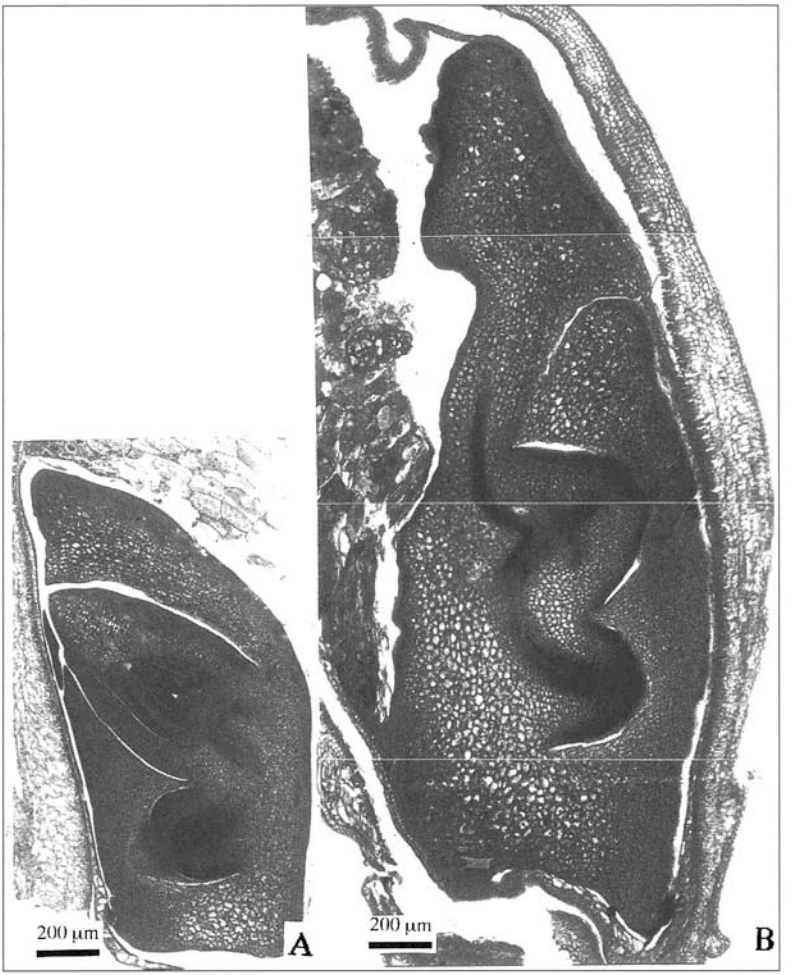

Fig. 2. Longitudinal sections of a normal embryo (a) and aborted embryo (b) with disturbed tissue differentiation due to the cross incompatibility reaction.

microspores? A possible explanation is that both genes modulate the cytoplasm of mega- and microspores. Another is genomic imprinting that the genic expression might be different at the early stage of development, depending on whether it transmitted through the maternal or the paternal parent.

Gene complex on chromosome 6 in the relatives

To compare the genetic divergence on the region. segments from the indica type (Patpak from India) and *O. glaberrima* (W025 from Guinea) were examined. The two strains seemed to carry no *Lcr* since plants with the segments showed cross compatibility

Table 2. The rate of success of crossing when cultivated and wild rice strains were pollinated by the *Lcr* female.

Cross		Strains tested (no.)	Rate of success % (range)	
Female[a]	Male			
T65	*Lcr/O. sativa japonica*	3	10.9	(5.1-15.4)
T65	*Lcr/O. sativa javanica*	2	15.8	(4.8-26.7)
T65	*Lcr/O. sativa indica*	4	62.1	(41.7-81.8)
T65	*Lcr/O. rufipogon*	2	59.8	(57.1-62.5)

[a]T65 *Lcr* is a NIL of Taichung 65 which carries a segment of chromosome 6 containing *Lcr* from *O. rufipogon* (W593 from Malaysia). Mean number of pollinated flowers per cross was 40.7.

with T65*wx*. Lines with varying segment lengths were established as in the segment from W593, and each of the lines was pollinated by the *Lcr* female. The results showed that in both cases, a gene controlling compatibility with *Lcr* was located near *Se1* as detected in W593.

To examine compatibility with the *Lcr* female in wild and cultivated rice strains, 11 strains were pollinated by the *Lcr* female (Table 2). Japonica and javanica types tended to give reduced seed setting compared with indica and the wild ancestor, suggesting that the latter forms might carry a gene(s) controlling the compatibility reaction in the male parent. *Lcr* has no adverse effect in the indica type and its presence in the indica type may not be recognized until it is pollinated by a japonica or a javanica (Okuno and Nakayama 1993).

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Genetic analysis of morphological mutations in rice spikelets

I. Takamure and T. Kinoshita

Five rice mutants were investigated for mode of inheritance and character expression of either grain size or malformed spikelets. N-179 and N-182 are spontaneous, long-grained mutants from breeding line Nagayama 77402 developed at the Kamikawa Agricultural Experiment Station, Japan. The long-grain character of N-179 is governed by a single recessive gene, *lk-na(t),* which is nonallelic to *LK-f* and *lk-i,* respectively. The mutant character of N-182 is controlled by an incomplete dominant gene, *Lk-nb(t),* which is nonallelic to *Lk-f,* and linked to sp located on chromosome 11 with a 19% recombination value. AT-130 is a small-grained mutant found among the progeny of double haploids after chronic gamma-ray irradiation of anther calli from cultivar Kitaake. A single recessive gene, *mi-k,* is responsible for the small-grained character of AT-130. Mutant-1 showed various modifications of lemma and palea—even within a panicle. This character is caused by a single recessive gene, *mls3,* located on chromosome 4, and character expressions are affected by different temperature conditions. N-76 has deformed lemma and palea, controlled by a single recessive gene, *lhs,* located on chromosome 3. Based on comparative observations of floral anatomy and peroxidase reaction, *lhs* might be related to the development of floral organs.

Many morphological mutations in rice spikelets center on variation in grain size and malformation of the floral organs (Kinoshita 1989). Grain size is a very important trait in rice breeding. Several major genes for large and small grains have been reported: *Lk-f (Takeda* and Saito 1980), *lk-i* (Takamure 1994), and *mi* (Takeda and Saito 1977). Although most malformed mutants have no use in actual breeding, they can be useful in studying morphogenesis of the floral organs.

Grain size

Two long-grained mutants, N-179 and N-182, were derived from breeding line Nagayama 77402 developed at the Kamikawa Agricultural Experiment Station. N-179 is characterized by low seed setting and reduced spikelet number. In the F_2 and F_3 populations from the cross N-179/A-58 (normal grain), the segregations for grain type satisfied the theoretical ratio caused by a single recessive gene designated as *lk-na(t).* Further, *lk-na(t)* appeared to be nonallelic to *Lk-f* and *lk-i,* respectively.

N-182 is characterized by reduced panicle and spikelet number. In a cross with ws-5 (normal grain), the F_2 and F_3 populations followed the inheritance mode expected from an incomplete dominant gene, *Lk-nb(t).* A nonallelic relation between *Lk-nb(t)* and *Lk-f* was also observed. *Lk-nb*(t) appears to be linked with *sp* on chromosome 11, with a 19% recombination value.

AT-130, a small-grained mutant, was found among the progeny of double haploids after chronic gamma-ray irradiation of anther calli from cultivar Kitaake. AT-130 is characterized by small grains, late heading, and low seed set. The F_2 and F_3 populations from the cross AT-130/Kitaake indicated that a single recessive gene designated as *mi-k* is responsible for the segregation of grain types, *mi-k* appears to be nonallelic to *Mi.*

Independent assortment and the additive gene effect of the combination of *lk-na(t)* and *mi-k* contributed to grain size. Combination of such genes is expected to increase the variability of this important breeding trait.

Malformed spikelets

A mutant line, Mutant-1, showed various degrees of malformation in the lemma and palea—even within a panicle. This character is caused by a single recessive gene designated as *mls3,* located on chromosome 4. Additional floral abnormalities can be caused by environmental conditions. For example, when Mutant-1 was grown in temperature-controlled growth chambers at either 20 or 28 °C during the growing season, the percentage of malformed spikelets prominently increased at the lower temperature. Seed fertility is also affected remarkably by the low temperature.

The mutant line N-76 has deformed lemma and palea, controlled by a single recessive gene, *lhs,* located on chromosome 3 (Kinoshita et a1 1977). When transverse sections of the leaf blade, leaf sheath, lemma, and palea of N-76 and the original strain, Sorachi, were observed, the lemma and palea of N-76 closely resembled the structure of the leaf sheath and consisted of many vascular bundles and lysigenous aerenchyma (Fig. 1). The expression of peroxidase is organ-specific among leaf blades, leaf sheaths, and glumes. In this experiment, both the glume and leaf sheath of N-76 showed the same zymogram. These results illustrate the possibility that *lhs* is responsible for the modification of the glume into the leaf sheath. According to the floral morphogenesis of higher plants, the glume (hull) and the leaf sheath belong to a homeologous organ. This mutant character might clarify the development of floral organs in rice.

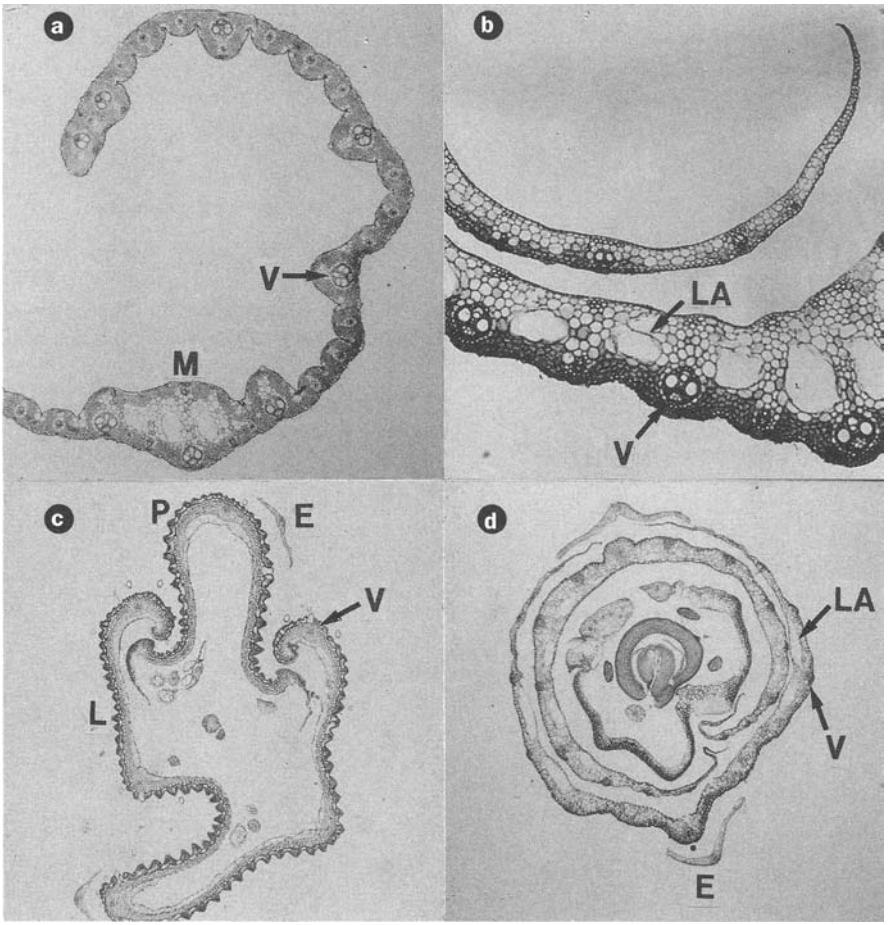

Fig. 1. Transverse sections of leaf blade, leaf sheath, lemma and palea of N-76 and original line Sorachi. a) leaf blade of N-76 (x40), b) leaf sheath of N-76 (x100), c) lemma and palea of Sorachi (x40), d) lemma and palea of N-76 (x40). V = vascular bundle, M = midrib, LA = lysigenous aerenchyma, E = empty glume, L = lemma, P = palea.

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Allelic variation at the gametophyte gene (*ga2*) locus in rice (*Oryza sativa* L.)

Sobrizal, A. Yoshimura, and N. Iwata

An allelic test of $ga2^+$ alleles that originated from five indica varieties was conducted by crossing five kinds of near-isogenic lines (NILs) having $ga2^+dl/ga2^+d/genotype$ with five kinds of NILs having $ga2^+dl^+/ga2^+dl^+$ genotype. The segregation of *dl* plants was observed in F_2 and BC_1F_1 populations. Segregation distortions were found in cross combinations between NILs possessing $ga2^+$ alleles either from HO 639 or TKM6 and from HO 1392, whereas the other cross combinations showed normal segregation. These results suggest that multiple alleles exist at the *ga2* locus. In this study, four kinds of alleles were identified at this locus. Moreover, observation of pollen development and segregation distortion in the progeny of crosses between NILs carrying $ga2^+$ of HO 639 and that of HO 1392 and between NILs carrying $ga2^+$ of HO 639 and that of HO 1097 suggests that the segregation distortions in this study were caused by the differential fertilization of male gametes.

The appearance of segregation distortions in wide crosses of rice has been frequently reported. Segregation distortion of drooping leaf (*dl*)—in which the frequency of *dl* plants was significantly lower than the Mendelian expected frequency—also was found in the progeny of crosses between japonica *dl* marker line and many indica varieties. Nakagahra (1972) reported that the *dl* gene was located on chromosome 3 and linked with the *ga2* gene. Segregation distortion of dl plants was assumed because of the differential fertilization between *ga2 dl* and $ga2^+dl^+$ male gametes.

Sobrizal (1995) reported that the chromosome segments carrying $ga2^+$ genes have been introgressed from five indica varieties into a japonica background, resulting in five kinds of near-isogenic lines (NILs). Furthermore, using these NILs, the $ga2^+$ genes from five indica varieties have been localized at the same locus. Since the *dl* gene has also been incorporated into the chromosome of these NILs, it would be possible to observe the effect of combining two kinds of indica alleles at the *ga2* locus

and to study the allelic relationship among the *ga2*$^{+}$ genes from various indica varieties. In addition, the mechanism of segregation distortion at the *ga2* locus was studied by observing pollen development and segregation distortion among NILs in the hybrids.

Materials and methods

To investigate the allelic relationship among the *ga2*$^{+}$ alleles of five indica varieties, five kinds of NILs having *dl* phenotype—NIL 639(*dl*), NIL 1091(*dl*), NIL 1097(*dl*), NIL 1392(*dl*), and NILTKM6 (*dl*)—were crossed with five kinds of NILs having *dl*$^{+}$ phenotype—NIL 639(*dl*$^{+}$), NIL 1091(*dl*$^{+}$), NIL 1097(*dl*$^{+}$), NIL 1392(*dl*$^{+}$), and NILTKM6(*dl*$^{+}$)—and the segregation of *dl* was examined in their F_2 populations. Reciprocal backcrosses between the F_1 plants and NIL(*dl*) plants were also performed and the segregation of *dl* was observed in the BC_1F_1 populations.

To study the mechanism of segregation distortion at the *ga2* locus, seed and pollen fertilities of F_1 hybrids of NIL 639(*dl*)/NIL 1392(*dl*$^{+}$), NIL 1392(*dl*)/NIL 639(*dl*$^{+}$), and NIL 639(*dl*)/NIL 1097(*dl*$^{+}$) were observed as described by Sobrizal (1995) and pollen germination was observed as described by Sitch (1990) with slight modification.

Results and discussion

The segregation of *dl* plants in the F_2 populations of 14 cross combinations between NIL(*dl*) and NIL(*dl*$^{+}$) is shown in Table 1. In the F_2 populations from NIL 639(*dl*)/NIL 1392(*dl*$^{+}$), the frequencies of *dl* plants were significantly higher than the normal frequency of 25%, but in the F_2 population from NIL 1392(*dl*)/NIL 639(*dl*$^{+}$), the frequencies of *dl* plants were significantly lower than normal. The same phenomenon was also found in the F_2populations from NILTKM6(*dl*)/NIL 1392(*dl*$^{+}$) and NIL 1392(*dl*)/NILTKM6(*dl*$^{+}$). On the other hand, in the F_2 populations from NIL 639(*dl*)/NILTKM6(*dl*$^{+}$) and NILTKM6(*dl*)/NIL 639(*dl*$^{+}$), the frequency of *dl* plants fitted well to 25%. Furthermore, when the NIL 1091(*dl*), NIL 1091(*dl*$^{+}$), NIL 1097(*dl*), and NIL 1097(*dl*$^{+}$) were used as the parental strains, normal segregation was found in the F_2 populations.

In BC_1F_1 populations, high-segregating type (where the frequency of *dl* plants was significantly higher than the normal frequency of 50%) was observed in backcross populations of NIL 639(*dl*)//NIL 639(*dl*)/NIL 1392(*dl*$^{+}$) and NILTKM6(*dl*)//NILTKM6(*dl*)/NIL 1392(*dl*$^{+}$). On the other hand, low-segregating type (where the frequency of *dl* plants was significantly lower than the normal frequency of 50%) was found in the backcross population of NIL 1392(*dl*)//NIL 1392(*dl*)/NIL 639(*dl*$^{+}$) (data not shown).

These results suggest that even though the *ga2*$^{+}$ from five indica varieties were located at the same locus, the alleles differed depending on the indica varieties. At least four kinds of alleles were present at this locus, including the *ga2* allele of japonica varieties that were used in this study. The *ga2*$^{+}$ alleles from HO 639 and TKM6 are designated as *ga2*$^{+A}$; the *ga2*$^{+}$ allele from HO 1392, *ga2*$^{+B}$; and the *ga2*$^{+}$ alleles from HO 1091 and HO 1097, *ga2*$^{+C}$. The allelic interactions of these alleles are shown in

Table 1. Segregation of *dl* plants in F_2 populations of crosses among NILs.

Cross combination	No. of crosses	Segregation			dl(%)	c^2 for 3:1
		dl^+	*dl*	Total		
NIL 639 *(dl)*/NIL 1091*(dl+)*	2	409	137	546	25.09	0.00
NIL 639 *(dl)*/NIL 1097*(dl+)*	4	766	266	1032	25.78	0.33
NIL 639 *(dl)*/NIL 1392 *(dl+)*	4	418	335	753	44.49	152.53***
NIL 639 *(dl)*/NILTKM6*(dl+)*	2	359	120	479	25.21	0.00
NIL 1091 *(dl)*/Nll 1392*(dl+)*	3	656	229	885	25.88	0.35
NIL 1097 *(dl)*/NIL 639*(dl+)*	10	1565	493	2058	23.96	1.20
NIL 1097 *(dl)*/NIL 1392*(dl+)*	3	400	136	536	25.37	0.04
NIL 1097 *(dl)*/NILTKM6*(dl+)*	3	553	168	721	23.30	1.11
NIL 1392 *(dl)*/NIL 639*(dl+)*	6	1213	141	1354	10.41	153.64***
NIL 1392 *(dl)*/NIL 1097*(dl+)*	4	646	184	830	22.17	3.55
NIL1392 *(dl)*/NILTKM6*(dl+)*	2	376	30	406	7.39	67.16***
NILTKM6 *(dl)*/NIL 639*(dl+)*	5	816	278	1094	25.41	0.10
NILTKM6 *(dl)*/NIL 1097*(dl+)*	2	301	99	400	24.75	0.01
NILTKM6 *(dl)*/NIL 1392*(dl+)*	2	240	184	424	43.40	76.53***

*** = significant at 0.1% level.

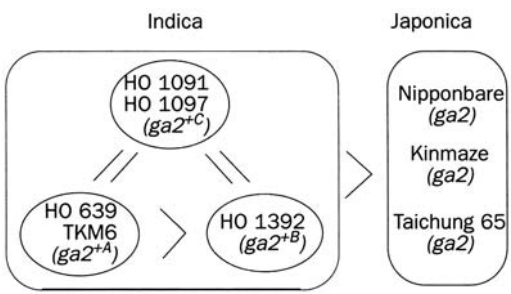

Fig. 1. Allelic interactions between indica-japonica and indica-indica alleles at the *ga2* locus. = no interaction for segregation distortion, > interaction for segregation distortion in which the allele on the left is stronger than the one on the right.

Figure 1. These alleles have been introgressed into five NILs of the japonica background and *dl* marker gene. These NILs would be excellent materials for the study of the evolution and differentiation of rice varieties.

Microscopic observations of pollen fertilities and pollen germinations were conducted in F_1 hybrids of crosses NIL 639(*dl*)/NIL 1392(dl^+), NIL 1392(*dl*)/NIL 639(dl^+), and NIL 639(*dl*)/ NIL 1097(dl^+) (Fig. 2). The pollen in the F_1 of the three crosses stained well, indicating that it was normal. Most of this pollen also germinated well. No difference in both pollen fertility and pollen germination among the three crosses was observed.

Data on pollen fertility and pollen germination of the F_1 of the three crosses are summarized in Table 2, including data on seed fertility and segregation distortion in

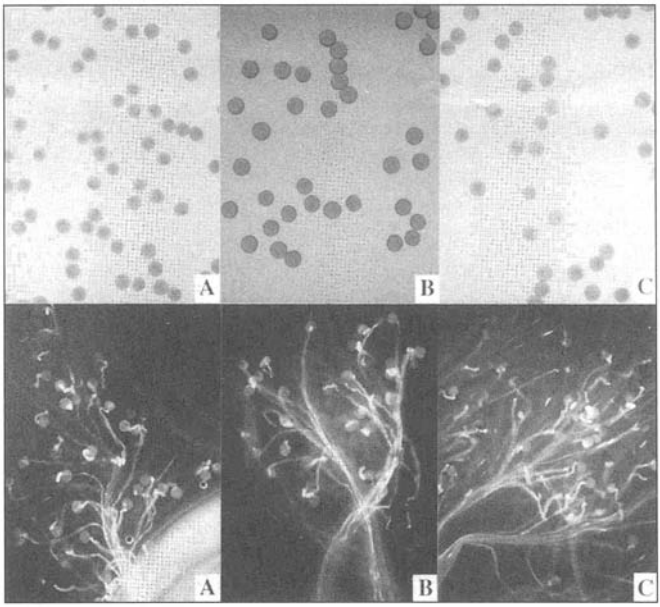

Fig. 2. Pollen fertility (top) and pollen germination (bottom) of F_1 hybrids. a) NIL 639(*dl*)/NIL 1392(dl^+), b) NIL 1392(*dl*)/ NIL 639 (dl^+), and c) NIL 639(*dl*)/NIL 1097 (dl^+).

Table 2. Seed and pollen fertilities, pollen germinability of F_1 plants of crosses NIL 639(*dl*)/NIL 1392(dl^+), NIL 1392(*dl*)/NIL 639)(dl^+), NIL 639(*dl*)/NIL 1097(dl^+), and segregation of *dl* plants in their F_2 populations.

Cross combination	F_1 fertility		Pollen germinability (%)	Segregation in F_2			*dl* (%)	c^2 for 3:1
	Seed (%)	Pollen (%)		dl^+	*dl*	Total		
NIL 639(*dl*)/NIL 1392(dl^+)	71.45	98.90	97.88	136	115	251	45.82	58.01***
NIL 1392(*dl*)/NIL 639(dl^+)	82.63	99.52	98.52	194	25	219	11.42	21.55***
NIL 639(*dl*)/NIL 1097(dl^+)	75.17	98.03	97.27	145	50	195	25.64	0.04

*** = significant at 0.1% level.

the F_2 populations. Even though pollen fertility and pollen germinability of these hybrids were normal, segregation distortion was found in the F_2 populations of crosses NIL 639(*dl*)/NIL 1392(dl^+) and NIL 1392(*dl*)/NIL 639(dl^+). On the other hand, the F_2 population of the cross NIL 639(*dl*)/NIL 1097(dl^+) showed normal segregation.

These results indicate that segregation distortion in this study was caused by neither the male gamete abortion gene as observed in indica-japonica hybrids (Lin and Ikehashi 1993, Lin et al 1992) nor by a pollen-killing gene as described in wheat (Loegering and Sears 1963) and *O. sativa* L. and *O. glaberrima* Steud hybrid (Sano 1983), nor by a gamete eliminator gene as reported in tomato (Rick 1966). The

segregation distortions in this experiment might be caused by the differential fertilization of male gametes in heterozygous plants as discussed by Nakagahra (1972).

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Notes

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The gametic lethal gene *gal:* activated only in the presence of the semidwarfing gene *d60* in rice

M. Tomita

The semidwarfism of rice mutant Hokuriku 100 is controlled by the single semidwarfing gene, *d60.* However, *d60* does have the pleiotropic effect of activating the gametic lethal gene, *gal.* The F_2 progeny between Hokuriku 100 (genotype *d60d60GalGal, Gal* is the gametic nonlethal allele) and original tall variety Koshihikari (*D60D60galgal, D60* is the tall allele) distortedly segregate into a ratio of 1 semidwarf (1 *d60d60GalGal*):2 tall and quarter sterile (2 D60d60Galgal):6 tall (2 D60d60GalGal:1 *D60D60GalGal:* 2 D60D60Galgal:1 *D60D60galgal)* because of the deterioration of the F_1 male and female gametes having both *gal* and *d60.* If *Gal* had not simultaneously mutated with *d60, d60* never could have been transmitted to express the semidwarfism of Hokuriku 100. To determine the presence or absence of *gal* and *d60* in other varieties, 30 semidwarf and dwarf varieties were crossed with three tester lines, Hokuriku 100, Koshihikari, and the D-60Gal line. All F_1 lines obtained from crossingwith Hokuriku 100 showed tallness and partial spikelet infertility, the latter increasing on the average by 27.2% from those obtained with Koshihikari and the D60Gal line. It must be emphasized that *gal* is a rare and valuable mutant gene essential in the transmission of *d60.*

Gametic abortions in rice plants, to date, have all associated with the “reproductive barrier” between distantly related cultivars. The semisterility that occurs in intersubspecific hybrids between indica and japonica varietal groups is caused by the duplication of the recessive gametophytic alleles, *s1* and *s2,* on different loci (Oka 1953,1957,1974) or the sporophytic gene reaction of heterozygous *S/Sa* to eliminate the gametes possessing the opposite allele Sa, namely the one-locus sporo-gametophytic (allelic) interaction (Kitamura 1962, Oka 1964, Ikehashi and Araki 1986). Gamete eliminator loci causing the sporo-gametophytic interaction are also found in the species hybrids of *Oryza sativa/ O. glaberrirna* and *O.sativa/O.rufipogon* (Sano

1983, 1990). Gametic abortions in remote crosses are attributed to the differentiation of the disordering allele among the macroscale gene pool.

The gametic lethal gene *gal* and its activator *d60* have been discovered not from the "reproductive barrier" but from the cross between semidwarf mutant Hokuriku 100 and its original tall japonica variety Koshihikari by the F_1 quarter sterility and a skewed segregation of semidwarfism in the F_2 (Tomita 1993). The induced semidwarfing gene *d60* of Hokuriku 100, which confers good erect stature about 15 cm shorter than Koshihikari, has great potential to become an alternative to the dominantly used semidwarfing gene *sd1*. However, *d60* pleiotropically causes the abortion of both sex gametes together with *gal* in the process of heredity. Therefore, the presence or absence of *gal* in cross parents has produced an intense interest in semidwarf breeding programs using *d60*.

Discovery of *gal*

Hokuriku 100 is the most promising semidwarf mutant selected from Koshihikari irradiated with ^{60}Co in a large-scale mutation breeding program to enhance the lodging resistance of Japanese variety Koshihikari (Samoto and Kanai 1975). Genetic information on the semidwarfism of Hokuriku was presented at the conferences of the Japanese Society of Breeding (Tomita et al 1986a,b; 1987a,b; 1988a,b; 1989).

The F_1 plants of Koshihikari (tall)/Hokuriku 100 (semidwarf) grew into tall phenotypes like Koshihikari, but they have, on average, 28.4% unfilled spikelets. The F_2 progeny showed a bimodal curve with regard to culm length distribution and they were clearly classified into 26 semidwarf plants and 241 tall plants. However, this segregation ratio significantly deviated from the 1 semidwarf:3 tall ratio expected from a single recessive gene segregation. In addition to this skewed segregation, 241 tall F_2 plants included 68 partially spikelet-sterile plants similar to those of the F_1 plants.

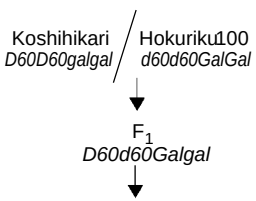
Pollen fertility was examined by the aceto carmine squash method using a few panicles sampled before anthesis from 10 F_1 plants and 40 randomly chosen F_2 plants. Small and empty pollen grains (averaging 24.5%) were observed as well as stainable mature pollen grains in all F_1 plants and eight F_2 plants with partial seed setting at maturity. Such a large amount of degraded pollen grains was not observed in the other 32 F_2 plants with nearly complete seed set. Because 75.5% of normal pollen grains of all F_1 plants and eight F_2 plants can fertilize well, their amount of unfilled spikelets (28.4%) must be caused by the infertility of the embryo sac. Consequently, a quarter of both sex gametes was found to abort in the F_1 plants and some of the tall F_2 plants of Koshihikari/Hokuriku 100.

As stated above, the F_2 population showed three phenotypes: tall and fertile like Koshihikari, tall and quarter-sterile like F_1, and semidwarf and fertile like Hokuriku 100. Since quarter sterility was thought to be a key to revealing the skewed segregation of the semidwarfness of Hokuriku 100, the author focused on the segregation of quarter sterility; 100 F_2 plants were randomly selected and progeny-tested in the F_3 (30 plants $line^{-1}$).

As a result, four phenotypic classes were observed in the F_3. Eleven F_3 lines derived from semidwarf F_2 plants were uniform for semidwarfness and normal fertility. Twenty-four F_3 lines derived from quarter-sterile F_2 plants segregated into tall plants, tall and quarter-sterile plants, and semidwarf plants as in the F_2. Sixty-five F_3 lines derived from tall and fertile F_2 plants were classified as 1) uniform for tallness and normal fertility (46 lines) or 2) segregated into semidwarf and tall plants in accordance with a 1:3 Mendelian ratio (19 lines). These data suggest that the semidwarfness of Hokuriku 100 is contolled by a single recessive gene and that the quarter sterility of tall parents results in the skewed segregation of semidwarfness of less than 25% in the next generation.

The semidwarfing allele and its opposite tall allele are designated as *d60* and *D60,* respectively. The presence of the gametic lethal gene *gal* in Koshihikari, which is activated by *d60,* and the induced opposite allele *Gal* in Hokuriku 100, which does not affect gametes (being a gametic nonlethal allele), was hypothesized. As Figure 1 indicates, such a hypothesis makes it possible for F_2 progeny of Koshihikari (*D60D60galgal*)/Hokuriku 100 (*d60d60GalGal*) to segregate into a ratio of 1 semidwarf (1 *d60d60GalGal*):2 tall and quarter-sterile (2 *D60d60Galgal*):6 tall (2 *D60d60GalGal:*1 *D60D60GalGal:*2 *D60D60Galgal:*1 *D60D60galgal)* because of the deterioration of the male and female gametes having both *gal* and *d60.* The observed segregation ratio of 11:24:19:46 on the F_3 classification showed a good fit to the theoretical ratio of 1:2:2:4 based on the above hypothesis (χ^2 =0.49, 0.75<*P*<0.90).

Thus, it has been proven that the semidwarfism of rice mutant Hokuriku 100 is controlled by a single recessive semidwarfing gene *d60* that activates the gametic lethal gene *gal* in the gamete having the two genes. Both *d60* and *gal* have been registered with the Committee on Rice Gene Symbolization. If *Gal* had not



		F_1 male gamete			
		D60gal	*D60Gal*	*d60gal*	*d60Gal*
F_1 female gamete	*D60 gal*	*D60D60galgal*	*D60D60Galgal*	No	*D60d60Galgal*
	D60Gal	*D60D60Galgal*	*D60D60GalGal*	No	*D60d60GalGal*
	d60gal	No	No	No	No
	d60Gal	*D60d60Galgal*	*D60d60GalGal*	No	*d60d60GalGal*

Fig. 1. Gametic lethal gene *gal* and its activator *d60* give a F_2 ratio of 1 semidwarf: 2 tall and quarter sterile: 6 tall in Koshihikari/Hokuriku 100. No = no zygotes are produced because of the deterioration of both sex gametes having *d60* and *gal.*

simultaneously mutated with *d60* in an M_1 primordium, *d60* could never have been transmitted to the M_2 plant that expressed the semidwarfism of Hokuriku 100—i.e., *Gal* is essential to the transmission of *d60.*

Universal distribution of *gal* in rice

Although the induced semidwarfing gene *d60* of Hokuriku 100 has great potential to become an alternative to the dominantly used semidwarfing gene *sd1, d60* pleiotropically causes the abortion of both sex gametes together with gal in the process of heredity. Therefore, to promote semidwarf breeding programs using d60, we need to know the presence or absence of *gal* in promising parents.

Thirty varieties bred in Japan, Korea, China, and USA were selected on the basis of historical and genotypical diversity for semidwarfness or dwarfness (Table 1). Tongil and Milyang 23 are derived from indica/japonica crosses. The other 28 varieties are japonicas. To determine the presence or absence of *gal* and *d60* in these 30 varieties, they were crossed with three tester lines, Hokuriku 100 *(d60d60GalGal),* Koshihikari *(D60D60galgal),* and D60Gal *(D60D60GalGal),* respectively. The D60Gal line was fixed in the F_4 of Koshihikari/Hokuriku 100. The D60Gal line/Hokuriku 100 gave an F_2 ratio of 1 semidwarf(1 *d60d60GalGal*):3 tall (2 *D60d60GalGal:* 1 *D60D-60GalGal*).

The analytical concept is as follows:

1) Assuming that a variety has *gal,* the F_1 line crossed with Hokuriku 100 will increase infertility, on average, by a quarter over that of the F_1 line crossed with the D-60Gal line.
2) Assuming that a variety has a semidwarfing gene that has the pleiotropic effect of activating *gal,* the F_1 line crossed with Koshihikari, will increase infertility, on average, by a quarter over that of the F_1 line crossed with D-60Gal.
3) Assuming that a variety has a semidwarfing gene allelic to *d60,* the F_1 line crossed with Hokuriku 100 will be significantly semidwarfed relative to the F_1 lines crossed with Koshihikari and D-60Gal.

The 5-10 F_1 plants and 30 parents of each cross were spaced at 10 × 30 cm and fertilized with 60 kg N ha^{-1}. Culm length and percentage spikelet fertility of each plant were recorded. Three panicles were harvested from each plant, and the numbers of filled and unfilled spikelets were counted for each panicle. Percentage spikelet fertility was calculated as the number of filled spikelets divided by the total number of spikelets multiplied by 100.

Table 1 shows the culm length and percentage spikelet fertility of each test-crossed F_1 line. When the 28 japonica varieties were crossed with Koshihikari and D-60Ga1, each F_1 showed nearly complete seed setting of more than 90%. On the other hand, when they were crossed with Hokuriku 100, each F_1 averaged 27.2% sterility (quarter sterility). These data indicate that gal does not act together with *sd1, dl, d2,* d6, *dl8k, d29, d30, d35(t), d49(t), d50(t),* and unknown genes involved in the 28 japonica varieties, and that the 28 japonica varieties all possess *gal* but not *d60.*

With respect to indica/japonica varieties, Milyang 23 and Tongil both have *sd1,* and the F_1 lines crossed with D-60Gal averaged 74.1 and 42.5% infertility, respectively.

Table 1. Percentage spikelet fertility (F) and culm length (L) of the F_1 lines each obtained by crosses between a variety and three testers, Hokuriku 100, Koshihikari, and the D60Gal line.

Variety	Dwarfing gene	a Hokuriku 100 d60Gal		b Koshihikari D60gal		c D-60Gal line D60Gal		t value[a] between a and c		t value[a] between b and c
		F	L	F	L	F	L	F	L	F
Jukkoku	*sd1*	73.6	85.1	97.3	89.3	96.8	88.0	27.80**	1.11	0.21
Shiranui	*sd1*	72.7	82.7	94.4	86.5	94.5	85.7	32.55**	1.55	0.67
M101	*sd1*	73.1	72.7	92.9	78.2	94.7	79.4	24.78**	2.97	0.42
Tongil	*sd1*	30.0	93.3	42.7	102.3	42.5	102.8	30.55**	2.26	0.88
Milyang 23	*sd1*	56.2	112.6	73.1	116.3	74.1	112.3	28.61**	0.18	0.14
Taichung 65 d-47	*sd1*	74.3	81.3	95.9	81.8	95.4	86.5	25.56**	1.98	0.24
Taichung 65		71.7	95.3							
Kinuhikari	*sd1*	71.4	84.3	96.5	88.6	95.5	88.2	24.65**	1.68	0.12
Reimei	*d49(t)*	73.0	72.0	94.6	78.5	93.3	78.0	26.88**	3.46	0.61
Fukei 71	*d50(t)*	74.5	83.0	94.3	90.3	93.3	87.5	23.55**	1.67	0.13
Shirosenbon		73.2	86.1	95.8	88.3	94.7	89.0	26.77**	1.89	0.77
Kinmaze		74.2	80.0	95.4	88.7	94.7	85.8	27.55**	2.09	0.33
Nipponbare		71.4	76.8	93.1	80.0	92.9	79.8	29.33**	3.22*	0.15
Nihonmasari		71.7	77.7	94.8	83.8	94.0	85.0	27.56**	3.43*	0.16
Daikoku	*d1*	74.1	100.0	93.3	105.0	94.9	105.8	30.13**	3.21*	0.15
Ebisu	*d2*	70.8	93.3	94.0	99.5	94.6	98.7	28.76**	3.16	0.78
Ebisumochi	*d6*	73.5	92.1	96.8	93.7	96.3	94.1	30.90**	0.77	0.12
Kotaketama-nishiki	*d18k*	72.5	100.8	96.8	106.2	96.9	108.5	28.26**	2.97	0.14
Dwarf Kyushu 1	*d29*	74.7	96.8	98.9	97.8	98.2	98.0	26.45**	0.29	0.26
Waiseishirasasa	*d30*	73.7	81.0	95.8	84.5	95.2	83.8	27.86**	1.12	0.36
Tanginbozu	*d35(t)*	73.0	88.5	96.1	95.3	96.0	97.0	29.36**	3.25*	0.19
Ginbozu		73.4	81.7							
HS90	*sd1*	73.9	74.5	94.0	76.3	92.6	75.9	25.89**	0.99	0.16
IM96		72.5	92.5	95.6	95.0	94.9	91.5	26.90**	0.17	0.14
IM181		71.7	80.0	94.9	84.7	95.2	84.4	25.60**	1.88	0.20
1M265		73.4	81.7	95.6	81.8	94.1	83.0	29.56**	1.99	0.17
EG1		71.0	90.3	94.6	86.6	96.2	85.5	28.75**	1.11	0.23
Norin 1		70.2	69.0							
Norin 22		72.0	75.5	95.3	78.3	95.1	77.6	29.94**	1.46	0.23
Koshihikari	*D60*	72.4	74.5	96.3	76.5	96.0	76.0	25.98**	0.88	0.17
Hokuriku 100	*d60*	95.2	61.5	71.7	75.3	95.8	76.2	0.13	13.56**	25.45**
Kanto 79	*D60*	71.9	75.3	98.5	76.8	96.7	76.5	28.94**	0.75	0.35

[a] * and ** = significant at the 5% and 1% level, respectively.

These F_1 infertility values are attributed to intersubspecific gamete elimination because D-60Gal does not carry both *d60* and *gal.* The F_1 lines crossed with Koshihikari also showed infertility equal to that of the F_1 lines crossed with D-60Ga1, indicating that Milyang 23 and Tongil do not have *d60.* On the other hand, the F_1 lines of Hokuriku 100/Milyang 23 averaged 56.2% infertility, and Hokuriku 100/Tongil, 30.0%, indicating that 24.2 and 29.4% of the spikelets became infertile besides the intersubspecific gamete elimination. The quarter sterility by *gal* and *d60* additively occurred in the F_1 crossed with Hokuriku 100, where *gal* must be carried by Milyang 23 and Tongil, respectively. It follows from the data so far presented that all 30 varieties possess *gal* but not *d60.*

Other than increasing infertility by a quarter, all F_1 lines crossed with Hokuriku 100 were morphologically as tall as each F_1 line crossed with Koshihikari and the D-60Gal line (Table 1). This means that the *d60*-homozygous F_1 line did not appear. These data indicate that the *d60* locus is not the allelic to those of *sd1, dl, d2, d6, dl8k, d29, d30, d35(t), d49(t), d50(t),* and unknown genes involved in the 30 varieties. Double dwarf lines possessing both *sd1* and *d60* were successfully obtained in the F_2 with Jukkoku, having *d47* identical with *sd1* (Kikuchi et al 1985), and in the F_2 with Kinuhikari, having *sd1* derived from IR8.

As previously mentioned, the semisterility that occurs in intersubspecific indica/japonica hybrids is explained by either one of two genetic mechanisms—the duplicate gametophytic lethal interaction (Oka 1953, 1957, 1974) or the one-locus sporo-gametophytic (allelic) interaction (Kitamura 1962. Oka 1964, Ikehashi and Araki 1986). Recently, several loci for hybrid sterility were reasonably identified based on the second mechanism. These are *S5* (Ikehashi and Araki 1986, Yanagihara et al 1995), *S7* (Yanagihara et al 1992), and *S8* (Wan et al 1993). Both *gal* and *d60* are clearly different from the genes responsible for intersubspecific hybrid sterility on the grounds that their effect of quarter sterility was added to hybrid sterility.

Conclusions

The genotype of the 30 varieties is *D60D60galgal,* which is identical with that of Koshihikari. The *d60* locus is not allelic to those of *sd1, dl, d2, d6, dl8k, d29, d30, d35(t), d49(t),* and *d50(t)* involved in the 30 varieties. *gal* is not activated together with the previously described semidwarf and dwarf genes other than *d60.* Both *gal* and *d60* are different from the genes responsible for intersubspecific hybrid sterility.

Taking into consideration the large genetic diversity of the 30 varieties, it is entirely fair to say that *gal* is universally distributed in rice plants throughout the world, with the exception of Hokuriku 100, the *d60* donor. It is important to note that gal has never been found to express the gametic lethal action unless *d60* and *Gal* were simultaneously induced by chance. *d60* and its transmitter *Gal* are rare and valuable mutant genes in semidwarf breeding, a good replacement for *sd1.*

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Differentiation of alleles at seven loci for hybrid sterility in cultivated rice (*Oryza sativa* L.)

H. Ikehashi and J. Wan

The semisterility in panicles in indica-japonica crosses of rice is due to an allelic interaction at a gamete abortion locus, *S-5*^{n}, on chromosome 6. The *S-5*^{n} allele is referred to as a wide compatibility gene. The donor of *S-5*^{n}, referred to as a wide compatible variety (WCV), has been used to overcome sterility in indica-japonica crosses. However, some WCVs showed hybrid sterility when crossed to other varieties. We have screened varieties that showed hybrid sterility in their cross to WCVs and analyzed their hybrid sterility gene loci (HSGLs) using standard testing methods. So far, seven HSGLs were identified on five out of 12 chromosomes. On the basis of the analyses, a set of standard testers was proposed to identify HSGLs in rice. Also, by testing mutants and their original varieties, mutant alleles were found at two HSGLs. Thus, mutational origin of alleles at HSGLs was suggested. Indian rices showed the highest diversity in terms of hybrid sterility alleles. Contrastingly, hybrid sterility in indica-japonica crosses was predominantly controlled by alleles at *S-5.*

Since the mid-1980s, sterility in indica-japonica crosses has been clearly explained by an allelic interaction at locus *S-5,* where indica and japonica varieties carry *S-5*^{i} and *S-5*^{j}, respectively, and some varieties carry the neutral allele *S-5*^{n}. The *S-5*^{i}/*S-5*^{j} genotype produces semisterile panicles due to partial abortion of female gametes carrying the *S-5*^{j} allele. Such abortion does not occur in *S-5*^{n}/*S-5*^{i} and *S-5*^{n}/*S-5*^{j} genotypes. The donor of *S-5*^{n} is referred to as a wide compatible variety (WCV) (Ikehashi and Araki 1986). This allele has been incorporated into indica or japonica varieties to overcome hybrid sterility in hybrid rice breeding (Ikehashi 1991, Yuan 1992, Zou et al 1992). So far, *S-5*^{n} has been effective in a large number of indica-japonica crosses. Out of more than 1,000 varieties from China, a few varieties showed hybrid sterility in their crosses to WCVs (Table 1). However, in wider ranges of varietal

Table 1. Applicability of WCG *(S-5^n)* to Chinese varieties.

Type	Source	F_1 fertility in crosses with tester varieties											
		Ketan Nangka			Dular			Nekkan 2			02428		
		Fer[a]	Ster[b]	(%)	Fer	Ster	(%)	Fer	Ster	(%)	Fer	Ster	(%)
Indica	Yunnan	158	5	(3.1)	163	0		157	6	(3.7)	141	22	(13.5)
	Tai-hu	127	1	(0.8)	128	0		127	1	(0.8)	128	2	(1.5)
	Modern	185	0	(0)	184	1	(0.5)	185	0		160	25	(13.5)
Japonica	Yunnan	113	7	(5.8)	120	0		112	8	(6.7)	113	7	(5.8)
	Tai-hu	275	9	(3.2)	284	0		274	10	(3.5)	278	6	(2.1)
	Modern	126	0	(0)	126	0		126	0		125	1	(0.8)
Total		984	22	(2.2)	1005	1	(0.01)	981	25	(2.5)	945	63	(6.3)

[a] Fer = panicles of a hybrid gave a fertility higher than 70%. [b] Ster = panicles of a hybrid gave a fertility lower than 70%.

testing, such WCVs exhibited hybrid sterility when crossed to varieties from the Indian subcontinent or to native rices in China. Further genetic analyses of hybrid sterility gene loci (HSGLs) were then conducted and results are reported in this paper.

Method for analyzing HSGLs

A large number of three-way crosses (A/B//C) were made after confirming that a hybrid A/C produced semisterile panicles and another hybrid B/C was fertile. The progeny of A/B//C segregated semisterile plants expected from A/C and fertile ones expected from B/C in a ratio of 1:1. When a backcross A/C//C was made, the progeny resulted in semisterile plants expected from A/C and fertile ones from C/C in a ratio of 1:1. Then, such genetic markers cosegregating with semisterility were surveyed to identify a locus for semisterility. In the backcrosses, F_1 plants were used as the female to find distortion of marker genotypes, which was caused by the abortion of the female gamete carrying one of the alleles. Examples of these analyses were shown for two hybrid populations (Table 2), where a new HSGL was identified on chromosome 12 and female gamete abortion was found to be caused at *S-5*.

Allelic differences at a new locus were estimated following the model of allelic interaction at *S-5*. For three given varieties, A, B, and N, if a hybrid A/B shows gamete abortion at HSGL *S-X* and if N/A and N/B do not show any distorted segregation of markers for *S-X*, variety N was determined to possess a neutral allele *S-X^n* at the new locus.

Table 2. Distribution of spikelet fertility classified by marker genotype in two hybrids.

Genotype	Number of plants in % spikelet fertility class										Total	Mean (%)
	10	20	30	40	50	60	70	80	90	100		
IR36/Dular//IR2061-628												
Sdh-1^1/Sdh-1^1	0	1[a]	1	5	6	4	6	8	24	12	67	70.7**[b]
Sdh-1^2/Sdh-1^1	0	0	6	12	13	5	8	8	5	1	58	53.6
Pox-2^1/Pox-2^1	0	0	0	2	1	0	13	13	23	12	64	79.5**
POX-2^0/POX-2^1	0	1	7	15	18	9	1	3	6	1	61	49.3
Est-2^2/Est-2^1	0	0	4	7	11	4	8	9	14	8	65	63.2
Est-2^1/Est-2^b	0	1	3	10	8	5	6	7	15	5	60	61.6
Akihikari/IR2061-628//Akihikari												
Sdh-1^2/Sdh-1^2	0	0	3	3	5	9	3	9	6	5	43	64.2
Sdh-1^1/Sdh-1^2	1	0	4	4	3	5	5	5	2	6	35	61.3
Est-2^0/Est-2^0	0	0	0	0	2	0	1	7	6	10	26**	76.8**
Est-2^2/Est-2^0	1	0	7	7	6	14	7	7	2	1	52	52.6

[a] Numbers underllned are assumed recombinants.[b]** Shows significant dlfference between two genotypes at 1%.

Identified HSGLs and markers

A number of new HSGLs were identified (Table 3). Locus *S-7* was detected in hybrids between aus varieties (summer rice on the Indian subcontinent) and some javanicas (Yanagihara et al 1992). Locus *S-8* was detected in a hybrid between a Korean indica variety and some javanicas (Wan et al 1993). Locus *S-9* was detected in hybrids between aus varieties and some javanicas (Wan et al 1995). Locus *S-15* was found in hybrids between aus variety Dular (WCV) and some IRRI lines (Wan et al 1995). *S-16* was identified near *Est-1* on chromosome 1 in hybrids between Ketan Nangka and local varieties in the Tai-hu Lake region of Yunnan Province in China (Wan and Ikehashi 1995). One more locus, *S-17* (t), was identified in crosses between Penuh Baru II and the japonicas (J. Wan and H. Ikehashi, Nanjing University, unpubl. data). The isozyme analyses mentioned above were conducted according to prescribed methods (Glaszmann 1988, Ishikawa et al 1989).

From these genetic analyses, a set of tester varieties is listed in Table 3. Since their alleles at HSGLs have been analyzed, these varieties may be used to identify respective alleles in other varieties.

The nature of hybrid sterility genes

An irradiated mutant Miyukimochi (Toda 1982) was analyzed together with its original variety Toyonishiki. The semisterility in hybrids derived from Toyonishiki and IR36 was caused only by the allelic interaction of *S-5^i/S-5^j* whereas the semisterility in F, hybrids derived from Miyukimochi and IR36 was attributed to allelic interactions by both *S-S^i/S-5^j* and *S-7^i/S-7^j*. Thus, the neutral allele *S-7^n* in Toyonishiki was found to be mutated into *S-7^j* by irradiation with ^{60}Co.

Table 3. Alleles at HSGL and markers in tester *varieties.*[a]

Tester variety	Marker gene and chromosome										
	Chromosome 6				Chromosome 7	Chromosome 4		Chromosome 12			Chromosome 1
	Amp3	*Est-2* (*S-5*)	*Cat-1*	*Pox-5* (*S-8*)	*Est-9* (*S-7*)	*Est-1*	*Mal-1* (*S-9*)	*Acp-1*	*Pox-2* (*S-15* & *S-17(t)*)	*Sdh-1*	*Est-5* (*S-16*)[b]
IR36	1	2(i)[c]	1	2(n)[d]	2(n)[d]	1	2(n)[d]	1	1(n)	1(n)	1(n)
Akihikari	1	0(j)	2	2(n)	1(n)	0	1(n)	2	0(n)	2(j)	1(n)
Ketan Nangka	2	1(n)	2	2(k)	1(kn)	0	1(kn)	2	1(n)	2(n)	1(kn)
Dular	2	1(n)	2	2(n)	1(n)	0	1(n)	2	0(du)	2(n)	1(n)
02428	2	1(n)	2	2(n)	1(kn)	0	1(kn)	2	0(n)	2(n)	1(kn)
CY85-26	2	2(n)	1	1(n)	2(kn)	1	2(n)	1	1(n)	1(n)	1(n)
Yeong Pung	1	2(i)	1	1(yp)	2(ai)	1	2(n)	1	1(n)	1(-)[e]	1(·)
N22	2	1(n)	2	2(n)	1(ai)	0	1(ai)	2	0(n)	2(n)	1()
IR2061-628	1	2(i)	1	1(n)	2(n)	1	2(n)	1	1(i)	1(-)	1(-)
Fengjingdao	1	0(j)	2	2(n)	1(ai)	0	1(n)	1	0(n)	2(n)	2(j)
Penuh Baru II	1	1(j)	2	2(pb)	1(kn)	1	1(n)	2	0(n)	2(pb)	1(-)
Panbira	2	1(n)	1	1(n)	2(ai)	1	2(n)	1	1(n)	1(n)	1()
DJ123	1	0(j)	2	2(n)	1(ai)	0	1(i)	2	0(n)	2(n)	1(n)

[a] The isozyme allele systems quoted from Morishima and Glaszmann 1991; [b] Seven hybrid sterility loci are shown under the marker loci; [c]( ) = allele at the hybrid sterility locus; [d] = not neutral to aus varieties: [e] (-) = no data.

An experimental line, 02428, was found to possess the $S\text{-}5^n$ allele. It was selected from a progeny population from a cross between Pangxiegu and Jibangdao, both irradiated with ^{60}Co and crossed at their M_1 stage (Zou 1992). Pangxiegu and Jibangdao were proved to possess $S\text{-}5^j$. Thus, the allele *S-5* identified in 02428 was found to be induced $S\text{-}5^j$. The fact that an allele at an HSGL mutated into another would explain the origin of hybrid sterility among different rice groups.

Three alleles were identified at the *S-5* locus. In the course of further analyses, however, allelic differentiation at HSGL was shown to form a number of alleles at a single locus. Especially in aus varieties, more than five alleles were identified at *S-7* using a set of testers. In a survey of diversity of alleles at HSGLs and isozymes in Chinese and aus varieties, the Indian rices showed the highest diversity in terms of alleles at HSGLs. In contrast, the hybrid sterility in indica-japonica crosses was predominantly controlled by alleles at *S-5* (Table 1).

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Genetics of hybrid sterility and hybrid breakdown in an interspecific rice (*Oryza sativa* L.) population

Z. Li, S.R.M. Pinson, A.H. Paterson, W.D. Park, and J.W. Stansel

Hybrid sterility (F_1 sterility) and hybrid breakdown (sterility and weakness of the F_2 and later generations) in crosses within cultivated rice (*Oryza sativa* L.) are common and genetically complicated. We used a restriction fragment length polymorphism linkage map and F_4 progeny testing to investigate hybrid sterility and hybrid breakdown in an intersubspecific cross between a "widely compatible" japonica variety, Lemont, from the southern United States and an indica variety, Teqing, from China. Our results imply different genetic mechanisms in hybrid sterility and hybrid breakdown, respectively. Hybrid sterility appeared to be due to the breakdown (by crossing over) of a number of putative differentiated "supergenes" in the rice genome, which may reflect cryptic structural rearrangements. The cytoplasmic genome had a large effect on fertility of both male and female gametes in the F_1 hybrids. There appeared a pair of putative complementary genes which had a very large effect on sterility and behaved like wide compatibility genes. We speculate that this pair of genes and the "gamete eliminator" (S_1) or "egg killer" (*S-5*) may influence the phenotypic effects of presumed "supergenes" in hybrids. Hybrid breakdown appeared to be due to the breakdown of coadapted indica and japonica gene complexes by random assortment of large numbers of unlinked complementary quantitative trait-modifying factors throughout the genome. These proposed mechanisms may partly account for the complicated nature of the post-reproductive barriers in rice.

The post-reproductive barriers (hybrid sterility and hybrid breakdown) in rice have been under intensive investigation but this subject remains controversial. Several mechanisms including chromosomal aberrations and major genes have been suggested to be responsible (Henderson et al 1958, Yao et al 1958, Delores et al 1975, Oka 1988, Ikehashi and Araki 1986).

Here, we investigate the genetic basis of post-reproductive barriers in rice. Using DNA markers, we sought to determine the number and locations of quantitative trait loci (QTLs) and quantitative trait-modifying factors (QTMFs), which were associated with hybrid sterility and hybrid breakdown in rice.

Materials and methods

Plant materials

Two *Oryza sativa* cultivars Lemont (widely compatible japonica from southern United States) and Teqing (indica from China) were used as parents. Reciprocal F_1 crosses were made. The 255 F_2 plants and the 2,418 F_2-F_4-derived lines were used as the mapping population.

RFLP marker genotyping, phenotyping, and data analysis

The genotyping of 255 F_2 plants for restriction fragment length polymorphism (RFLP) marker loci and the field experiment for progeny testing of 2,418 F_4 lines were described previously (Li et al 1995). Spikelet sterility (SS) was assayed on 10 different plants in each of the F_4 lines. Plant height (PH) was assayed on each of the F_4 lines (Li et al 1995). PH was used as a trait associated with hybrid weakness (Oka 1988). The breeding value of each of the 255 F_2 plants for these traits was obtained by averaging the 10 F_4 lines. In addition, the parents and the reciprocal F_1 plants were planted in the field and grown in the greenhouse for SS and pollen fertility assay using I_2KI staining. Mapping of QTLs and QTMFs affecting SS was done using methods described by Li et al (1995).

Results

Cytoplasmic effect on fertility of F_1 plants

Highly significant differences for SS and pollen fertility were detected between the reciprocal F_1 hybrids, indicating that the gene(s) in Lemont's cytoplasmic genome has played an important role in its wide compatibility.

Mapping of SS QTLs

Four QTLs and three putative QTLs affecting SS were mapped to 6 of the 12 chromosomes (Fig. 1). Two putative underdominant QTLs on chromosomes 2 and 5 had virtually zero additive effects but very large effects (10.8 and 12.8%) for increased SS.

Identification of QTMFs affecting SS and PH

We identified 43 and 20 interactions between unlinked QTMFs affecting SS and PH, respectively. The mean R^2 explained by 43 interactions affecting SS was 6.09±1.28%, approximately the same as that of the four SS QTLs (6.05±1.85%). There were 81 additive epistatic effects (47.1%), which were significantly different from zero ($P<0.05$), The mean additive epistatic effect was 4.7±2.4%, which was equivalent to

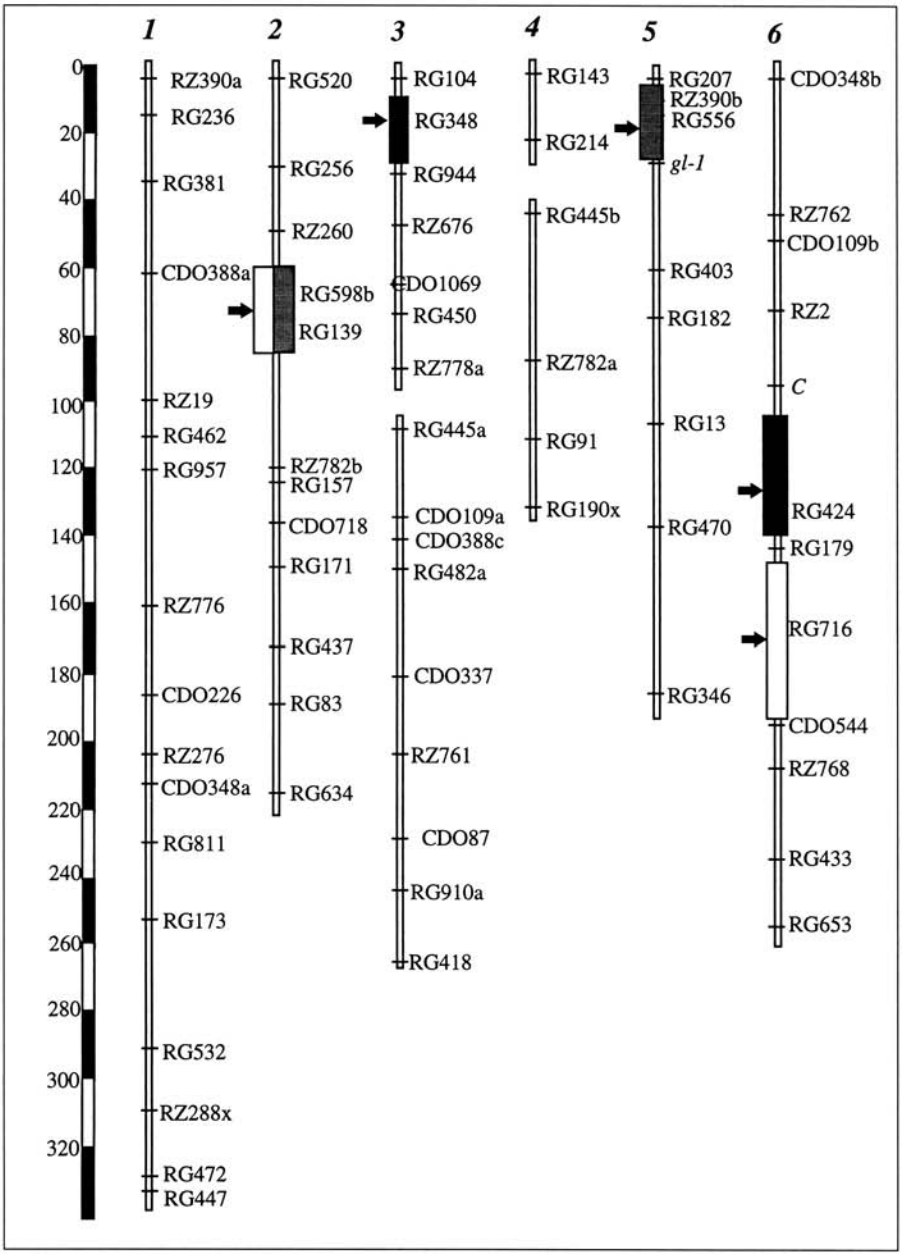
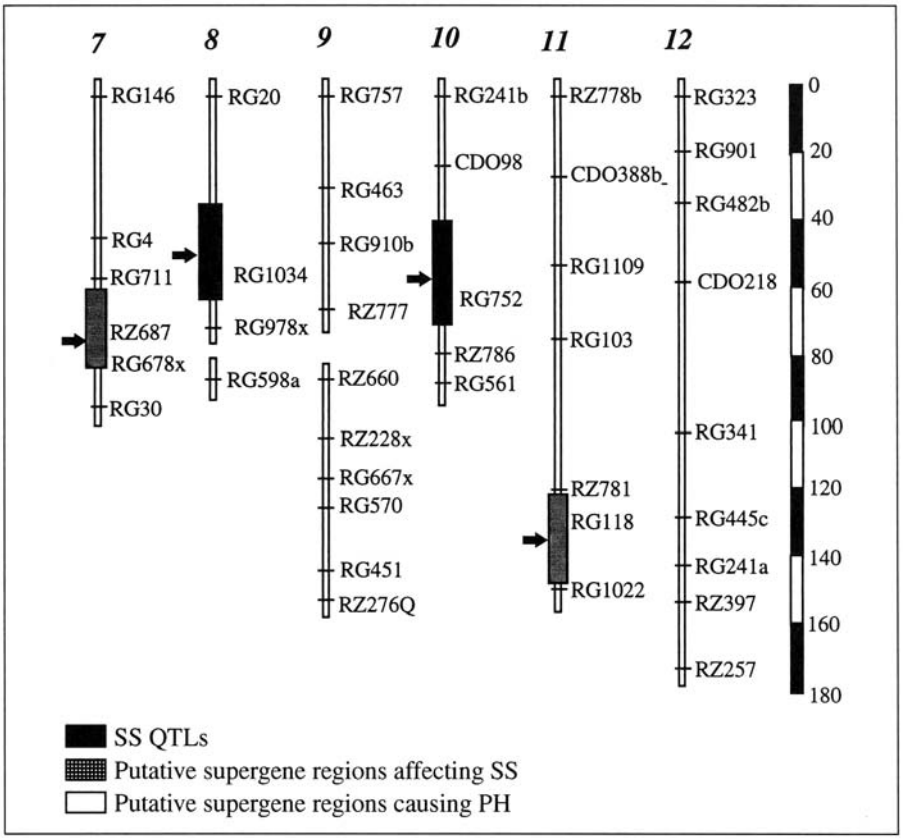


Fig. 1. Chromosomal locations of QTLs and putative supergene regions affecting spikelet sterility (SS) and plant height (PH). The boxes cover QTLs at one log of odds (LOD) confidence interval.

Fig. 1 continued.



the doubled mean additive effect (4.9±0.4%) of the SS QTLs. No interactions were detected between the SS QTLs. On average, each of the 46 QTMFs was involved in 1.87 interactions. Table 1 shows that increased SS was due to the incompatibilities between alleles at many QTMF (complementary) loci from different parents. Similar patterns were observed for PH; the recombinant type of interactions tended to result in reduced height (Table 1).

The most notable interaction affecting SS occurred between CDO348b of chromosome 6 and RZ660 of chromosome 9. The observed genotypic frequencies of Lemont/Lemont, Lemont/Teqing, Teqing/Lemont, and Teqing/Teqing (nonwidely compatible homozygote, which had increased sterility by 21.2%) were 11:5:12:1, which deviated significantly from the expected 1:1:1:1 ratio ($c^2 = 11.14$, $P = 0.011$).

Table 1. Characterization of significant additive digenic epistatic effects (t_{ij}) on spikelet sterility and plant height from interactions between QTMFs in the Lemont/ Teqing cross.

			Spikelet sterility		Plant height	
			# of t_{ij}	%	# of t_{ij}	%
Parental types	Lemont	+[a]	6	30.0	9	100.0
		-	14	70.0	0	0.0
	Teqing	+	5	31.3	6	85.7
		-	11	68.7	1	14.3
		+	11	30.6	15	93.7
	Total	-	25	69.4	1	6.3
Recombinants		+	30	66.7	6	40.0
		-	15	33.3	9	60.0

[a] '+' and '-' represent the parameters for increased and decreased trait value, respectively.

Putative supergenes affecting SS and PH

Table 2 shows three (SS) and two (PH) genomic regions where significant interactions were detected between linked markers. In these cases, dramatically increased SS and reduced PH were almost exclusively associated with recombinant genotypes resulting from infrequent crossing-over events within these genomic regions.

Discussion

Our results from QTL mapping and interaction analyses suggest that several genetic mechanisms may be responsible for hybrid sterility and hybrid breakdown in rice.

Genetic mechanisms for hybrid sterility

Our results suggest that three different genetic mechanisnls—putative differentiated "supergenes," cytoplasmic gene(s), and a pair of putative complementary *Rm* genes —appeared to be involved in hybrid sterility.

First, we identified five genomic regions on chromosomes 2, 5, 6, 7, and 11 (including the two underdominant QTLs within which crossing-over caused sterility). While the overall genomic heterozygosity (estimated from all 115 markers) of individual F_2 plants did not correlate with SS (r=0.013), the heterozygosity at the putative supergene regions (flanking markers) was significantly associated with SS (r=0.435, $P<0.0001$). We also found that these putative differentiated supergene regions might have cryptic chromosomal rearrangements in nature (Stebbins 1958). as suggested by a comparison between our RFLP map and that from the Cornell group (Causse et al 1994) (Fig. 2).

Second, SS of the reciprocal Lemont/Teqing F_1 hybrids differed by as much as 33.9%. Such a big difference in SS was best accounted for by the cytoplasmic gene(s) of Lemont on the female gametes.

Table 2. Large phenotypic effects associated with the recombinants arising from crossing-over within certain genomic regions on spikelet sterility (SS, in%) and plant height (PH) revealed putative 'supergene' regions in the Lemont (L)/Teqing (T) rice cross.

Genomic regions			Interval	R^2	Digenic genotypes[a]							
Character	Flanking markers		(r)	(%)	IL/2L	1L/2T	1T/2L	1T/2T	1L/2H	1T/2H	1H/2L	1H/2T
Spikelet sterility												
5	RG207	*gl1*	0.14	4.20	-0.9	3.8**	14.6****	1.0	2.3	0.3	1.5	2.1
5	RG556	*gl1*	0.13	4.43	-1.1	3.4**	-	1.3	2.3	1.7	3.2**	4.0**
7	RG4	RG678b	0.19	4.40	0.0	11.7****	-	-1.2	-1.1	1.5	0.1	0.1
7	RG711	RG678b	0.15	4.62	-0.7	16.1****	-3.9**	-0.7	0.2	1.3	1.9	1.8
7	RG711	RG30	0.26	5.64	-2.2	15.1****	-0.3	-1.0	1.3	0.6	2.7*	0.8
11	RZ781	RG1022	0.18	8.04	-2.6	6.2****	-5.1***	2.0	3.0*	0.4	3.2*	-0.4
Plant height												
2	RG256	RG139	0.19	5.52	4.1*	4.4*	-9.4***	-1.1	-4.0	1.6	0.3	-1.0
2	RG256	RG598b	0.27	4.51	2.1	3.1	-10.9****	-0.8	-1.3	1.9	2.7	-0.8
6	RG424	CD0544	0.26	3.50	-2.6	-2.5	-17.1****	-1.0	1.4	1.2	2.1	2.3
6	RG179	CD0544	0.24	5.34	-3.8	-6.1**	-17.4****	0.1	2.3	0.9	3.8	0.9
6	RG179	RZ768	0.29	5.43	-2.9	-3.8*	-16.5****	-1.0	2.9	1.4	3.1	1.9
6	RG716	RZ768	0.24	6.71	-1.2	-4.3*	-18.2****	-0.6	3.3	0.3	2.3	3.2

[a]L, T, and H represent the homozygous Lemont alleles, the Teqing alleles, and the heterozygotes at the flanking markers and *,**,***, and **** indicate that the parameters are different from zero at P£0.05, 0.01, 0.001, and 0.0001, respectively.

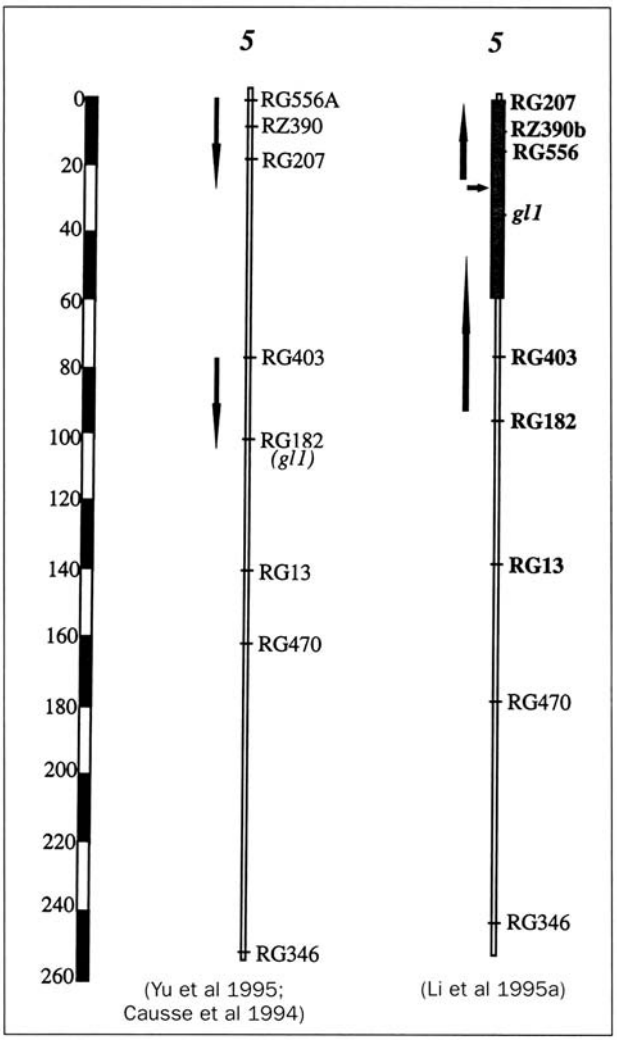


Fig. 2. Comparison of the Cornell RFLP map of chromosome 5 (Causse et al 1994, Yu et al 1995) and that from the Lemont/Teqing population (Li et al 1995a) suggests two inversions around *gl1.* We noted that the inference of an inversion relies in part on data for *gl1,* a morphological marker which has been assigned to different locations in the two studies. Our *gl1* data were based on phenotyping 7-11 F_4 plants from each of 255 plants. The *gl1* data of Yu et al(1995) were based on phenotyping of 60 F_2 plants in four crosses. Based on our data, the location of *gl1* reported by Yu et al (1995) is 24-LOD less likely than the location we report. Moreover, the marker order reported by Causse et al (1994) is four-LOD less likely than the order shown in our population.

Third, we detected an interaction between a pair of putative complementary genes (mapped near CDO348b on chromosome 6 and RZ660 on chromosome 9), which behaved like wide compatibility genes in the Lemont/Teqing cross. CDO348b mapped near the location (reported on chromosome 6) of the gamete eliminator S_1 causing hybrid SS of interspecific crosses between *O. sativa* and *O. glaberrima* (Sano 1990), and *S-5* affecting intergroup hybrid SS between japonica and indica varieties (Ikehashi and Araki 1986). We speculate that the *S_1/S-5* and the pair of complementary genes might be recombination-modulating genes based on their impacts on recombination frequencies in their neighboring regions (Sano 1990, Ikehashi and Araki 1986). If so, they are expected to be able to either promote or inhibit the phenotypic effects on sterility of the differentiated supergene regions in hybrids.

Genetic mechanisms causing hybrid breakdown in rice

Hybrid breakdown, defined as sterility and weakness in F_2 and later generations (Stebbins 1958), is much more complicated than F_1 sterility since this property is influenced by the environment and the many genes functioning in both gametophytic and sporophytic stages. Our results indicated that the primary mechanism causing hybrid breakdown in rice is the breakdown of coadapted indica and/or japonica gene complexes by recombination, referred to as the complementary gene system (Dobzhansky 1936, Muller 1940, Stebbins 1958, Oka 1988).

In heterogeneous backgrounds, alleles at many QTMF loci from the same parents tended to interact favorably, while the recombinant alleles interacted unfavorably and resulted in reduced fitness (increased SS and reduced height). The presumed coadapted gene complexes appear to have large impacts on hybrid breakdown but little impact on hybrid sterility since the F_1 plants were largely fertile and vigorous. Our results indicate that the indica and japonica gene complexes included large numbers of unlinked loci throughout the genome and that epistasis is the primary factor in maintaining the integrity of these gene complexes.

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Genetics of F_1 pollen sterility in *Oryza sativa*

G. Zhang and Y. Lu

A series of isogenic F_1 sterile lines (ISLs) of Taichung 65 was used for isogenic analysis of F_1 pollen sterility. Three loci of the F_1 pollen sterility genes, *S-a, S-b,* and *S-c,* were identified in the ISLs. Using the ISLs as testers, the genotypes at the three loci in 45 varieties including indica, japonica, intermediate, and wide-compatibility types were tested. Three other loci of F_1 pollen sterility genes, *S-d, S-e,* and *S-f,* were found in crosses Taichung 65/Karang Serang, Taichung 65/N29, and Yongshanxiaobaigu/Guangluai 4, respectively. The action of the F_1 pollen sterility genes was in the one-locus sporo-gametophytic interaction model. The allelic interaction of S^i/Y^j at the loci caused abortion of the male gametes carrying the Y allele. In F_1 hybrids, the more heterozygous the six loci were, the higher the pollen sterility and spikelet sterility. Spikelet sterility in the F_1 hybrids was caused by pollen sterility. Therefore, F_1 sterility in rice is mainly pollen sterility. Understandingthe genetic basis of F_1 sterility is an important step toward overcoming the F_1 sterility barrier between distantly related varieties in rice.

Cultivated rice (*Oryza sativa*) has numerous varieties that are classified into different types and distributed over a wide area. Hybrid sterility occurs in crosses between distantly related taxa. Reports on the genetic basis of hybrid sterility in rice have been reviewed by Oka (1988). To study the genetic basis of F_1 pollen sterility, we used isogenic F_1 sterile lines (ISLs) to analyze F_1 pollen sterility genes individually. This paper gives a general overview of the main results in addition to new results. Detailed results have been published elsewhere (Zhang and Lu 1989, 1993; Zhang et aL 1993, 1994).

Genic model

Taichung 65 (T65) and five ISLs provided by Dr. Oka were used for isogenic analysis of F_1 pollen sterility in this study. Three F_1 pollen sterility genes were identified in the five ISLs of T65 (TISLs): *S-a* in TISL3 (E3) and TISL4 (E4), *S-b* in TISL2 (E2), and *S-c* in TISL5 (E5) and TISL6 (E6). The action of the three S genes is in the one-locus sporo-gametophytic interaction model. TISL2 carries S^i/S^i at the *S-b* locus and S^j/S^j at the *S-a* and *S-c* loci; TISL3 and TISL4 carry S^i/S^i at the *S-a* locus and S^j/S^j at the *S-b* and *S-c* loci; and TISL5 and TISL6 carry S^i/S^i at the *S-c* locus and S^j/S^j at the *S-a* and *S-b* loci; while T65 carries S^j/S^j at the three loci. The allelic interaction of S^i/S^j at the three loci caused the male gametes carrying S^j allele to be aborted. The abortive pollens with S^j at the *S-a* locus are empty abortive pollens (EAPs) which failed to be stained by 1% I_2-KI solution and are small in size. Those with S^j at the *S-b* and *S-c* loci are stainable abortive pollens (SAPs) which are stainable by 1% I_2-IK solution and are also small (Zhang and Lu 1989).

Recently, the *S-c* locus was mapped on chromosome 3 by the use of restriction fragment length polymorphism (RFLP) markers. In the F_2 population of the cross T65/TISL5, the fertile plants and semisterile plants segregated in the ratio of 1:1. At the two flanking RFLP marker loci which are closely linked to the *S-c* locus, the genotypes of TISL5 and heterozygotes also segregated in the ratio of 1:1. No genotypes of T65 were found at the two marker loci except for one plant carrying the genotype at one marker locus (Zhuang et al, pers. commun.). The results indicate that the male gametes carrying the S^j allele from T65 were aborted in the F_2 plants of the cross. This evidence supports the hypothesis that the action of the F_1 pollen sterility genes occurs in the one-locus model.

Gene pyramiding

Using TISL2, TISL4, and TISL5 as donors for S^i genes, three TISLs carrying the S^i genes at the two loci were established by crossing the TISLs with each other. TISL24 carries the S^i genes at the *S-a* and *S-b* loci, TISL25 carries the S^i genes at the *S-b* and *S-c* loci, and TISL45 carries the S^i genes at the *S-a* and *S-c* loci. The segregation pattern of pollen fertility in the F_2 population of the cross TISL24/TISL25 (Table 1) is the same as that in the cross TISL4/TISL5 (Zhang and Lu 1993). From the cross TISL24/TISL25, a TISL, TISL245, carrying S^i genes at the *S-a, S-b,* and *S-c* loci, was established.

A series of TISLs was established completely, consisting of all genotype combinations at the *S-a, S-6,* and *S-c* loci. The degree of pollen sterility caused by the allelic interaction at the loci varied with the loci and locus numbers (Table 2). Designating pollen sterility caused by allelic interaction at a locus as *s*, pollen fertility (F) in heterozygotes with *n* heterozygous loci is $F = (1-s_1)*(1-s_2)* ...*(1-s_n)$, or $F = f_1*f_2*...*f_n$, where $f = 1-s$ (Zhang et al 1994).

Table 1. Segregation of pollen fertility in the F_2 population of the cross TISL24/TISL25.

Genotype	No. of plants in different pollen fertility classes									Total	Pollen fertility[a] (%±sin$^{-1}\sqrt{\%}$)			c^2 (1:1:1:1)
	10	20	30	40	50	60	70	80	90 - 100%		FP	SAP	EAP	
Homozygote									50	50	96.4±4.08	1.2±4.14	2.4±3.63	
Heterozygote at														
S-a					11	12	17	5		45	68.8±5.57	1.9±4.08	29.2±5.40	1.986
S-c			3	16	31	3				53	50.6±3.52	46.7±3.15	2.7±4.26	(P>0.50)
S-a & *S-c*	2	18	28	11						59	33.2±4.85	33.3±5.30	33.5±5.53	
Total	2	18	31	27	42	15	17	5	50	207				

[a]FP = fertile pollen; SAP = stainable abortive pollen; EAP = empty abortive pollen.

Table 2. Pollen fertility in F_1s of testcrosses of isogenic F_1 sterile lines (ISLs) carrying different genotypes with recurrent parent Taichung 65.

ISL	Genotype[a]			Pollen fertility in F_1s of the testcrosses with Taichung 65 (%±sin$^{-1}\sqrt{\%}$)[b]			
	S-a	*S-b*	*S-c*	Plant (no.)	FP	SAP	EAP
TISL2	-	+	-	19	22.8±2.98	71.1±2.91	6.0±2.60
TISL4	+	-	-	29	50.4±2.05	0.5±2.11	49.1±2.01
TISL5	-	-	+	31	47.3±2.21	49.1±2.30	3.6±2.74
TISL24	+	+	-	13	11.8±3.23	38.0±3.74	50.2±3.23
TISL25	-	+	+	29	6.7±2.53	83.6±3.21	9.7±4.57
TISL45	+	-	+	101	21.6±5.22	28.5±2.83	49.9±4.25
TISL245	+	+	+	218	4.5±4.79	41.3±3.66	54.2±3.05

[a]+= S^i/S^i ,- = S^j/S^j; [b]FP = fertile pollen, SAP = stainable abortive pollen, EAP = empty abortive pollen.

Gene frequency

The genotypes at *S-a, S-b,* and *S-c* loci in 45 varieties were tested by using TISLs as testers. At the *S-a* locus, all varieties tested have S^j/S^j except for Dular, which has S^i/S^i. At the *S-b* and *S-c* loci, the averages of S^i frequencies are 0.667 in Hsien (indica), 0.200 in Keng (japonica), and 0.500 in intermediate (including five wide-compatibility types) (Zhang et al 1993).

The *S-d, S-e,* and *S-f* loci

The *S-d* locus was identified in the F_2 population from the cross T65/Karang Serang 55. Karang Serang 55 is a javanica variety from Indonesia. At the *S-d* locus, T65 carries S^j/S^j and Karang Serang 55 carries S^i/S^i. The *S-e* locus was identified in the F_2 population from the cross T65/N29. N29 is also a javanica variety from Indonesia. At the *S-e* locus, T65 carries S^j/S^j and N29 carries S^i/S^i. The *S-f* locus was identified in the F_2 population from the cross Yongshanxiaobaigu/Guangluai 4. Yongshanxiaobaigu is a Keng variety and Guangluai 4 is a Hsien variety, both from China. At the *S-f* locus, Yongshanxiaobaigu carries S^j/S^j and Guangluai 4 carries S^i/S^i. The allelic interaction at the three loci caused the male gametes carrying S^j to be aborted, which became stainable abortive pollens (Zhang et al 1994).

Heterozygosity and sterility

For F_1 pollen sterility, six loci of F_1 pollen sterility genes were identified in rice. The genotypes at the six loci were analyzed in 11 varieties. Using the 11 varieties and 5 TISLs as parents, 97 crosses were made. The pollen fertility and spikelet fertility in the F_1 hybrids from the 97 crosses were observed. The F_1 hybrids carried genotype S^i/S^j at 1-6 loci and their pollen fertility covered all classes. In the F_1 hybrids, the more heterozygous the loci were, the lower the pollen fertility and spikelet fertility (Table 3).

Table 3. Pollen fertility and spikelet fertility in the F_1s with different numbers of heterozygous loci (S^i/S^j) at the *S-a, S-b, S-c, S-d, S-e,* and *S-f* loci (Zhang et al 1994).

No. of loci with S^i/S^j	No. of crosses in different F_1 pollen fertility classes										Total	Fertility[a] (%±sin$^{-1}\sqrt{\%}$)	
	0 - 10	- 20	- 30	- 40	- 50	- 60	- 70	- 80	- 90	- 100%		Pollen	Spikelet
1			2	4	6	3	5	3			25	67.9±12.90	83.8±8.30
2	1	1	6	9	4	6	1	3	2		33	43.4±12.75	63.7±14.83
3		2	3	4	6	1	2				18	37.9±8.78	52.3±9.59
4	3	3	6	1		1					14	21.7±9.26	27.0±14.96
5	4	1									5	7.3±3.93	8.1±3.19
6	2										2	3.0±0.12	0.3±0.75

Pollen and spikelet fertility

The regressions between spikelet fertility (Y) and pollen fertility (X) in F_1 hybrids from 271 crosses were analyzed. At the interval of 10.1-50.0 $\sin^{-1}\sqrt{\%}$ of the pollen fertility, the regression equation is Y = -9.34+1.48X, r = 0.90**, and at the interval of 50.1-90.0 $\sin^{-1}\sqrt{\%}$, Y = 53.35+0.26X, r = 0.48**. It indicates that spikelet fertility in the F_1 hybrids varied with pollen fertility. Therefore, F_1 sterility in rice is mainly pollen sterility.

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Effects of three genes that control heading date (*Se1, E1,* and *Ef1*) in four varietal groups of japonica rice

Y. Okumoto, H. Inoue, K. Ichitani, and T. Tanisaka

The genes that control heading date in each of the four groups of japonica rice varieties cultivated in Japan and Taiwan, China, were analyzed by using 12 tester lines for the loci of *E1, E2, E3, Se1,* and *Ef1.* Results showed that the *E1, E2, E3,* and *Se1* loci control photoperiod sensitivity (PS), while the *Ef1* locus controls basic vegetative growth (BVG). Each group had its own fundamental genotype for the *E1, Se1,* and *Ef1* loci. The difference in PS among the four groups was caused by the difference in genotype of the *Se1* and *E1* loci, while the difference in BVG among the four groups was ascribable to the difference in genotype of the *Se1* and *Ef1* loci. The BVG phase is remarkably prolonged by the complementary effects of *ef1* and $Se1^e$.

Japonica rice varieties are widely cultivated throughout temperate Asia. According to previous works on geographical analysis of heading traits of japonica varieties cultivated in Japan and Taiwan, China (Sakamoto and Toriyama 1967), these varieties are classified into four groups: group A (high photoperiod sensitivity [PS], short basic vegetative growth [BVG]), group B (low PS, intermediate BVG), group C (extremely low PS, short BVG), and group D (low PS, long BVG). Groups A-C consist of varieties cultivated mainly in Japan; group D varieties are cultivated mainly in Taiwan. In this study, the genes controlling heading date (HD) that characterize each group were analyzed by using 12 tester lines for the loci of *E1, E2, E3, Se1,* and *Ef1.* It has been shown that the *E1, E2, E3,* and *Se1* loci mainly control PS (Yokoo et al 1980,Yamagata et al 1986) and that the *Ef1* locus controls BVG (Tsai 1986).

Materials and methods

The genotypes for HD of representative varieties in each group were estimated through segregation analysis of progeny from crosses made with 12 tester lines that control

Table 1. Genotypes for heading date of 12 tester lines.

Tester line	Genotype				
EGO	*e1e1*	*e2e2*	*e3e3*	*$Se1^nSe1^n$*	*Ef1Ef1*
EG1	*E1E1*	*e2e2*	*e3e3*	*$Se1^nSe1^n$*	*Ef1Ef1*
EG2	*e1e1*	*E2E2*	*e3e3*	*$Se1^nSe1^n$*	*Ef1Ef1*
EG3	*e1e1*	*e2e2*	*E3E3*	*$Se1^nSe1^n$*	*Ef1Ef1*
EG4	*E1E1*	*E2E2*	*e3e3*	*$Se1^nSe1^n$*	*Ef1EF1*
EG5	*e1e1*	*E2E2*	*E3E3*	*$Se1^nSe1^n$*	*Ef1EF1*
EG6	*E1E1*	*e2e2*	*E3E3*	*$Se1^nSe1^n$*	*Ef1Ef1*
EG7	*E1E1*	*E2E2*	*E3E3*	*$Se1^nSe1^n$*	*Ef1EF1*
ER[a]	*E1E1*	*e2e2*	*E3E3*	*$Se1^eSe1^e$*	*Ef1Ef1*
LR[a]	*E1E1*	*e2e2*	*E3E3*	*$Se1^uSe1^u$*	*Ef1Ef1*
T65[b]	*E1E1*	*E2E2*	*E3E3*	*$Se1^eSe1^e$*	*ef1ef1*
T65ef[b]	*E1E1*	*E2E2*	*E3E3*	*$Se1^eSe1^e$*	*Ef1Ef1*

[a]Provided by Dr. M. Yokoo.[b]Provided by Dr. K.H. Tsai.

HD (Table 1). The F_2 populations underwent gene analysis for HD. From each cross, about 300 F_2 plants were grown in the field at Kyoto (N35°01') along with the parents.

The genotype relationships for HD with PS and BVG were examined by using eight tester lines controlling HD that are different from each other in genotype for the five loci. All lines were grown under five different daylengths—10, 13, 14, 15, and 16 h.

Results

The results of gene analysis are summarized in Table 2. Each group has its own fundamental genotype (FG) for the *E1, Se1,* and *Ef1* loci. The FG of group A is *E1E1 $Se1^nSe1^n$ Ef1Ef1,* and that of Group B is *E1E1 $Se1^eSe1^e$ Ef1Ef1.* For Group C, there are two FGs: *e1e1 $Se1^nSe1^n$ Ef1Ef1* and *e1e1 $Se1^eSe1^e$ Ef1Ef1.* It appears that the difference in HD among Groups A, B, and C largely depends on the difference in gene constitution of the *E1* and *Se1* loci. The FG of Group D is estimated to be *E1E1 $Se1^eSe1^e$ ef1ef1,* suggesting that the large difference in BVG between Japanese and Taiwanese varieties is due to the difference of alleles at the *Ef1* locus. There is a somewhat large variation within groups, possibly due to the difference in gene constitution of the *E2* and *E3* loci. In fact, it is obvious that *E2* and *E3,* which slightly enhance photoperiod sensitivity, are distributed in all four groups (Table 2).

Figures 1 and 2 show the photoperiod-response curves of the tester lines controlling HD. The tester lines carrying both *E1* and *$Se1^u$*(or *$Se1^n$*) (LR, EG6, EL30, and EG7) have high PS, while those not carrying one of the two genes (ER, EG3, T65, and T65Ef) have a low PS. Our results support the findings of Okumoto et al (199 1) that high photoperiod sensitivity is due to the complementarity between El and *$Se\text{-}1^u$* (or *$Se\text{-}1^n$*). In our study, the length of BVG was approximately expressed by the days to heading under 10-h of daylength. The difference in BVG between EL30 (*ef1ef1*

Table 2. Gene constitution of loci that control heading date of four varietal groups classified according to BVG and PS.

Varietal group				Genotype	
Group A	*E1E1*	*e2e2*	*e3e3*	$Se1^nSe1^n$	*Ef1Ef1*
	E1E1	*E2E2*	*e3e3*	$Se1^nSe1^n$	*Ef1Ef1*
	E1E1	*e2e2*	*E3E3*	$Se1^nSe1^n$	*Ef1Ef1*
Group B	*E1E1*	*e2e2*	*e3e3*	$Se1^eSe1^e$	*Ef1Ef1*
	E1E1	*e2e2*	*E3E3*	$Se1^eSe1^e$	*Ef1EF1*
Group C	*e1e1*	*e2e2*	*e3e3*	$Se1^eSe1^e$	*Ef1EF1*
	e1e1	*E2E2*	*e3e3*	$Se1^eSe1^e$	*Ef1Ef1*
	e1e1	*E2E2*	*E3E3*	$Se1^nSe1^n$	*Ef1EF1*
Group D	*E1E1*	*E2E2*	*E3E3*	$Se1^eSe1^e$	*ef1ef1*
	E1E1	*e2e2*	*E3E3*	$Se1^eSe1^e$	*ef1ef1*

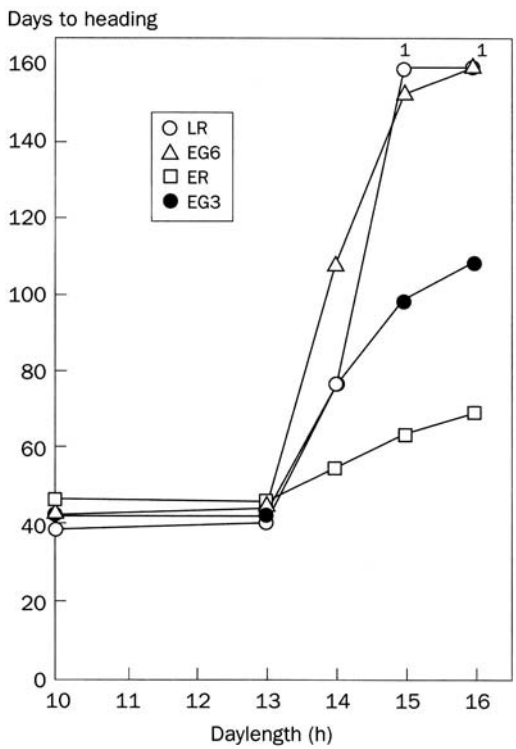

Fig. 1. Photoperiodic response of four lines differing in genotype for the *Se1* and *E1* loci. 1 no heading as of 160 d after sowing.

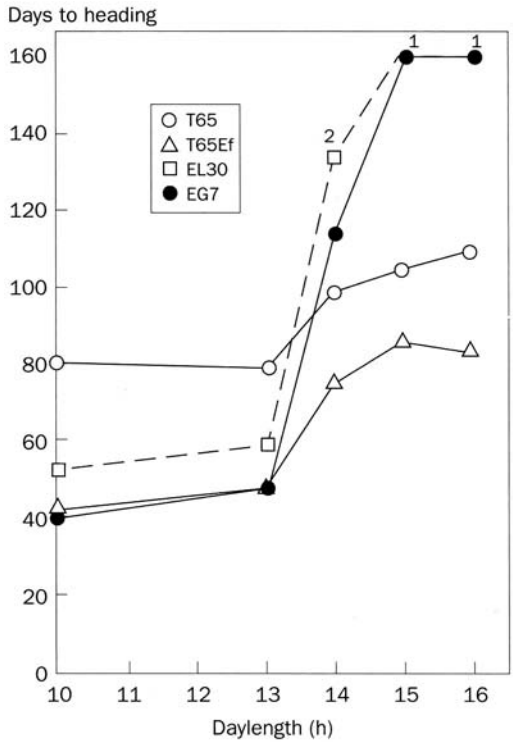

Fig. 2. Photoperiodic response of four lines differing in genotype for the *Se1* and *Ef1* loci. 1: no heading as of 160 d after sowing. 2: most plants were dead before heading due to defects. *EL30: E1E1 E2E2 E3E3 $Se1^nSe1^n$ ef1ef1.*

$Se1^nSe1^n$) and EG7 (*Ef1Ef1* $Se1^nSe1^n$) shows that the recessive allele, *ef1*, at the *Ef1* locus increases BVG. On the other hand, the difference in BVG between T65 and T65Ef shows that *ef-1* remarkably increases BVG. These facts indicate that the effect of *ef1* on BVG is enhanced by $Se1^e$.

Discussion

The varieties in Groups A, B, and C are cultivated primarily in southwestern Japan (N31-36°), around Tohoku (N36-41°), and on Hokkaido island (N42-45°), respectively. This indicates that photoperiod sensitivity in Japanese rices is highly associated with latitude. The experimental results revealed that the large differences in PS among Groups A, B, and C are caused by the differences in gene constitution of the two photoperiod-sensitivity loci, *E1* and *Se1*. Since the extremely early-maturing varieties of Group C and the early-maturing varieties of Group B are also cultivated in northeastern China and the southern Korean peninsula, respectively, *E1* and *Se1* are

considered very important loci related to the differentiation of japonica rice varieties cultivated in high-latitude areas.

This study also verifies that the effect of *ef1* is enhanced by $Se1^e$. Such a complementary effect can be seen in varieties cultivated in the lower latitudes of Taiwan. Accordingly, it can be concluded that the gene constitution of the *E1*, *Se1*, and *Ef1* loci determines the regional adaptability of japonica rice varieties cultivated in the temperate zone.

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Identifying cold tolerance gene(s) in rice at the booting stage

M. Maekawa

To identify gene sources of cold tolerance at the booting stage in rice (*Oryza sativa*), the possibility of trapping cold tolerance gene(s) from the wild species *O. longistaminata* was examined in BC_6F_2 populations using the cross *O. longistaminata*/Shiokari in 1992 and 1993. Variety Mpunga wa Majani, collected in Mombasa, Kenya, has rhizome and long anthers (4.83 mm). A BC_5F_1 plant and BC_6F_1 plants, except those of No. 2 and No. 4, showed high fertility in 1993. In 10 populations grown, No. 7 and No. 9 populations did not show any segregation for low fertility in 1992; they segregated into high fertility and low fertility groups in 1993. Of the total plants in No. 7, 34% showed high fertility in 1993. This result indicates that cold tolerance gene(s) at the booting stage segregate in the population and that such gene(s) could be identified using anther length.

Cool weather at the booting stage in rice causes pollen sterility, resulting in severe yield reduction. In 1993, Tohoku and Hokkaido in Japan had a cool summer and rice production was severely affected. To avoid or reduce severe damage in rice by the cool temperature, breeding projects have been carried out in Tohoku and Hokkaido to improve cold tolerance of rice. As a result, many cold-tolerant varieties have been released. However, the gene sources of cold tolerance were limited to such Japanese native varieties as Aikoku and Shinriki (Sasaki and Matsunaga 1985). Introduction of alien cold tolerance genes from foreign varieties has recently been made and a few parental lines for cold tolerance were bred (Abe et al 1989). To make varieties in Hokkaido and Tohoku cold-tolerant, it is important to identify sources of genes for cold tolerance.

Although the physiological mechanism for cold tolerance at the booting stage remains unclear, it has been reported that a long anther is correlated with cold tolerance (Suzuki 1981, Satake 1986, Takeda 1990). Thus, an attempt was made to introduce *O. longistaminata*'s long anther characteristic into Shiokari, a Japanese variety, by

backcrossing. The present study aims to confirm the possibility of trapping cold tolerance gene(s) from *O. longistaminata* based on anther length.

Materials and methods

O. longistaminata used in the experiment was collected in a valley located 10 km north of Mombasa, Kenya. Called Mpunga wa Majani (MwM), this variety has long anthers (4.83 mm) and rhizome (Table 1). Figure 1 indicates the process of producing BC_6F_2 populations from the Shiokari/MwM cross. Although anther length was used as a selection marker in the BC_1 and BC_2 generations, good fertility in the greenhouse during winter was adopted in later generations. Shiokari/MwM BC_6F_2 populations

Table 1. Spikelet length, anther length, and pollen and spikelet fertility of MwM, T65, Shiokari, and selected F_1 and F_2 plants.

	Selected plants				
Item	MwM	MwM/T65 F_1	T65	F_2	Shiokari
Spikelet length (mm)	7.83	7.45	6.40	7.60	5.80
Anther length (mm)	4.83	3.58	2.03	3.51	1.71
Pollen fertility (%)	-	73.5	91.6	83.4	92.1
Spikelet fertility (%)	-	44.7	88.7	0.0	83.6

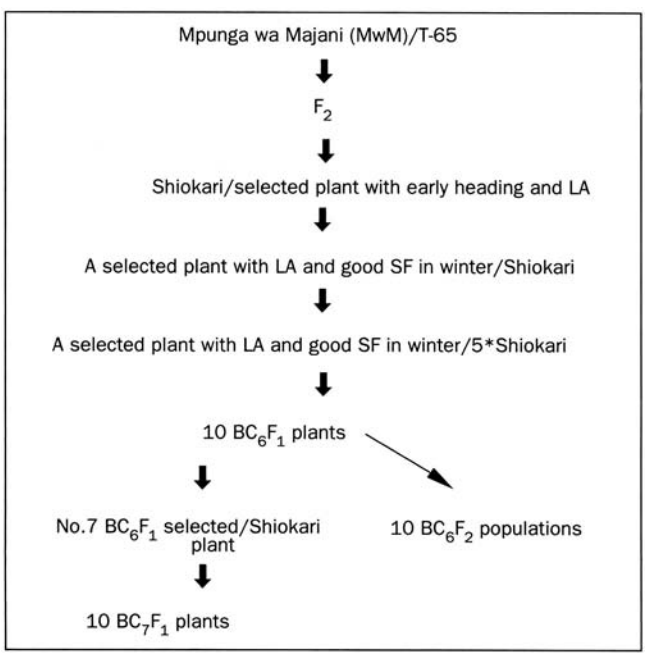

Fig. 1. Breeding of Shiokari/MwM near-isogenic lines. LA = long anther. SF = spikelet fertility.

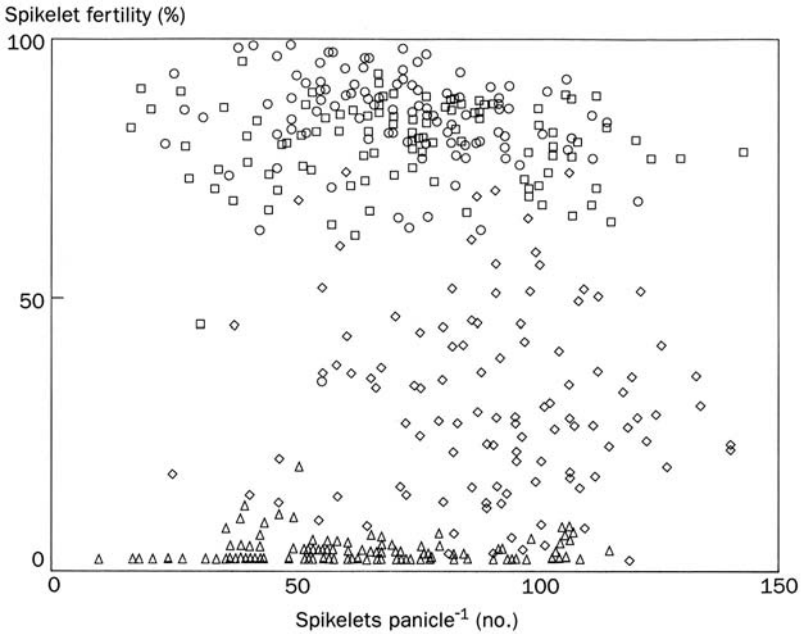

Fig. 2. Distribution of spikelet fertility in each panicle of Hayayuki (○), Yukihikari (□), Norin 20 (◇) and Shiokari (△) in 1993.

were grown at the Agricultural Experiment Farm, Hokkaido University, Sapporo, in 1992 and 1993. In 1993, rice production was affected severely by the cool summer but extent of damage varied with varieties (Fig. 2), indicating that cold tolerance could be assessed under natural conditions in 1993 and that Shiokari was extremely sensitive to cool temperature. At the same time, BC_5F_1, and BC_6F_1 ratoon plants were grown.

Spikelet fertility was expressed as the percentage of number of fertile spikelets to total number of spikelets of three normally developed panicles. Spikelets of the first primary branch at heading were fixed and the lengths of anthers and spikelets (except those of second spikelets) of the primary branch were measured by a profile projector (Nikon Co. Ltd. 6C).

Results and discussion

Frequency distributions of 10 BC_6F_2 populations grown in 1992 and 1993 are presented together with those of BC_5F_1 and BC_6F_1 plants and Shiokari in Figure 3. Although spikelet fertility of Shiokari was below 10% in 1993, it was also damaged in 1992. A BC_5F_1 plant and BC_6F_1 plants (except No. 2 and No. 4 populations) showed high fertility in 1993. This result indicates that the BC_5F_1 plant was heterozygous for cold tolerance gene(s) and the gene(s) was dominant. On the other hand, BC_6F_1 plants, from which No. 2 and No. 4 BC_6F_2 populations were derived, were homozygous for

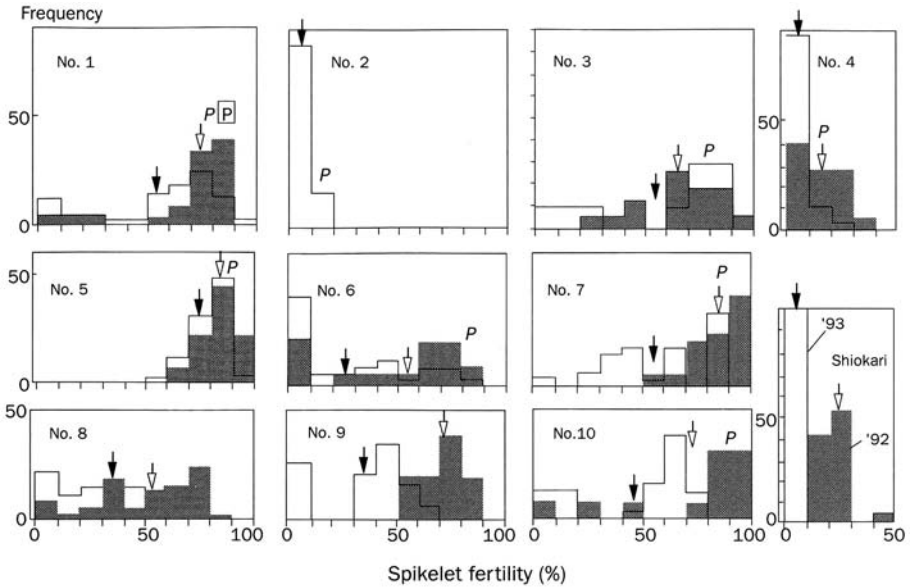

Fig. 3. Frequency distribution of spikelet fertilities in Shiokari/MwM BC_6F_2 populations in 1992 and 1993.

P = Spikelet fertility of BC_5F_1 plant in 1993.

***P* = Spikelet fertility of BC_6F_1 plant in 1993.**

↓ = Mean spikelet fertility of BC_6F_2 plants in 1992.

↓ = Mean spikelet fertility of BC_6F_2 plants in 1993.

Table 2. Mean days to heading in Shiokari/MwM BC_6F_2 populations and Shiokari.[a]

	Population no.	1992	1993	Difference
MwM BC_6F_2	1	103.3± 4.34 (25)	115.2 ± 4.05 (42)	-11.9
	2		106.0 ± 4.15 (6)	
	3	100.1± 4.03 (15)	114.7 ± 4.79 (10)	-14.6
	4	93.3± 2.50 (18)	102.6 ± 5.51 (35)	-9.3
	5	106.5± 1.94 (13)	118.9 ± 1.62 (40)	-12.4
	6	105.6± 4.52 (16)	119.3 ± 6.81 (31)	-13.7
	7	100.3± 3.86 (19)	115.0 ± 3.54 (32)	-14.7
	8	95.3± 2.69 (36)	109.8 ± 2.92 (55)	-14.5
	9	94.6± 1.30 (5)	110.8 ± 4.59 (20)	-16.2
	10	104.2± 5.23 (11)	117.7 ± 3.05 (23)	-13.5
Shiokari		91.5± 1.23 (17)	103.1 ± 3.87 (16)	-11.6

[a] Figures in parentheses indicate number of plants.

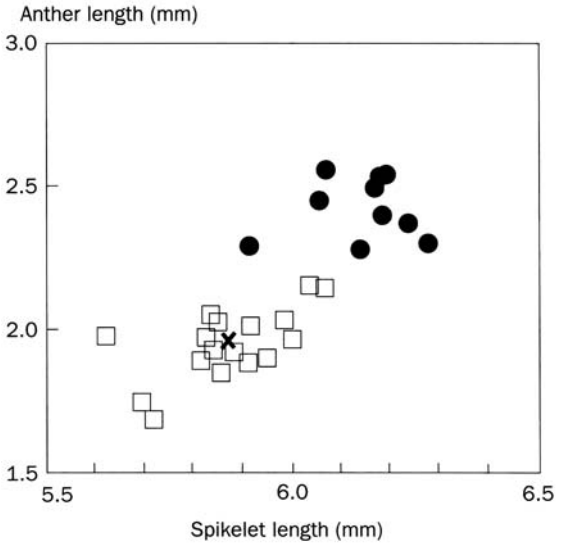

Fig. 4. Relationship between spikelet length and anther length in Shiokari/MwM BC_7F_1 plants (●) and Shiokari (□). X means average in Shiokari.

the recessive allele(s). Therefore, No. 2 and No. 4 populations were fixed at low fertility in 1993. In contrast, the No. 5 population was fixed at high fertility in the 2-yr study. Although this result suggests that cold tolerance gene(s) was fixed, the cause remains unknown. Population nos. 1, 3, 6, 8, and 10 segregated into high and low fertility groups in 1992 and 1993. In 1993, plants showing low fertility increased. Mean spikelet fertility in 1993 was observed to be lower than that in 1992. On the other hand, No. 7 and No. 9 populations did not show any segregation for low fertility in 1992 and segregated into high fertility and low fertility groups in 1993. It is especially interesting to note that 34% of the plants showed spikelet fertility above 80% in 1993. This result shows that cold tolerance gene(s) segregated in the two populations. On this basis, BC_6F_1 plants of the No. 7 population were considered to possess promising cold tolerance gene(s) and were backcrossed again with Shiokari. Figure 4 illustrates the relationship between spikelet length and anther length in Shiokari and Shiokari/MwM BC_7F_1 plants. All BC_7F_1 plants clearly have longer anthers and longer spikelets than Shiokari.

On the other hand, earliness is another important character that varieties in northern Japan must carry. On this point, mean days to heading in all BC_6F_2 populations were increased compared with Shiokari in 1992 and the differences between mean days to heading in 1992 and 1993 were amplified in the BC_6F_2 populations (Table 2). Sato et al (1994) have pointed out that the long anther character might bring about later heading in BC_6F_2 populations. Further breeding of isogenic lines with the same earliness but longer anther than Shiokari is needed to determine whether long anther improves cold

tolerance in rice. These results suggest that cold tolerance genes could be identified using the long anther character from *O. longistaminata* as a criterion.

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Genotypic variability in nitrate assimilation in rice

E.A. Barlaan and M. Ichii

In nitrate assimilation, reduction of nitrate to ammonia is catalyzed by nitrate reductase (NR) and nitrite reductase (NiR). To determine the activities of these enzymes in rice, 53 cultivars from six varietal groups based on isozyme variations were randomly selected. Seedlings were analyzed in vitro for nitrate reductase activity (NRA) and nitrite reductase activity (NiRA) and nitrate content. Significant differences in NADH-NRA, NADPH-NRA, NiRA, and nitrate content among rice cultivars and six varietal groups were observed. Differences in NRA and NiRA among cultivars within a group were significant for some groups but not so in nitrate content. Among rice genotypes, threefold differences in NADH-NRA and NiRA were found. Based on NADH-NRA, rice genotypes could be classified into three NRA categories—high, moderate, and low. NRA expresses the ability of the plants to use nitrate. Results suggest that certain cultivars and varietal groups were good nitrate assimilators. Cultivars with high NRA were identified. Of the six varietal groups, Groups II , V, and I (indica) had higher NRA than japonica, floating, and deepwater rices. In all cultivars, activity of NADH-NR was higher than that of NADPH-NR. In separate experiments, 14 cultivars with varying NRA levels were evaluated under irrigated lowland conditions. Significant and positive correlations were observed between NRA and grain yield and between NRA and biomass.

In biological systems, inorganic N is converted to organic form usually by nitrate assimilation. The process involves reduction of nitrate to ammonia catalyzed by nitrate reductase (NR) and nitrite reductase (NiR) and assimilation of ammonia into the amino acid pool. The importance of N in increasing crop yield is well recognized. For most higher plants, especially cereal and grain crops, nitrate is the major source of N. Rice is grown in different ecosystems, and under these conditions, it uses both ammonium and nitrate. The goal is to improve the efficiency of nitrate assimilation in rice, which

could be integrated in rice improvement programs in different ecosystems. However, there only have been a few studies on nitrate assimilation relative to NRA and NiRA in this crop and only a few cultivars were evaluated. Thus, a broader perspective on the activities of these enzymes and nitrate utilization is lacking. These studies investigated a number of cultivars for NADH-NRA, NADPH-NRA, NiRA, and nitrate content and evaluated genotypes with varying NRA levels under irrigated lowland conditions.

Materials and methods

Fifty-three cultivars representative of six varietal groups based on isozyme variation were randomly selected (see Glaszmann [1987] for group description). Seedlings were hydroponically cultured in full strength of Kimura's Solution B in a growth chamber programmed for 14-h light and 10-h darkness at 30±2°C. Two-wk-old seedlings were placed in an induction solution containing 2 mM KNO_3 and 500 µM $CaSO_4 \cdot 2H_2O$ (pH 5). After 24 h, seedlings were harvested and stored in -8 °C freezer. NRA of rice shoots was assayed in vitro by the procedure of Kleinhofs et al (1986). NiRA was determined by the reduction of NO_2^- from the assay mixture using the dithionite-methyl viologen method described by Ida and Morita (1973). Nitrate content was analyzed using the procedure of Steven (1986). The experiment was carried out using randomized complete block design with three replications. Cultivar effects were partitioned into among-group and within-group components.

To evaluate enzyme activities and yield and agronomic performance, genotypes with varying levels of NRA were grown under irrigated lowland conditions. Fourteen entries consisted of cultivars previously screened for relatively high or low NRA; NR mutant line M819 and original cultivar Norin 8; NR827, NR676, and original cultivar Nipponbare; C25, C27, C32, C33, and original cultivar IR30. The experiment was laid in a randomized complete block design with three replications. A single dose of 70 kg NPK ha^{-1} was applied basally. Twenty-eight-day-old seedlings were transplanted at 10- × 30-cm spacing with 1 seedling/hill in a six-row plot. Fifteen days after transplanting, expanded leaves of young plants were collected to determine the NADH-NRA, NADPH-NRA, and NiRA. Enzyme analyses were done using previously described methods. Grain and biomass yields were obtained from the two middle rows.

Results

Significant differences in NRA, NiRA, and nitrate content were observed among cultivars (Table 1). Mean values of NADH-NRA, NADPH-NRA, NiRA, and nitrate content of rice cultivars are presented in Table 2. Among rice genotypes, a threefold difference in NADH-NRA was obtained. The cultivar with the lowest NADH-NRA had 29% of the activity of the cultivar with the highest NADH-NRA. Based on NADH-NRA, rice genotypes could be classified into three categories—high, moderate, and low. The classification needs to be standardized since NRA is highly influenced by

Table 1. Mean squares from the analyses of variance for NR and NiR activities and nitrate content of rice cultivars.

Source of variation	df	Mean squares[a] NADH-NRA	NADPH-NRA	NiRA	NO_3^- content
Replication	2	67470.8	1377.7	26.1	97.0
Cultivars	52	31511.7**	54.7**	1024.7**	77.4*
Within group I	(9)	45396.9**	34.6 ns	692.6 ns	20.6 ns
Within group II	(9)	27136.3**	60.2*	1343.2*	22.9 ns
Within group III	(5)	20458.5**	36.8 ns	444.7 ns	16.3 ns
Within group IV	(6)	9917.2 ns	114.8**	185.7 ns	19.8 ns
Within group V	(9)	16913.3*	14.0 ns	661.1 ns	29.4 ns
Within group VI	(9)	25197.3**	46.5 ns	1371.6*	28.4 ns
Among groups	(5)	89003.8**	115.0**	2666.1**	583.1**
Error	104	7001.4	29.3	537.0	20.1
CV(%)		23.3	57.6	26.0	7.4

[a]ns = not significant, * = significant 5% level, ** = significant at 1% level.

many factors such as plant age, plant parts, growing conditions, induction, and bioassay procedures (in vivo vs in vitro), and other environmental conditions. In rice seedlings, two types of NR were identified: NADH-NR and NAD(P)H-NR. It was found that more NADH-NR was obtained than NAD(P)H-NR (Shen et al 1976). Likewise, results of the present study indicate a consistently much higher activity of NADH-NR than NADPH-NR in all cultivars. In NiRA, as in NADH-NRA, a threefold difference was observed among rice cultivars. The cultivar with the lowest NiRA had 30% of the activity of the cultivar with the highest NiRA. In nitrate content, a difference of 26% was observed between cultivars with the highest and lowest nitrate content.

While there were significant within-group differences in NADH-NRA, NADPH-NRA, and NiRA among cultivars for some groups, none was observed in nitrate content (Table 1). The sample size in each varietal group is acceptable enough as shown by differential significance in within-group differences.

Variation in NADH-NRA, NADPH-NRA, NiRA, and nitrate content existed among six varietal groups. Groups II, V, and I showed higher NADH-NRA than Groups III (deepwater rices), IV (floating rices), and VI (Fig. 1a). Groups IV and V were significantly different from Groups I and VI in NADPH-NRA (Fig. 1b). Groups I, II, IV, and V reduced more nitrate than did Groups III and VI (Fig. 1c). Groups III and IV had the highest nitrate content. In rice, it has been hypothesized that japonica and indica may have different nitrate assimilatory ability. From mean values of the typical indica types in Group I (n=9) and japonica in Group VI (n=6) in Table 2, indica has significantly higher NADH-NRA and NiRA than japonica. A 30% difference was observed between the two subspecies in both enzyme activities. However, no significant differences in NADPH-NRA and nitrate content were observed.

Under irrigated lowland conditions, significant differences in NADH-NRA, NiRA, grain yield, and biomass were observed among cultivars and lines (Table 3). Genotypes with high NRA also exhibited high grain yield and biomass. Significant and positive

Table 2. Mean values of NR and NiR activities and nitrate content of rice cultivars from six varietal groups.

Varietal group	Cultivar	Conventional group	NADH-NRA[a] (nmol NO_2^- min^{-1} g fresh $weight^{-1}$)	NADPH-NRA[a]	NiRA[a]	NO_3^- content[a] (µmol NO_3^- g $freshweight^{-1}$)
I	Bhasamanik	Indica	334.6 f-r	8.7 b-i	99.6 a-f	54.0 lm
	BPI 76 (Bicol strain)	Indica	467.7 a-g	9.4 b-i	97.6 a-f	61.0 b-n
	BR1	Indica	286.8 k-r	4.6 ghi	105.9 a-d	57.6 g-n
	British Guiana 79	Indica	605.0 a	12.8 b-h	89.6 a-f	57.0 g-n
	Kwang Lu Ai 4	Indica	386.8 c-n	5.9 d-i	89.3 a-f	56.6 g-n
	LEB Mue Nahng 111	Indica	336.2 f-r	3.2 hi	114.9 ab	54.3 k-n
	Mudgo	Indica	176.7 r	1.6 i	82.0 a-g	58.8 d-n
	Padi Bali	Javanica	209.6 pqr	6.6 d-i	71.3 b-g	54.6 j-n
	PTB10	Indica	389.5 c-n	10.1 b-i	93.3 a-f	57.4 g-n
	Suweon 287	Indica	376.7 c-o	6.0 d-i	122.6 a	52.1 n
II	Boro 1	Indica	504.2 a-e	8.4 b-i	99.6 a-f	56.3 h-n
	Chinsurah Boro 1	Indica	451.4 a-j	7.3 d-i	100.9 a-f	56.5 g-n
	Dharial	Indica	527.3 abc	9.8 b-i	59.0 efg	55.5 n
	Jhona 349	Indica	494.0 a-f	12.8 b-h	110.9 abc	54.6 j-n
	Kalamkati (aus)	Indica	302.8 h-r	8.9 b-i	97.0 a-f	56.3 h-n
	Kalu Balawee	Indica	272.9 l-r	5.5 e-i	121.9 a	52.8 g-n
	Kele	Indica	551.6 ab	14.9 b-f	90.6 a-f	55.7 i-n
	M142	Indica	412.1 b-l	18.3 ab	91.0 a-f	53.1 mn
	N22	Indica	379.4 c-o	5.7 d-i	55.3 fg	62.1 a-l
	Surjamukhi	Indica	371.4 c-p	4.4 ghi	105.6 a-d	52.5 n
III	Aswina	Indica	279.1 k-r	5.7 d-i	72.3 b-g	68.2 ab
	Bamoia 341	Indica	180.6 qr	5.9 d-i	65.6 c-g	68.1 ab
	Bhadoia 233	Indica	399.8 b-m	10.3 b-i	96.3 a-f	63.3 a-k
	Goai	Indica	238.9 m-r	7.8 c-i	73.6 b-g	64.9 a-h
	Laki	Indica	363.6 d-p	15.1 b-e	92.6 a-f	69.5 ab
	Taothabi	Indica	341.0 f-q	9.2 b-i	83.6 a-f	66.8 a-e
IV	Rayada 16-02	Indica	329.1 g-r	5.7 d-i	114.6 ab	67.2 a-d
	Rayada 16-03	Indica	340.4 f-q	11.7 b-h	101.6 a-f	64.0 a-i
	Rayada 16-04	Indica	254.0 i-r	9.6 b-i	88.0 a-f	66.8 a-e
	Rayada 16-08	Indica	299.6 i-r	14.4 b-g	97.0 a-f	68.6 ab
	Rayada 16-10	Indica	410.8 b-l	24.7 a	100.0 a-f	70.5 a
	Rayada 16-13	Indica	249.4 l-r	15.6 bcd	99.3 a-f	63.5 a-j
V	Atte	Indica	319.8 g-r	12.4 b-h	69.0 b-g	59.0 d-n
	Basmati	Indica	437.3 b-k	9.4 b-i	101.3 a-f	62.8 a-l
	Dome Siyah	Indica	461.5 a-i	14.4 b-g	122.9 a	61.9 a-m
	Dome Zard	Indica	510.8 a-d	10.5 b-i	86.6 a-f	58.8 d-n
	Firooze	Indica	526.6 a-d	10.5 b-i	93.3 a-f	58.5 d-n
	Hansraj	Indica	345.6 e-p	10.8 b-i	77.0 a-g	56.9 g-n
	Kataribhog	Indica	398.8 b-m	7.6 c-i	88.6 a-f	57.8 f-n
	Mehr	Indica	464.7 a-h	12.8 b-h	90.3 a-f	63.4 a-j
	Randhuni Pagal	Indica	325.5 g-r	7.6 c-i	82.3 a-g	62.3 a-l
	T3	Indica	371.9 c-p	11.4 b-i	99.3 a-f	66.9 a-e
VI	Azucena	Indica	294.8 j-r	5.0 f-i	105.6 a-d	56.5 h-n
	Chikushi	Japonica	221.7 o-r	4.8 ghi	102.8 a-e	57.3 g-n
	E425	Indica	385.6 c-n	17.4 abc	63.6 d-g	58.0 e-n
	RAT10	Intermediate	385.2 c-n	4.8 ghi	75.0 b-g	59.2 c-n
	Kotobouki Mochi	Japonica	212.3 pqr	4.8 ghi	64.3 d-g	56.3 h-n
	Nankeng 31	Japonica	231.8 n-r	6.0 d-i	36.6 g	58.0 e-n
	Sung Liao 2	Japonica	292.8 j-r	10.0 b-i	58.6 efg	65.4 a-g
	T9	Indica	493.3 a-f	9.6 b-i	90.3 a-f	63.0 a-k
	Taichung 65	Japonica	288.2 k-r	8.2 c-i	77.3 a-g	56.5 g-n
	Takao Mochi	Japonica	229.0 n-r	8.9 b-i	61.0 d-g	60.8 b-n

[a]Means followed by a common letter within the trait are not significantly different at the 5% level by DMRT.

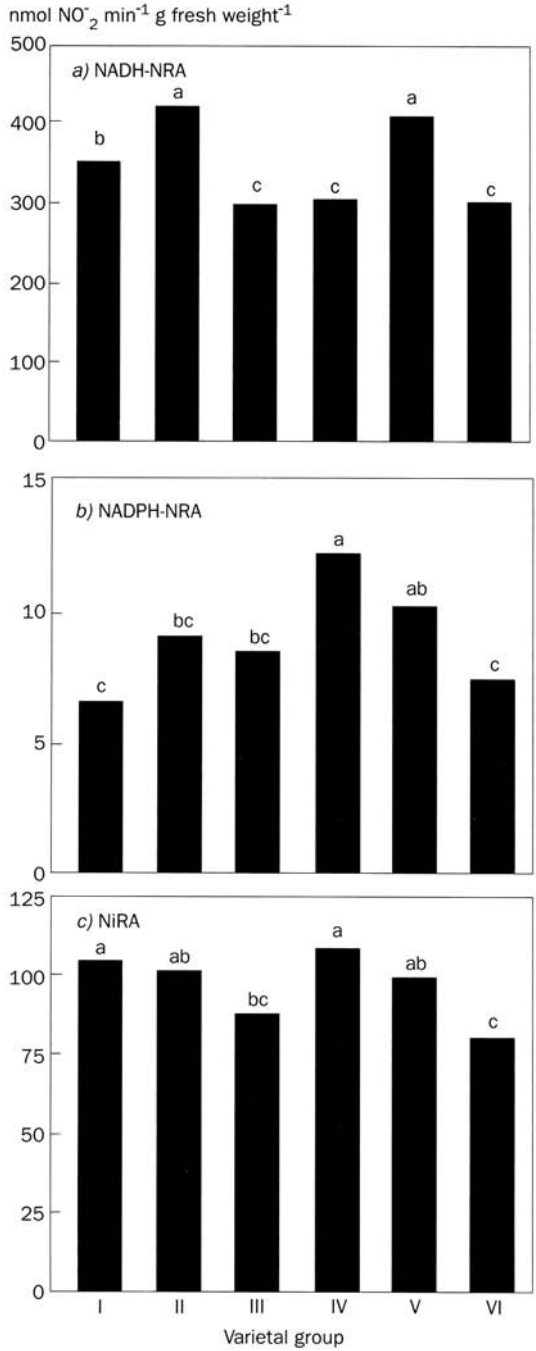

Fig. 1. Mean comparison among six varietal groups of rice in (a) NADH-NR, (b) NADPH-NR, and (c) NiR activities.

Table 3. Means of leaf NR and NiR activities at 15 d after transplanting, grain yield, and biomass of rice cultivars and lines evaluated under irrigated lowland condition.

Cultivar/ line	NADH-NRA	NADPH-NRA (nmol NO_2^- min^{-1} g fresh $weight^{-1}$)	NiR	Grain yield (g m^{-2})	Biomass (g m^{-2})
British Guiana 79	282.7	5.8	42.7	666.2	1880.3
Firooze	239.6	6.0	44.7	598.6	1514.9
Mudgo	245.0	7.6	32.7	682.2	2396.1
Dharial	126.6	6.2	39.0	621.8	1475.8
Norin 8	177.2	5.7	31.3	605.5	1445.9
M819	3.0	5.7	53.0	441.2	1336.0
Nipponbare	181.4	5.3	44.7	604.2	1463.5
NR827	89.2	4.8	20.7	323.2	642.2
NR676	87.8	5.2	42.3	279.7	559.1
IR30	247.1	6.0	22.7	523.7	1193.2
C25	7.5	5.0	64.3	339.8	1175.4
C27	25.8	5.3	43.3	235.0	1004.9
C32	38.9	5.0	64.7	301.0	1182.1
C33	31.8	5.8	65.3	139.4	987.1
Mean	127.4	5.7	43.7	454.4	1304.0
LSD .05	44.9	2.4	22.3	138.3	231.7
P (F-test)	**[a]	ns[b]	**	**	**
Cv(%)	21.0	24.9	30.4	18.1	10.6

[a] ***significant*** at the P = 0.01 level. [b] ns = not significant.

correlations between NADH-NRA and grain yield and biomass (r=0.82** and r=0.62*, respectively) and between NADPH-NRA and grain yield and biomass (r=0.60* and r=0.80**, respectively) were observed. NiRA was not related to both characters.

Discussion

Variability in nitrate assimilation exists in rice. Significant differences were observed in activities of NADH-NR, NADPH-NR, and NiR among rice cultivars and among varietal groups. Based on the differential enzymatic activities among cultivars and varietal groups, results of this study suggest that certain cultivars or varietal groups were good nitrate assimilators. NR is nitrate-inducible. Its activity expresses the ability of the plants to use nitrate (Chirkova and Belonogova 1991). Results may have implications on the selection of genotypes that would efficiently use nitrate in the soil and on the use of rice varieties that are suitable for nitrate-containing fertilizers. The indicas and japonicas are the most exploited cultivars in nutrient utilization and comparative fertilizer studies (De Datta 1981). Indicas belong to Group I and japonicas belong to Group VI; they have relatively lower NRA than those under Groups II and V. These areas, however, need further investigation. Rice prefers ammonium to nitrate under flooded field conditions. However, results suggest that cultivars with improved nitrate assimilation would also perform better under lowland conditions. A positive

and significant relationship between NRA and grain yield and biomass has been shown. Thus, selection of genotypes for improved nitrate assimilation is practically feasible.

The data also support the existence of six varietal groups based on differential enzyme activities. The classification of rice based on isozyme polymorphism provided a description of the organization of *Oryza sativa* in Asia and a possible explanation for the restrictions to recombination among rice cultivars (Glaszmann 1987). Only a few studies, however, were initiated to investigate its biological significance. Studies based on reactions to bacterial leaf blight (Ogawa et al 1991), restriction fragment length polymorphism (Wang and Tanksley 1989), and morphological traits and phenol reactions (Oka 1988) appeared to correspond with the six enzymatic varietal groups. Our results support its existence at the physiological level.

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Introducing lodging resistance into long-culm rices

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We compared dry matter production and yield of Tainung 67, a high-yielding rice cultivar (long-culm, ear-weight type) in Taiwan, China, with those of Nipponbare, a standard Japanese cultivar (short-culm, ear-number type). We found that the higher dry matter production and yield of Tainung 67 was due to the maintenance of higher CO_2 concentration inside crop stands, which provides a high CO_2 supply for the photosynthesizing leaves. Before using the beneficial traits of long-culm rices in a breeding program, it is important to understand varietal differences in physical characteristics of the culm as related to lodging resistance. In an analysis of 17 cultivars with wide genetic variation, we found high varietal differences in breaking strength of the basal internode related to lodging resistance, which can be associated with culm thickness, culm stiffness, and reinforcement of leaf sheath cover. Our analysis of the cross Koshihikari/Chugoku 17 revealed the possibility that lodging resistance could be introduced into long-culm cultivars.

Semidwarf type rice cultivars with short and erect leaves have been widely cultivated in Asia to get higher yields because of their high lodging resistance and low intercepting characteristics of the canopy. However, the maximum yield in rice has remained practically constant. It is necessary to improve the capacity of dry matter production notably for breeding higher yielding cultivars. The potential for dry matter production and yield is lower in short-culm cultivars than in long-culm cultivars (Takeda et al 1983). To obtain higher yield and dry matter production, the physiological and ecological characteristics in long-culm, ear-weight type cultivars for producing higher biomass must be studied. This paper examines the differences in yield, dry matter production, and the ecophysiological characteristics between a high-yielding rice cultivar (long-culm, ear-weight type) and a standard cultivar (short-culm, ear-number type). It also investigates the varietal difference in physical characteristics of the culm

related to lodging resistance and the possibility of introducing lodging resistance into long-culm cultivars.

Ecophysiological characteristics of dry matter production

Long-culm Tainung 67 was 18 cm taller than short-culm Nipponbare and its grain number m^{-2} was 38% higher because of more grains ear^{-1} in spite of fewer ears m^{-2}. Tainung 67's yield was 6.8 t ha^{-1}—19% higher than that of Nipponbare. The whole plant weight of Tainung 67 was 11 % heavier than that of Nipponbare at harvest. This was due to the higher crop growth rate (CGR) of Tainung 67 after heading, which was caused by higher net assimilation rate (NAR).

To clarify the cause of the difference in NAR between the two cultivars after heading, we compared canopy structure, light intercepting characteristics, and leaf area density (LAD, leaf area index divided by the plant height) related to the diffusive efficiency (CO_2, H_2O) inside the crop stands. In the canopy structure of Tainung 67, the leaves were distributed over a wide range due to its tall stature—from 20 to 150 cm above the ground. The light extinction coefficient, which reflects light intercepting characteristics, was a bit lower in Tainung 67 than in Nipponbare. The most notable characteristic in Tainung 67 was a smaller LAD due to its taller stature in spite of its higher leaf area index as compared with that of Nipponbare.

The evaporation rates inside the stands were measured using the improved piche evaporimeter to clarify the difference of the gas diffusion rate due to the difference of the LAD between the two cultivars. The evaporation rates of Tainung 67 were higher than those of Nipponbare. A higher CO_2 concentration inside the Tainung 67 stand was maintained during the daytime in spite its higher leaf photosynthetic rate. Thus, one of the factors contributing to the higher NAR in Tainung 67 was the higher gas diffusive efficiency inside the stands, which provides a high CO_2 supply for the photosynthesizing leaves.

Since a large difference in single-leaf photosynthetic rate has been found among leaves located at equivalent positions on the stem (Kuroda et al 1989), we compared the photosynthetic rates among the synchronously expanded leaves on the main stem and tillers of Tainung 67 and Nipponbare.

Immediately after the leaves had fully expanded, little difference was detected between the photosynthetic rates of the leaf on the main stem and the synchronously expanded leaves on the primary tillers in both cultivars.

Two or three weeks after expansion, the photosynthetic rates of the leaf on the main stem of Nipponbare were higher than those of the synchronously expanded leaves on the primary tillers. The extent of decrease in photosynthetic rates with leaf aging was higher in the leaves on the primary tillers than that on the main stem. In Tainung 67, however, the photosynthetic rates were not different between the synchronously expanded leaves on the main stem and the primary tillers. The extent of the decrease in the photosynthetic rates with leaf aging was not different between the leaf on the main stem and the leaves on the primary tiller. This characteristic of

Tainung 67 was considered to be important for maintaining a high photosynthetic rate within the canopy.

It is clear from these results that the maintenance of 1) higher CO_2 concentration inside the crop stands and 2) the same photosynthetic rates of the senescing leaves on the tillers and on the main stem provide an advantage for producing high yield and dry matter in long-culm cultivars.

Varietal differences in physical characteristics of the culm related to lodging resistance

To breed superior-yielding cultivars using the beneficial characteristics of the long-culm rices, lodging resistance should be introduced. Hence, we investigated the varietal differences in physical characteristics of the culm related to lodging resistance in 17 cultivars with wide genetic variation.

Severe lodging was observed in Japanese (Koshihikari, Fujimori) and American long-culm cultivars. However, some long-culm cultivars (Chogoku 117, Taichung 189, and Tainung 67) did not lodge. The lodging index—which is the ratio between the bending moment of the basal internode with leaf sheaths at breaking (MLS, breaking strength) and the bending moment by the whole plant added to the basal internode (WP)—is closely correlated with the degree of lodging among cultivars. Thus, we examined the relationship between the MLS and the WP among different cultivars. In Japanese long-culm cultivars, the lodging indices were high due to a small MLS. On the other hand, the lodging indices in the long-culm cultivars such as Chugoku 117, Taichung 189, and Tainung 67, were low due to the high MLS in spite of the high WP. In japonica short-culm cultivars such as Nipponbare, Reimei, and M-302, lodging indices were low because both the MLS and WP were low. In short-culm, high-yielding cultivars such as Akenohoshi and Milyang 23, the lodging indices were low because both the MLS and WP were high (Table 1).

Since MLS is a function of the degree of the reinforcement due to leaf sheath covering (RLS) and the bending moment of the basal internode at breaking (M), these parameters were further assessed. A higher RLS was found in Akenohoshi. The MLS of Akenohoshi, which has a large number of green leaf sheaths, was nearly double that of Koshihikari, which has a low number of green leaf sheaths (Table 1). On the other hand, M is the product of the section modulus (culm thickness) and the bending stress (culm stiffness). Values were found to be small in Japanese japonicas such as Koshihikari because of small section modulus. By contrast, Tainung 67 and Taichung 189 had a higher M due to the high bending stress, although the section modulus was slightly higher than that of Koshihikari. The high bending stresses in Tainung 67 and Taichung 189 were closely related to the high density of lignin (Ookawa et al 1993). In Milyang 23, M was highest because the section modulus was double that of Koshihikari.

These results suggest that it would be difficult to develop long-culm cultivars with lodging resistance in a breeding program that used only Japanese japonica cultivars mated among assorted genetic lines. Our data indicate that a large section modulus

Table 1. Comparison of lodging index and characteristics related to lodging resistance at 20 d after heading.

Cultivars	Lodging index	WP[a] (g·cm)	MLS[b] (g·cm)	M[c] (g·cm)	Section modulus (mm^3)	Bending stress ($g \cdot mm^{-2}$)	RLS[d] (%)	Green leaf sheath (no.)
Long-culm								
Chugoku 117	1.12	2482	2203	1450	17.8	823	34.2	6.0
Taichung 189	1.17	2474	2178	1655	12.3	1342	24.0	5.1
Tainung 67	1.04	2205	2122	1573	10.8	1469	25.9	5.1
Zenith	2.43	4120	1713	1538	24.0	640	10.2	2.7
Blue Bonnet	2.13	3499	1648	1565	24.1	655	5.0	3.3
Jaguary	2.32	3398	1478	1369	23.5	586	7.4	3.0
Fujiminori	1.58	1876	1190	973	11.0	894	8.3	4.9
Kamenoo	1.72	1849	1168	898	5.8	1565	23.1	4.6
Aikoku	1.71	1815	1073	913	8.0	1159	14.9	3.6
Koshihikari	1.72	1737	1018	815	7.6	1081	19.9	3.8
Short-culm								
Milyang 23	0.95	2495	2633	2119	21.8	976	19.5	4.9
Akenohoshi	1.02	2134	2100	1070	8.3	1308	49.0	5.6
Lemont	0.74	1221	1654	1245	12.9	969	24.7	4.5
Nanjing 11	1.26	1867	1520	1240	12.9	969	18.4	4.5
M-302	1.10	1462	1335	953	12.2	778	28.7	4.1
Nipponbare	1.04	1316	1268	843	5.5	1547	33.6	4.6
Reimei	1.16	1263	1093	848	8.1	1056	22.4	4.8

[a]WP = bending moment by whole plant added to the basal internode. [b]MLS = bending moment of the basal internode with leaf sheaths at breaking. [c]M = bending moment of the basal internode at breaking. [d]RLS = degree of reinforcement by leaf sheath covering.

(Milyang 23, Chugoku 117), high bending stress related to the high density of lignin (Tainung 67, Taichung 189), and a high degree of reinforcement of the leaf sheath covering related to delayed senescence (Akenohoshi) would confer high lodging resistance. Hence, if the lodging resistance in Chugoku 117, Tainung 67, Taichung 189, Akenohoshi, and Milyang 23 were introduced to the long-culm cultivars, the advantageous characters of long-culm cultivars could be fully utilized.

Introducing lodging resistance into long-culm cultivars

To investigate the possibility of introducing lodging resistance into the long-culm cultivars, we have examined the quantitative characteristics related to lodging resistance in a cross between Koshihikari (small section modulus) and Chugoku 117 (large section modulus) and their progeny.

In the F_1, the mean section modulus was much higher than that of Koshihikari. The section modulus in the F_2 ranged widely from 8 to 25 mm^3 and showed a trimodal frequency distribution. The ratio of 30:29:28 for [8-12 mm^3]:[12-19 mm^3]:[19-25 mm^3] lines did not fit the expected 1:2:1 ratio for one-gene segregation. However, this distribution suggests that some major genes were related to the section modulus. The section modulus showed a high heritability value (0.69) in the F_2 so that this character

was expected to be effective for selection. Despite negative genotypic correlation (Rg = -0.70) between section modulus and bending stress, three plants in the F_2 had a very high bending moment of the basal internode at breaking (M) due to the large section modulus and the large bending stress. The M and the section modulus in the two F_3 lines were almost large corresponding to the selected F_2 parental plants. From these results, there is a good possibility of introducing lodging resistance into long-culm cultivars by individual selection.

We need to study further the genetic analysis of quantitative characters (section modulus, bending stress, reinforcement of leaf sheath covering) related to lodging resistance. Furthermore, we need to examine the regulatory mechanism of the lignin biosynthesis in stem related to culm stiffness and the leaf senescence related to reinforcement due to leaf sheath covering.

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Genetic analysis of blast resistance in tropical rice cultivars using near-isogenic lines

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A set of near-isogenic lines (NILs) for rice blast resistance was previously developed in the genetic background of the indica cultivar CO 39. Allelism between the resistance genes in the CO 39 NILs and Kiyosawa's differentials was analyzed. *Pi1*(t) was closely linked to *Pi-k* on chromosome 11. *Pi2*(t) was allelic to *Piz* on chromosome 6. *Pi3*(t) was closely linked to *Pi-i. Pi4*^{a}(t) was identical to *Pita* on chromosome 12. To analyze *Pi5*(t), *Pi7*(t), and *Pi12*(t) in a durably resistant cultivar Moroberekan, we used an approach for developing pre-isogenic lines from a recombinant inbred population that has been used for molecular mapping of those resistance genes. *Pi7*(t) was allelic or closely linked to *Pi1.* The genetic analyses of *Pi5*(t) and *Pi12*(t) are currently under way.

Rice blast, caused by *Pyricularia grisea* Sacc., is one of the most destructive diseases of rice. Systematic genetic studies of blast resistance in the tropics have been hindered by the diversity of the host and pathogen and the presence of many resistance genes in rice cultivars. These conditions have prevented the establishment of a suitable differential set for discrimination of pathogen virulences in the tropics. The development of a set of near-isogenic lines (NILs) carrying single resistance genes is the most appropriate approach.

A set of 22 NILs for blast resistance was previously developed in the genetic background of indica cultivar CO 39 (Mackill and Bonman 1992). The donor parents of the CO 39 NILs were resistant indica cultivars Tetep and 5173 and resistant japonica cultivars Pai-kan-tao and LAC23. We showed that only five resistance genes *(Pi1*(t), *Pi2*(t), *Pi3*(t), *Pi4*^{a}(t), and *Pi-?)* were present in the 22 CO 39 NILs. We further determined the allelism relationships between those resistance genes and already known resistance genes in Kiyosawa's differentials (Table 1) (Inukai et al 1994a). One of the CO 39 NILs, C105TTP-4L23, was found to carry both *Pi4*^{a}(t) and an additional and unidentified resistance gene (referred to as *"Pi-?"* in this paper). We are now developing a line carrying only *Pi-?*. The gene symbols for the resistance genes analyzed here

Table 1. Complete blast resistance genes in CO 39 near-isogenic lines (NILs) and pre-isogenic lines (PILs) selected from recombinant inbred lines (RILs) of a cross between CO 39 and Moroberekan.

Line	Donor cultivar	Gene symbol		Chromosomal location	Relationship with known genes
		Original	Revised		
C101LAC	LAC23	*Pi1(t)*	*Pi1*	11	Linked to *Pi-k*
C101A51	5173	*Pi2(t)*	*Pi-z*^{5}	6	Allelic to *Pi-z*
C104PKT	Pai-kan-tao	*Pi3(t)*	*Pi3*	?	Linked to *Pi-i*
C101PKT	Pai-kan-tao	*Pi4*^{a}*(t)*	*Pi-ta*	12	Identical to *Pi-ta*
C105TTP-4L23	Tetep	*Pi4*^{b}*(t)*	*Pi-ta* *Pi-?*	12	Identical to *Pi-ta*
RIL 249	Moroberekan	*Pi5(t)?*		4?	Allelic or closely linked to *Pi3*
RIL 29	Moroberekan	*Pi7(t)*		11	Allelic or closely linked to *Pil*
RIL 10	Moroberekan	*Pi12(t)*		?	?

were revised based on the rules of gene nomenclature (Kinoshita 1993), and the new symbols are indicated in Table 1.

The available set of CO 39 NILs for blast resistance should be increased because the number of resistance genes in the set is insufficient. The available CO 39 NILs allow few pathogen races to be discriminated, compared with other differentials such as the international differentials (Inukai et al 1994b). Backcrossing breeding is a general method to develop NILs and is effective for the transfer of single genes for resistance. However, it may not be efficient when multiple genes for resistance are present in the donor cultivar. If multiple genes for resistance are not differentiated by the isolates used for selection, some of the loci may be "lost."

To solve this problem, we present here an approach for developing NILs from populations of fixed segregants that have been used for molecular mapping of resistance genes. All the resistance genes present in a donor cultivar can be detected because every chromosomal segment of the donor parent is present in at least one member of a permanent population of fixed lines (e.g., recombinant inbred [RI] lines or doubled haploid lines). Because the lines are fixed, the population can be analyzed in replicated experiments and with multiple pathogen isolates. Molecular marker data and phenotypic information obtained during the gene mapping process can be used to select lines carrying particular resistance genes and few other loci from the donor parent. We term such lines "pre-isogenic lines" (PILs) in the sense that they could be used as intermediates in the production of NILs.

We have used this approach to develop PILs for blast resistance genes originating from the durably resistant cultivar Moroberekan. Wang et al (1994) mapped multiple genes from conferring complete and partial resistance to blast using an RI population derived from a cross between Moroberekan and CO 39. Moroberekan was shown to have at least two genes for complete resistance to Philippine isolate PO6-6. As a first step toward the development of NILs carrying these resistance genes, we developed PILs for those resistance genes and subjected them to genetic analysis. Two putative major genes, designated *Pi5*(t) and *Pi7*(t), were located by Wang et al (1994) on

chromosomes 4 and 11, respectively. We also identified an additional resistance gene in Moroberekan using Philippine isolate Ca65 and temporarily designated it *Pi12*(t). To analyze individual resistance genes, we selected three lines (RIL 249, RIL 29, and RIL 10) from the RI population as PIL for *Pi5*(t), *Pi7*(t). and *Pi12*(t), respectively. Because skewed segregation in the RI population favored CO 39 (indica) alleles, these PILs carried 10.3-17.5% of Moroberekan alleles (the proportion expected after two or three backcrosses).

RIL 249, putatively carrying *Pi5*(t), showed a similar reaction pattern to the CO 39 NIL C104PKT carrying *Pi3* (Table 2). RIL 249 was shown to carry a single gene for resistance to PO6-6, which was further found to be allelic or closely linked to *Pi3* (Table 3). In progeny of a cross between RIL 249 and CO 39, resistance was not found to cosegregate with RFLP marker RG788 on chromosome 4, a marker which was shown to be closely linked to *Pi5*(t) in the previous study (Wang et al 1994) (data not shown). This result suggested that the gene in RIL 249 was not the same as *Pi5*(t) (this would imply that Moroberekan carries an additional resistance gene to isolate

Table 2. Reaction pattern of CO 39 NILs and PILs selected from RILs of a cross between CO 39 and Moroberekan to *Pyricularia grisea* isolates.

Line	Pi gene	Reaction to isolates[a]				
		PO6-6	IK81-3	IK81-25	V86010	Ca65
CO 39		S	S	S	S	S
C101IAC	*Pi1*	R	R	S	R	-
C101A51	*Pi-z*^{5}	R	R	R	R	-
C104PKT	*Pi3*	R	S	S	S	-
C101PKT	*Pi-ta*	S	R	R	S	-
C105TTP4L23	*Pi-ta, Pi-?*	S	R	R	R	-
RIL 249	*Pi5(t)?*	R	S	S	I	S
RIL 29	*Pi7(t)*	R	R	S	R	S
RIL 10	*Pi12(t)*	S	S	S	R	R

[a] R = resistant; I = intermediate; S = susceptible.

Table 3. Reaction of F_2 populations of crosses between PILs and CO 39 NILs to *Pyricularia grisea* isolates.

Cross	Test isolate	F_2 plants observed for each class (no.)		Expected ratio (R:S)	Probability
		R	S		
CO 39/RIL 249	PO6-6	61	19	3:1	0.75-0.90
CO 39/RIL 29	PO6-6	88	32	3:1	0.50-0.75
CO 39/RIL 10	V86010	66	33	3:1	0.05-0.10
RIL 29/RIL 249	PO6-6	227	11	15:1	0.25-0.50
C104PKT/RIL 249	PO3-82-51	466	0	1:0	
RIL 29/C101LAC	PO6-6	235	0	1:0	

PO6-6), or that *Pi5*(t) is actually not linked to the RG788 locus. Further analysis of the *Pi5*(t) locus is being conducted using a larger number of RI lines (G. Wang, pers. commun.).

RIL 29, putatively carrying *Pi7*(t). showed a similar reaction pattern to the CO 39 NIL C101LAC carrying *Pi1* (Table 2). The resistance gene in RIL 29 was confirmed to be allelic or closely linked to *Pi1* located on chromosome 11 (Table 3). The reaction pattern of RIL 10 carrying *Pi12*(t) to the four test isolates was clearly different from that of the existing CO 39 NILs, or any of the identified PILs carrying resistance genes to isolate PO6-6 (Table 2). The genetic analysis of *Pi12*(t) is currently under way.

The set of NILs will not only be a powerful tool for analyzing resistance genes and pathogen races but will also serve as donors for resistance breeding. Some of the genes in the CO 39 NILs have been tagged with molecular markers (e.g., Yu et al 1991) and could be transferred from the CO 39 NILs by marker-aided selection.

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Blast resistance genes from Teqing and Lemont

R.E. Tabien, S.R.M. Pinson, M.A. Marchetti, Z. Li, W.D. Park, A.H. Paterson, and J.W. Stansel

To determine the number of major genes that impart resistance to rice blast, parental, F_1, and F_2 seedlings of Lemont/Teqing and Rosemont/Teqing crosses were prick-inoculated with IB49, IB54, IC17, IG1, and IE1 races of *Pyricularia grisea.* In addition, a replicated set of Lemont/Teqing recombinant inbred lines (RILs) was spray-inoculated in the greenhouse as well as evaluated in a field nursery. Single-race and two-race reaction data suggested that Teqing has at least four blast resistance genes—two genes each effective against three races and two genes each conferring resistance to a single race. Lemont apparently has one recessive gene and at least one dominant gene. The reaction of RILs in the blast nursery was generally consistent with greenhouse inoculation tests. RILs that were resistant to all five races in the greenhouse study generally had smaller percent diseased leaf area (%DLA) in the field study. However, some RILs that were susceptible to all races as well as some that were resistant to only a single race had similar %DLA in the field as RILs that were resistant to two or more races in the greenhouse, suggesting the presence of rate-reducing or partial resistance genes. Also discussed is the current restriction fragment length polymorphism genotyping of RILs to map and tag resistance genes in Lemont and Teqing.

Use of resistant varieties has been the most economical and effective method of managing rice blast caused by *Pyricularia grisea,* but their development requires years of breeding. Closely linked markers could hasten selection of desired phenotypes and allow pyramiding of genes that would otherwise mask each other. At least 34 genes/loci have been identified and mapped using both classical and molecular approaches, two-thirds of which were major genes while one-third were quantitative trait loci. Although the genes were distributed on all chromosomes except chromosome 10, blast resistance genes were often found clustered (McCouch et al 1994). Multiple alleles for resistance have been reported at *Pi-k, Pi-z,* and *Pi-ta.* Blast has been epidemic

in the U.S. in recent years. Sixteen genes have been reported in U.S. breeding programs, some of which may be new, but allelism and gene locations are not yet fully known.

Materials and methods

Parental, F_1, and F_2 seedlings from Rosemont (susceptible)/Teqing and Lemont/Teqing crosses were planted in aluminum trays and were inoculated with five blast races (IB49, IB54, IC17, IEI, and IG1) using the pricking method (Iwano 1977) starting 3 wk after seeding. Two or three leaves were pricked for each race. To inoculate the same seedlings with all races, plants were initially inoculated with two races, scored 8 d later, then reinoculated with additional races 4-5 d after scoring. Inoculated plants were incubated in a dew chamber for 16-24 h (Marchetti et al 1987). Varieties having scores of 1-3 were considered resistant (R) while those with scores of 4-5 were susceptible (S) (Mackill and Bonman 1992). R-S ratios were analyzed using c^2.

Five sets of 245 recombinant inbred lines (RILs) (F_8) from Lemont/Teqing, one for each blast race, with three replications each, were seeded at weekly intervals and grown in the greenhouse. Seeds were planted into autoclaved field soil with 10-15 seeds $hill^{-1}$, 20 hills aluminum $tray^{-1}$ (23 × 11 × 11 cm). Each tray contained Lemont, Teqing, M-201 (S check), and 17 test lines. The 21-d-old seedlings were spray-inoculated and incubated (Marchetti et al 1987). Scoring was done as above. RILs were also evaluated in replicated blast nursery plots (Marchetti 1983). Disease development was evaluated weekly for 6 wk starting 21 d after seeding and was based on a visual estimation of percent diseased leaf area (%DLA) within a single-row plot.

Results and discussion

Lemont/Teqing F_1 plants were resistant to all races indicating that resistance is dominant over susceptibility. Single-race reaction data from both F_2 populations indicated that Teqing, the more resistant parent, has one major dominant gene for IB54 and two duplicate dominant genes each for IC17, IE1, and IG1. Rosemont/Teqing F_2 data indicated one gene for resistance to IB49 while Lemont/Teqing F_2 data indicated segregation for one dominant plus one recessive gene. The additional recessive gene was presumably from Lemont, but apparently not expressed since Lemont was susceptible to IB49. This gene may be masked by another gene in Lemont as was hypothesized in a case involving *Pi-z* (Marchetti 1983). Lemont is resistant to IBS4 and IGI while Rosemont is not. However, the segregation data did not differ between the two F_2 populations for these races.

The Lemont/Teqing RIL population segregated for two genes each for all five races. The recessive gene for resistance to IB49 detected in the F_2s was accounted for in the RILs, which segregated into 3R:1S, the expected ratio from two genes now fixed in their homozygous state. RIL data revealed a gene from Lemont for resistance to race IB54 that was not detected from F_2 data. The discrepancy between F_2 and RIL ratios may be attributed to differences in the inoculation methods used (Kiyosawa 1970). The gene from Lemont might be effective against spore attachment or peg

penetration of the fungi, which are bypassed with the pricking method. Again, Lemont is resistant to IG1, but the RIL data did not reveal any resistance genes other than those from Teqing for this race. The Lemont gene may be segregating along with one of the Teqing genes. Allelism and close linkage have been reported for several blast resistance loci (McCouch et al 1994).

To test the effectiveness of the detected genes to additional races, tests for commonality or independence were conducted in two-race combinations (Table 1).

Table 1. Segregation of F_2 plants from Rosemont/Teqing and RILs from Lemont/ Teqing based on two-race reaction at greenhouse inoculation test.

Race combination[a]	Reaction group				Remarks
	RR	RS	SR	SS	
Rosemont/Teqing (F_2)					
IB49 (1)/IC17 (2)	63	0	13	4	One common gene
IB49 (1)/IB54 (1)	47	16	10	7	Two independent genes
IB49 (1)/IEl (2)	63	0	13	4	One common gene
IB49 (1)/IG1 (2)	56	7	15	2	Three independent genes
IC17 (2)/IB54 (1)	59	19	0	4	One common gene
IC17 (2)/IE1 (2)	74	2	2	2	One common gene
IC17 (2)/IG1 (2)	69	7	2	2	One common gene
IB54 (1)/IE1 (2)	56	1	20	3	Three independent genes
IB54 (1)/IG1 (2)	53	4	18	5	Three independent genes
IG1 (2)/IE1 (2)	68	8	3	1	Four independent genes
Lemont/Teqing (RIL) (selected combinations shown)					
IC17 (2)/IB49 (2)	123	0	14	35	Few recombinants
IB49 (2)/IE1 (2)	120	17	0	35	Few recombinants
IC17 (2)/IG1 (2)	115	8	26	23	Favor resistance
IB54 (2)/IG1 (2)	127	9	14	22	Favor resistance
IB49 (2)/1854 (2)	117	20	19	16	Has additional common gene
IB49 (2)/IG1 (2)	123	14	18	17	Has additional common gene

[a]Numbers Inslde parentheses are estimated number of genes involved.

Table 2. Blast resistance genes identified from Teqing and Lemont and their interaction[a] with five races of *Pyricularia grisea.*

Gene	Race				
	IC17	IB49	IE1	IB54	IG1
Teqing					
Pi-q1	-	-	-	+	+
Pi-q2	-	+	+	-	-
Pi-q3	+	+	-	+	+
Pi-q4	+	+	+	+	-
Lemont					
Pi-b1	+	-	+	+	+
Pi-b2	+	+	+	-	-?

[a](+) = positive interaction (susceptible), (-) = negative interaction (resistant), (-?) = may be another gene for IG1.

Segregation in the Rosemont/Teqing F_2s showed that Teqing has four dominant genes: two genes each effective against three races and two genes each conferring resistance to a single race (Table 2). Two-race combination analysis of Lemont/Teqing F_2 data was restricted to the three races to which Lemont is susceptible. Segregation patterns mirrored those from the Rosemont/Teqing progeny except where the additional recessive gene from Lemont against IB49 was concerned. confirming that Teqing has four resistance genes. Data from spray inoculation of the RILs deviated from all expectations except for the IC17/IB54 race combination (Table 1). Linkage appeared to be involved because two-race analyses for IC17, IB49, and IE1 showed few or no recombinant types with an excess of individuals that were resistant or susceptible to both races. Gene interaction may have caused the increased proportion of resistant plants observed in other combinations, an issue to be addressed by our molecular studies.

Table 2 summarizes the genes identified from Teqing and Lemont. Considering the pedigrees of Teqing and Lemont, some of these genes are likely to be identical to previously reported genes. Tetep contains *Pi4* and is a progenitor of Teqing. Marchetti (1994) suggested that Lemont has *Pi-k*^{h}, which is effective against all races except IC17, IB1, and IB49. However, some RILs were resistant or susceptible to IB54 alone indicating that Lemont may contain another gene. Lemont may also have minor genes for blast resistance. This variety is only moderately susceptible in farmers' fields suggesting it has horizontal resistance. The reaction of some RILs in the blast nursery also suggested the presence of genes for partial resistance. Most lines were rated as generally resistant with only four lines reacting as susceptible as the check M-101. Additionally, some lines that were susceptible to all five races in greenhouse studies showed slower disease development than M-101.

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Monitoring race distribution and identification of genes for resistance to bacterial leaf blight

T. Ogawa

For controlling bacterial leaf blight of rice caused by *Xanthomonas campestris* pv. *oryzae,* the use of resistant cultivars is the most economical and effective method. Effective bactericidal agents are scarce and usually contain chemicals harmful to the environment. A method and strategy for monitoring race distribution and identification of new resistance genes is presented. A set of near-isogenic lines (NILs) developed using IR24, Milyang 23, and Toyonishiki as recurrent parents is proposed as international differentials for monitoring the race distribution of the pathogen. The resistance reactions of these NILs to Japanese and Philippine races of the pathogen are also classified.

An initial set of near-isogenic lines (NILs) (more advanced generation, the BC_4F_4) was developed using IR24, Milyang 23, and Toyonishiki as recurrent parents (Ogawa et al 1991). The NILs were developed with IR24 for use as international differentials to bacterial leaf blight (BLB) caused by *Xanthomonas campestris* pv. *oryzae* and as testers to identify new resistance genes to the disease. However, the genes recently identified are not related to these lines because the breeding of NILs carrying the new resistance genes is currently under way. Therefore, 18 lines and cultivars are recommended for monitoring the race distribution of the BLB pathogen (Table 1). By using this germplasm, it is likely that most races could be identified and effective resistance genes could be recognized in respective countries.

Table 1. Proposed international differentials for monitoring the race distribution of the bacterial leaf blight pathogen.

Differential	Ecotype	Resistance gene
Toyonishiki	Japonica	*Xa18*
IR24	Indica	*Xa16*
IR-BB 1	Indica	*Xa1, Xa12*
IR-BB 2	Indica	*Xa2*
IR-BB 3	Indica	*Xa3*
IR-BB 4	Indica	*Xa4*
IR-BB 5	Indica	*Xa5*
IR-BB 7	Indica	*Xa7*
IR-BB 8	Indica	*xa8*
IR-BB-10	Indica	*Xa10*
IR-BB 11	Indica	*Xa11*
IR-BB 21	Indica	*Xa21*
BJ1	Indica	*xa5, xa13*
Taichung Native 1	Indica	*Xa14*
Asominori	Japonica	*Xa17*
Optional		
M41	Japonica	*(xa15)*
XM5	Indica	*xa19*
XM6	Indica	*xa20*

Identification of a new resistance gene

For the identification of a new gene for resistance to BLB, the following steps are recommended.

Step 1 — comparison of the reaction pattern

The 18 differentials should be subjected to inoculation tests along with the resistant cultivar where the resistance gene is expected to be identified. If the reaction of the resistant cultivar is clearly different from that of the differentials and if it is confirmed that the resistant cultivar carries only one resistance gene in Step 3, the resistant cultivar can be assumed to carry a new resistance gene.

Step 2 — hybridization

It is always necessary to obtain the F_1 hybrids between resistant and susceptible cultivars.

Step 3 — determining the number of resistance genes

By breeding the F_2s derived from susceptible/resistant cross, the number of resistance genes in the resistant cultivar should be estimated from the ratio of segregation in the population.

Step 4 — allelic testing with dominant genes

When the F_1 hybrids obtained in Step 2 are resistant to the isolates, it is necessary to develop F_2 hybrids from crosses between the resistant cultivar and the 18 differentials that carry the dominant genes and show a similar reaction to that of the tested resistant cultivar. If susceptible plants are detected in every F_2 population, the resistant cultivar can be assumed to carry a new resistance gene.

Step 5 — allelic testing with recessive genes

When the F_1 hybrids obtained in Step 2 are susceptible, the resistant cultivar should be crossed with the differentials that carry a recessive gene and show a similar reaction to that of the resistant cultivar. If every F_1 hybrid is susceptible to the isolates, it is assumed that the resistant cultivar carries a new resistance gene.

If there is more than one resistance gene in the tested resistant cultivar in Step 3, a similar procedure should be adopted from Step 1 onward except for Step 3 after obtaining segregants carrying only one resistance gene from the hybridization between susceptible resistant cultivars.

Resistance reactions of cultivars

There are various kinds of resistance reactions to the BLB pathogen in rice cultivars (Table 2). Usually, it is recommended to observe rice plants from 2 to 4 wk after inoculation by applying the clipping method. Suitable concentration of bacteria for inoculation may be 10 or 8 cells ml^{-1}. The resistant plants show a lesion length below about 6 cm 3 wk after inoculation. However, the reactions vary depending on the virulence of the inoculum, weather conditions, plant age, etc.

The resistance reactions of cultivars can be classified into the following categories: 1) Highly resistant: no or minimal lesion development after inoculation (reaction of cultivar with *Xa10* gene to Philippine race 2).

Table 2. Reaction of NILs to Japanese and Philippine races of the BLB pathogen.[a]

NILs	Japanese race[b]						Philippine race[b]					
	IA	IB	II	IIIA	IIIB	IV	1	2	3	4	5	6
IR-BB 1	HR	M	S	S	S	S	S	S	S	S	S	S
IR-BB 2	HR	HR	S	S	S	S	S	S	S	S	S	S
IR-BB 3	RB	RB	RB	RB	RB	S	RB	RB	RB	RB	RB	S
IR-BB 4	R	R	R	R	R	R	R	S	S	M	R	S
IR-BB 5	R	R	R	R	R	R	R	R	R	M	R	S
IR-BB 7	HR	HR	HR	HR	HR	HR	R	HR	HR	S	HR	S
IR-BB 8	R	HR	HR	HR	R	R	R	R	R	M	R	M
IR-BB 10	S	S	S	S	S	S	S	R	S	S	R	S
IR-BB 11	S	R	R	R	S	S	S	S	S	S	S	S

[a]Reaction at booting stage: HR = highly resistant, RB = resistant with browning margin, R = resistant, M = moderately susceptible to moderately resistant, S = susceptible. [b]Standard isolates for each race are as follows: T7174 for race IA, T7156 for race IB, T7147 for race II, T7133 for race IIIA, 46803 for race IIIB, H75373 for race IV, PX061 for race 1, PX086 for race 2, PX079 for race 3, PX071 for race 4, PX0112 for race 5, PX099 for race 6. Reaction to Japanese race V(H75304) is not included due to its low virulence.

2) Resistant: lesion length below about 6 cm 3 wk after inoculation (reaction of cultivar with *Xa4* gene to Philippine race 1).
3) Resistant with lesions showing a brown margin: lesions are less than about 6 cm long at 3 wk after inoculation and characterized by a brown margin or spots around the lesions (reaction of cultivar with *Xa3* gene to Philippine races 1 to 4).
4) Moderately resistant or susceptible, i.e., variable: lesions continue to develop after inoculation, but more slowly than in the case of the susceptible cultivars (reaction of cultivars with *Xa4* and *Xa5* genes to Philippine race 4).
5) Susceptible: lesions continue to develop after inoculation.

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Molecular variation as a diverse source of disease resistance in cultivated rice

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Isozyme diversity in Asian cultivated rice allows for identification of various varietal groups, whose correspondence with classical morphological groups is only partial. The same global structure is revealed when larger numbers of other molecular markers are surveyed. To rationalize comparative diversity studies, we chose 261 accessions to represent the whole array of variation for several parameters, including geographic origin, culture type, and the classification based on isozymes. Within the framework of international collaboration, we looked at the reactions of these accessions to three major diseases after artificial inoculation: rice blast, using 13 strains of the fungus *Pyricularia grisea* for the inoculations; rice bacterial leaf blight, using six different strains of the bacteria *Xanthomonas oryzae* to inoculate the varieties; and rice tungro virus disease. The different groups under the isozyme classification scheme were compared. They displayed contrasting responses, with major differences in resistance frequencies and strong specialization of certain reference strains, particularly for blast. The minor varietal groups appeared especially rich in resistant materials. The structuring of cultivated rices into varietal groups thus appears essential for understanding host-pathogen interactions and for searching diverse sources of resistance to diseases. This is a good example where neutral markers have a predictive value for diversifying a germplasm collection for traits of agricultural interest.

One of the primary goals of rice improvement programs worldwide is to produce disease-resistant cultivars. Identification by rice pathologists of diverse sources of resistance requires a rational access to the huge array of germplasm available. Subspecific groups of *Oryza sativa* have been recognized empirically for centuries in China. They were found to reflect the species structure in most other regions of the world (Oka 1958) and were named indica and japonica. Isozyme diversity confirms

the existence of two major types (Second 1982). which might be related to two independent domestications (Second 1985). Finer analysis of isozyme diversity among Asian cultivars (Glaszmann 1987) showed that several specific types coexist with the two major groups. Their evolutionary origin is still unclear.

A few years ago, we constructed a restricted sample of germplasm consisting of 288 accessions that were selected to best represent the *O. sativa* species. The bases for the sampling were various parameters such as geographic origin, culture type, and position in the isozyme classification. In a collaborative effort between IRRI, Centre de coopération internationale en recherche agronomique pour le développement (CIRAD), and the Institute of Agricultural Sciences (IAS), these accessions were purified (a single plant homozygous for all isozymes was selfed) to study the diversity of responses to various pathogens. The distribution of reactions to leaf scald disease in this sample has been reported (Bonman et al 1990a). We also studied the reactions to other diseases by using 261 of the initial 288 accessions (Bonman et al 1990b). This paper illustrates how isozyme data, though representing neutral markers, contributed to widening the spectrum of resistance accessible in our sample.

Isozyme and other molecular markers

Isozyme classification recognizes six varietal groups (I to VI)—two are major groups corresponding to indica (I) and japonica (VI) (Oka 1958), two are minor groups distributed along the Himalayan foothills (II and V), and two are minute groups localized in Bangladesh and northeast India. Although isozymes are more restricted in number than other molecular markers, they give access to a structure of the species that was largely confirmed by subsequent analyses with other molecular markers, including random amplified polymorphic DNAs (MacKill 1995, Virk et al 1995) and restriction fragment length polymorphisms (Wang and Tanksley 1989, Second and Ghesquière 1994). Therefore, the sampling of diversity with isozymes may provide access to the diversity that can be revealed with other molecular markers.

Blast

Inoculations were conducted in greenhouses with six isolates from the Philippines, two from Korea, and one each from China, Japan, Cameroon, Brazil, and Panama. Disease was scored 6-7 d after inoculation, using a 0-5 scale (Bonman et al 1987) and the formula of Zhang et al (1987) for calculating a disease index (DI). A complete data set was obtained for 257 accessions. Various multivariate analyses were conducted on the data: some used the quantitative raw data whereas others used data converted to qualitative parameters to separate complete resistance from partial resistance. Only a principal component analysis (PCA) using quantitative data is presented here, for it synthetically illustrates the distinctive general pattern of reactions in the various varietal groups.

The PCA identified two major axes that accounted for 53.1 and 10.6% of the whole variation, respectively. Axis 1 separated the accessions resistant to most isolates

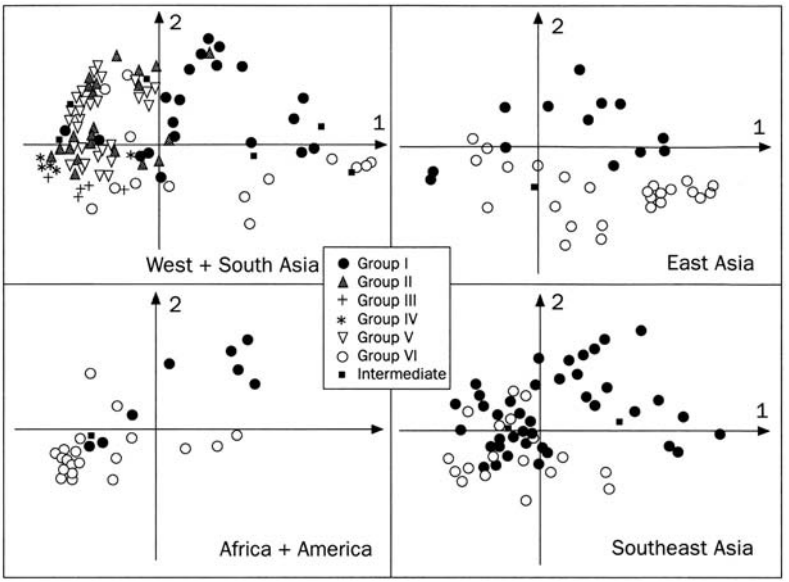

Fig. 1. Resistance to blast among rice varieties representing six varietal groups: distribution of 257 accessions on a plane (1,2) of a principal component analysis of disease index scored after inoculation of 13 strains of *Pyricularia grisea* separately.

(negative values) from the susceptible ones (positive values). Axis 2 separated the accessions susceptible to three isolates from China, Japan, and Korea and resistant to two isolates from Panama and the Philippines (negative values) from accessions with the symmetrical reactions (positive values). The distribution of the accessions on a plane (1,2) is given in Figure 1.

Clearly, accessions of groups II,III, IV, and V (present mainly in West and South Asia) cluster in the left portion of the plane, indicating general resistance to most isolates. Accessions of groups I and VI are scattered in the whole plane, indicating existence of resistances as well as susceptibilities in these groups. Among the accessions with susceptibilities (right portion of the plane), the distribution along axis 2 separates those of group I, in the higher portion of the plane, from those of group VI.

West Asia and South Asia are the regions where marker variation among accessions is most extensive. Within these regions, the diversity of reactions to blast is strongly related to the varietal groups. Accessions of groups II, III, IV, and V are restricted to the left portion of the plane, indicating general resistance; groups III and IV are localized within the lower left portion due to their higher resistance to the isolates from Panama and Cameroon. Accessions of groups I and VI spread along axis 1, of which majority are on the positive side and are separated along axis 2.

Macrogeographic patterns appear when considering only accessions of major groups I and VI. Among the four regions that were distinguished, Southeast Asia and Africa and America contain larger numbers of resistant accessions than West and

South Asia and East Asia. This geographic pattern is particularly marked for accessions of group VI, in which the most susceptible accessions are from West, South, and East Asia. It corresponds well with a "temperate" versus "tropical" distinction within group VI. Indeed, group VI varieties in West and South Asia are mostly grown at high altitudes and those in East Asia are mostly grown at high latitudes, whereas group VI varieties in Southeast Asia and Africa and America are mostly typical tropical varieties.

Bacterial blight

The 261 accessions were tested at IRRI for resistance to six isolates representing the six races of the pathogen (Mew 1987). They were scored based on percent leaf area infection (Baw 1984).

A PCA on raw data led to a pattern very similar to that derived from blast data. The first axis separated resistant vs susceptible accessions and accession of groups II, III, IV, and V were located on the side of resistant accessions. The second axis revealed a slight differentiation between groups I and VI. Unlike for blast, no global macrogeographic pattern was observed, other than a slightly higher resistance of the tropical forms of group VI compared with its temperate forms.

Tungro disease

All 261 accessions were tested at IRRI for resistance to tungro using a greenhouse mass-screening method (Hasanuddin et al 1988). The accessions were scored using a 1-9 scale for symptom severity. The distribution of the disease severity scores in the different varietal groups is shown in Figure 2. The diversity of reaction within each group spans from very resistant to very susceptible. However, there exist major differences in the frequency distribution of the different scores. Marked resistance is found essentially in groups IV and V. Despite their similar geographic distribution, groups II and V exhibit very contrasting responses to the tungro virus.

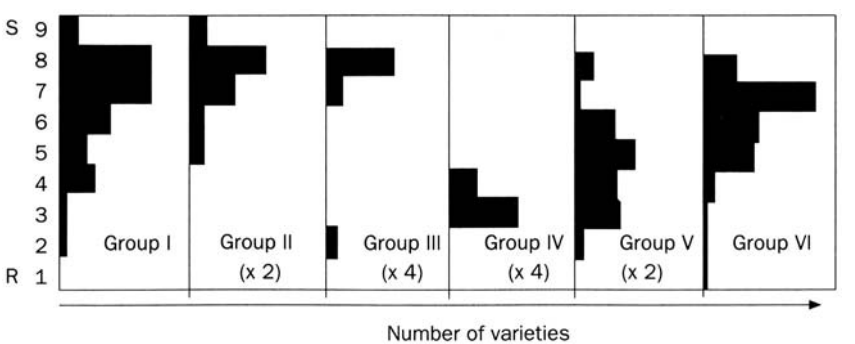

Fig. 2. Resistance to tungro virus in six varietal groups: distribution pattern in the different groups of the disease severity scores (from 1—resistant to 9—susceptible).

Discussion

Few studies have focused on the distribution of disease resistance in distinct varietal groups. A pioneering study showed that strains of the blast fungus tend to cluster according to their pathogenicity toward indica and japonica rices (Morishima 1969). A more recent study on bacterial blight included several hundred accessions representing groups I, II, V, and VI (IRRI 1987, Busto et al 1990) and illustrated very well the differentiation between groups. Among known resistance genes, *Xa3* is found mainly in groups V and VI, *Xa4* in group I, and *Xa5* in group II.

The present report does not attempt to discuss the genetics of the various patterns observed but it illustrates how passport data and simple descriptors can help to diversify a restricted sample of materials for useful traits. From our data, resistance to the three diseases obviously varies with the subspecific cultivar group. It also varies with the geographic origin of the rice accessions, but some situations allowed separating these two effects, showing marked differences between groups in a given region.

The basis of parallel differentiations for characters that are highly subjected to natural selection, such as disease resistance, and the expected neutral markers that enabled one to establish varietal groups must be searched in the evolutionary past of the crop. Before a full understanding of this history is available, it is possible to take neutral markers into account for preselecting the germplasm to be thoroughly evaluated and used in breeding. This applies not only for disease resistance, but also for many other traits whose diversity can be used by breeders.

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Physical mapping of several genes in rice using fluorescent in situ hybridization

N. Ohmido, H. Ohtsubo, E. Ohtsubo, and K. Fukui

Fluorescent in situ hybridization (FISH) was used to localize repeated DNA sequences on the mitotic chromosomes. Tandem repeat sequence (TrsA) was used as the probe. The TrsA was detected adjacent to the telomeric regions of the chromosomes in all the materials used—Oryza *sativa* (Nipponbare and IR36), *O. glaberrima,* and *O. meridionalis.* The number of TrsA sites varied among the A-genome species. In *O. meridionalis,* the TrsA sites were detected in the interstitial region of chromosome 4. Two repeated sequences, TrsB and TrsC, were isolated from *O. officinalis* (C genome) and *O. brachyantha* (F genome), respectively. The TrsB and TrsC were physically mapped on the interstitial region of a pair of *O. brachyantha* chromosomes and the distal ends of *O. officinalis* chromosomes, respectively. The distribution patterns of repeated sequences and genes using FISH offer indispensable information for understanding the genome organization and the dynamics of the chromosomes and nuclei.

In situ hybridization in rice uses a radioactive probe (Fukui et a1 1987) that produces an enzymatic color reaction (Fukui 1990, Fukui and Iijima 1991). In rice, however, the chromosomes are very small and the preparation of good chromosome samples is relatively difficult. Thus, we developed an improved method—flourescent in situ hybridization (FISH)—to localize genes on the rice chromosomes (Fukui et a1 1994, Ohmido and Fukui 1995).

In this study, we localized three repeated sequences of TrsA, TrsB, and TrsC isolated from *O. sativa, O. brachyantha,* and *O. officinalis,* respectively, on rice chromosomes using FISH.

Materials and methods

Nipponbare (japonica) and IR36 (indica) were used as representative cultivars of *Oryza sativa.* An African cultivated rice of *O. glaberrima* (W025), two wild rices of *O. officinalis* (C genome, W0002), and a cultivar of *O. brachyantha* (F genome, W1401) were also used. These materials were supplied by the National Institute of Genetics, Japan, except for *O. sativa.*

Root tips about 1 cm in length were excised and fixed in a fixative (1 ethanol:1 acetic acid) without any pretreatment. Chromosome samples were prepared using a modified enzymatic maceration/air drying method (Fukui and Iijima 1991). The enzymatic mixture was composed of 1% cellulase Onozuka RS, 0.75% pectolyase Y-23, 0.15% macroenzyme R200, 0.5 mM disodium ethylene diamine tetraacetate, and pH 4.2. Good chromosome samples at the prometaphase stages were stained with Giemsa solution. All rice chromosomes were identified prior to FISH based on the condensation pattern (Fukui and Mukai 1988, Iijima et al 1991, Fukui and Iijima 1992).

FISH guarantees clear and reproducible signals on the rice chromosomes (Fukui et al 1994). Three improvements characterize FISH: 1) post-treatments remove the thin fluorescence covering the chromosome samples. 2) a flat plate thermal cycler provides a strict temperature control, and 3) imaging techniques analyze the FISH signals digitally.

Tandem repeated DNA sequences were isolated from *O. sativa* (TrsA, 355-bp long, Ohtsubo et al 1991). Two repeated sequences, TrsB (159 bp) and TrsC (366 bp), were isolated from *O. brachyantha* and *O. officinalis,* respectively (Zhao et al 1989, Nakajima 1995). They were biotin-labeled by the direct-labeling method (Fukui et al 1994, Ohmido and Fukui 1995).

Results and discussion

Physical mapping of TrsA on somatic prometaphase chromosomes was carried out using variety Nipponbare. Two chromosomes—chromosomes 6 and 12—showed fluorescent signals on the distal end of their long arms. Twelve signals were detected on the chromosomes of IR36. Three pairs of hybridization signals were physically localized on the chromosomes of *O. glaberrima.* Clear fluorescent signals were detected at the distal end of the long arms of its chromosomes. Interestingly, in the case of *O. meridionalis,* the two bright hybridization signals were observed at the interstitial region of chromosome 4, while 22 signals were found at the ends of other chromosomes.

It is estimated that the rice genome consists of approximately 50% repeated DNA sequences (Deshpande and Ranjekar 1980). Two kinds of repeated sequences—tandem repeat sequences and interspersed repeat sequences—exist in the rice genome. In this study, three kinds of tandem repeat sequences have been successfully mapped on the rice chromosomes of six rice species.

Tandem repeat sequences were characterized by the flanking nucleotide sequences (Ohtsubo and Ohtsubo 1994). Near the TrsA sites. there are the GC-rich sequences

within the region of the AT-rich sequences. The GC-rich sequences have also been observed in the subtelomeric regions of chromosomes in the mouse genome. This suggests the TrsA sites are also located at the subtelomeric regions in the rice genome. The FISH results unequivocally demonstrate that the TrsA sites are located at the subtelomeric regions. The copy number of TrsA was shown to be different even among A-genome species by Southern blot analysis. The number of TrsA sites revealed by FISH analysis depends on the copy number estimated by Southern blot analysis.

TrsB was physically localized on the interstitial region of a pair of *O. brachyanrha* chromosomes. TrsC sites were detected at the subtelomeric region of *O. officinalis* similar to those of TrsA in *O. sativa.* This reflects the similar accumulation of repeated sequences at the subtelomeric regions. Tandem repeat sequences in rye have also been observed, especially at the subtelomeric regions, and showed high modification (Bedbrook et al 1980). This study demonstrates that tandem repeat sequences are specifically amplified after the differentiation of rice and that they vary in copy number.

Information on the distribution of genes and repeated DNA sequences is important to understand rice genome organization and the biological meaning of the repeated DNA sequences in the genome. It is also useful to localize DNAs with low copy numbers using FISH on the rice chromosomes.

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Chromosomal location of genes encoding glutelin polypeptides in rice

Y.J. Uemura, H. Satoh, M. Ogawa, H. Suehisa,
T.C. Katayama, and A. Yoshimura

Two cultivars, Kinmaze (japonica) and IR36 (indica), were used for the electrophoretic characterization of glutelin polypeptides. The glutelin subunits were separated by isoelectric focusing electrophoresis (IEF) using a horizontal slab gel system, yielding 11 bands for the acidic (α) glutelin subunit and nine bands for the basic (ß) glutelin subunit. Almost all IEF bands of glutelin subunits were detected as single spots in the two-dimensional electrophoresis (IEF/SDS-PAGE), indicating that the IEF system is very useful for detecting mutants for glutelin polypeptides and for genetic analysis of these polypeptides. A wide variation of IEF bands for glutelin subunits was found among local rice cultivars. The genetic analysis of these bands demonstrate that the variations of glutelin polypeptides are caused by spontaneous mutation and that almost all of the individual bands are controlled by incomplete single dominant genes and some of the genes cluster on specific chromosomes. Restriction fragment length polymorphism analysis using recombinant inbred lines confirmed that the genes controlling the α-2 band are located into a cluster on chromosome 1 and genes controlling the α-3 and ß-2 bands are located in close linkage on chromosome 2.

Glutelin, the major protein in the rice endosperm, is synthesized as a 57-kDa precursor polypeptide. Then, it is cleaved into two subunits, the a and b (Yamagata et al 1982). The polypeptide subunits show microheterogeneity (Wen and Luthe 1985). The nucleotide sequences for glutelin polypeptides have been clarified, showing that the genes encoding glutelin polypeptides consist of two or three subfamilies (Takaiwa et al 1987, Okita et al 1989). So far, little is known about the genetic properties of the glutelin genes due to the lack of effective methods to analyze individual glutelin polypeptides exactly, resulting in poor collection of materials necessary for their genetic analysis.

Recently, we established the electrophoretic method to analyze individual glutelin polypeptides derived from a single seed. This report deals with the electrophoretic characterization of glutelin polypeptides — the genetic analysis of glutelin genes.

Electrophoretic characterization of glutelin polypeptides

Japonica cultivar Kinmaze and indica cultivar IR36 were used for the electrophoretic characterization of glutelin polypeptides. Their seed storage proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a gradient gel. Glutelin subunits a and ß were separated into four and three bands, respectively. The a bands were designated as a -l (39 kDa), a - 2 (38 kDa), a - 3 (37.5 kDa and 37 kDa), and a - 4 (34 kDa and 33 kDa) and the ß bands were designated as ß–1 (23 kDa), ß-2 (22.5 kDa), and ß-3 (22 kDa). The a -3 band of Kinmaze was smaller in MMr than that of IR36. On the other hand, the a - 4 band of Kinmaze (34 kDa) was larger in MMr than that of IR36 (33 kDa).

After extraction from a single seed using 1% lactic acid, the glutelins of the two cultivars were analyzed by IEF using a horizontal slab gel system. They were separated into 11 bands ranging from pH 6.2 to 7.5 for the a submit. and nine bands ranging from pH 7.8 to 8.4 for the ß subunit (Fig. 1). The two-dimensional electrophoresis (IEF/SDS-PAGE) showed that the respective SDS-PAGE bands consisted of several IEF bands and almost all IEF bands were detected as single spots. This indicates that the IEF analysis using the horizontal slab gel system is very useful to detect mutants for glutelin polypeptides and is also effective for the genetic analysis of these polypeptides. Accordingly, individual glutelin polypeptides were specified by using

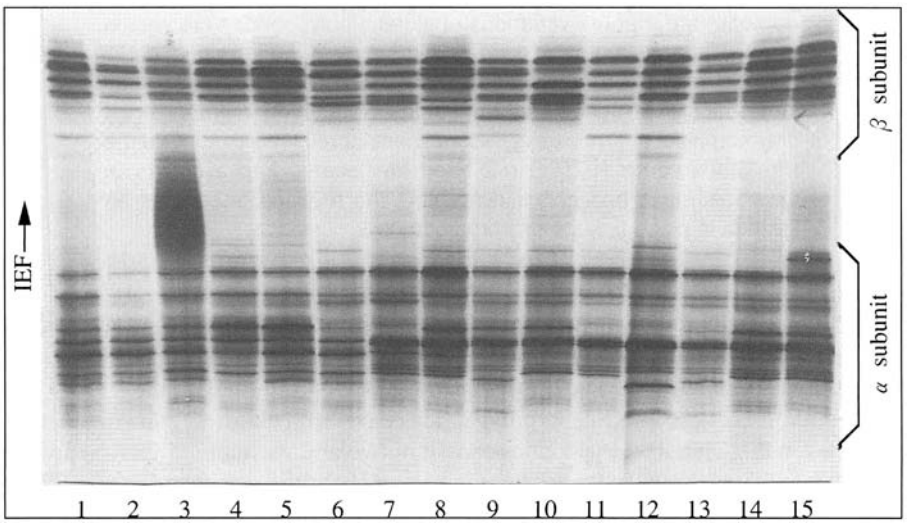

Fig. 1. IEF analysis of the rice glutelins of 15 rice varieties. 1: Kinmaze, 2: H023, 3: H0473, 4: H0621-1, 5: H0870, 6: H01177, 7: AL01001, 8: H0461, 9: H01195, 10: H01201, 11: H01233, 12: H01421, 13: H01438, 14: IR36, 15: IR24.

their own isoelectric point (pI) and MMr as Glu-6.71/38 (pI/MMr). The glutelin of Kinmaze is characterized by four unique IEF bands—Glu-6.50/38, Glu-6.71/38, Glu-8.13/22.5, and Glu-8.24/22.5, while IR36 has four specific IEF bands—Glu-6.59/38, Glu-6.80/38, Glu-8.37/22.5, and Glu-8.45/22.5. The Glu-7.19/37 band of Kinmaze was stained more intensively than that of IR36.

Genetic variations of glutelin polypeptides

The seed storage proteins of about 1,400 rice cultivars in Asia were analyzed by SDS-PAGE. The remarkable variation was found on the MMr of the a-3 and a-4 bands of these cultivars. Some cultivars such as IR24 have two a-3 bands with MMrs corresponding to 37.5 kDa and 37 kDa. In the ß subunit, a few cultivars have the ß-4 band (21 kDa). However, SDS-PAGE analysis did not clearly elucidate the genetic traits for glutelin polypeptides because each of the SDS-PAGE bands consists of more than two IEF bands.

The glutelins of 140 cultivars were analyzed by IEF. A wide range of variations was observed in the IEF band pattern of both subunits among the cultivars, such as the number of bands, the presence or absence of bands, and the staining intensity. In the b subunit, the pI and the number of bands differed among the cultivars. A close relationship was not observed in the band pattern between the a and the b subunits.

Genetic analysis of variations of glutelin polypeptides

Genetic behavior

Kinmaze and IR24 were used for the genetic analysis of glutelin polypeptides using the IEF system. IR24 has one more band, Glu-7.52/37, than IR36 (Fig. 1, 2). The glutelins of the F_1s were separated into 13 IEF bands for the a subunit and 11 IEF bands for the ß subunit. The gene dosage effect was observed on all of the unique bands in the reciprocal F_1 seeds from the cross Kinmaze/IR24 (Fig. 2). In the F_2 seeds, segregation of each band fitted the 1:1:1:1 (or 3:1) ratio, indicating that almost all of individual bands were controlled by incomplete single dominant genes. The recombinants were observed between most of the IEF bands, except for between Glu-6.71/38 and Glu-6.80/38, Glu-8.13/22.5 and Glu-8.24/22.5, and Glu-8.37/22.5 and Glu-8.45/22.5. Also, some of them were linked closely, suggesting that the variations of glutelin polypeptides are caused by a mutation occurring spontaneously for the structural genes coding glutelin polypeptides and that some of the genes cluster on the specific chromosomes.

RFLP mapping of glutelin genes

The glutelin genes were mapped on the chromosomes by using the F_9 seeds of 71 lines derived from the cross IR24/Asominori. The IEF band pattern of Asominori is similar to that of Kinmaze. RFLP analysis using recombinant inbred lines showed that the genes controlling the a-2 band are located into a cluster on chromosome 1

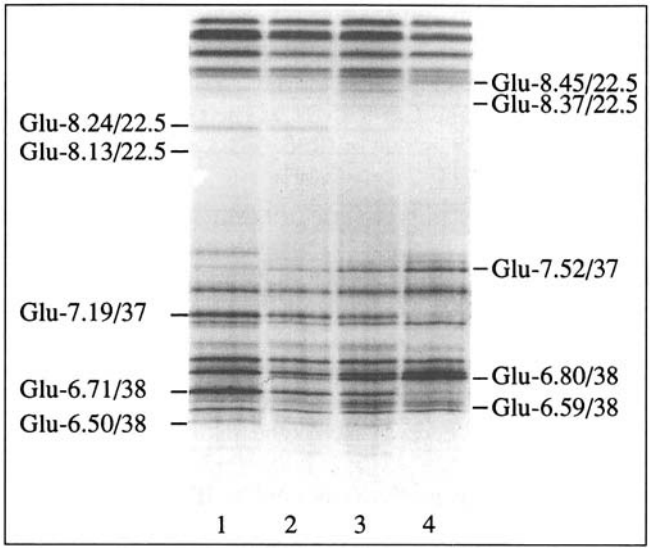

Fig. 2. IEF analysis of the glutelin in F_1 seeds of the cross Kinmaze/IR24. 1: Kinmaze (P1), 2: P1/P2, 3: P2/P1, 4: IR24 (P2).

and those controlling the a-3 and b-2 bands are located in close linkage on chromosome 2 (Fig. 3).

F_2 and RFLP analyses indicated the possibility that 1) Glu-6.50/38 and Glu-6.71/38 as well as Glu-6.59/38 and Glu-6.80/38 are the products of the same genes, or the genes coding them are linked tightly to each other, and 2) a gene coding Glu-6.71/38 is linked closely with a gene coding Glu-6.80/38, or the former is allelic to the latter and they inherit codominantly. It is also possible that Glu-8.13/22.5 and Glu-8.24/22.5 as well as Glu-8.37/22.5 and Glu-8.45/22.5 are the products of the same genes or the genes coding them are linked tightly to each other. Therefore, the genes for Glu-6.50/38 and Glu-6.71/38, and Glu-6.59/38 and Glu-6.80/38 are designated as *Glu1*^{a} and *Glu1*^{b}, respectively. The genes coding Glu-7.19/37 and Glu-7.52/37 are designated as *Glu2* and *Glu3*, respectively. The gene coding Glu-8.13/22.5 and Glu-8.24/22.5 is designated as *Glu4*, and the gene coding Glu-8.37/22.5 and Glu-8.45/22.5 is designated as *Glu5*.

Our results coincide with those obtained by in situ hybridization (Suzuki et al 1991). The *Glu1* gene reported by Nakamura et al (1995) is most likely the same *Glu1* gene in our study on the basis of the two-dimensional electrophoresis and the genetic analysis. We found a wide variation of glutelin polypeptides in the pI among local rice cultivars by using the IEF system. These variations will offer valuable information on the genetic analysis of the glutelin genes.

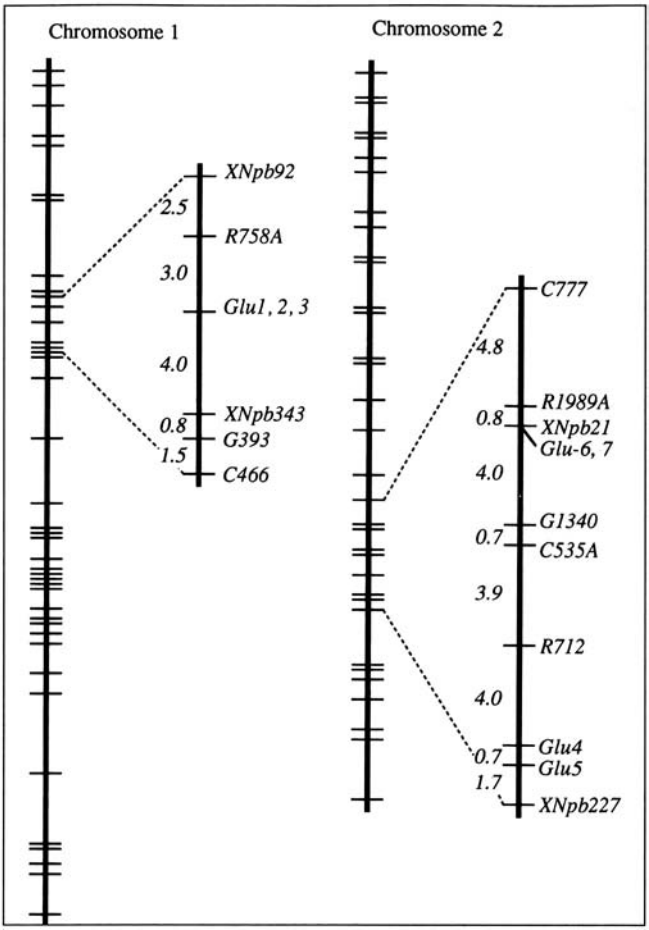

Fig. 3. Tentative locations of the genes encoding glutelin polypeptides. Seven loci of the genes encoding glutelin polypeptides are listed on the right and the map distances in the recombination values (%) are on the left. *Glu1:* Glu-6.71/38 and Glu-6.50/38 (*Glu1*^{a}); Glu-6.80/38 and Glu-6.59/38 (*Glu1*^{b}); *Glu2:* Glu-7.19/37; *Glu3:* Glu-7.52/37; *Glu4:* Glu-8.13/22.5 and Glu-8.24/22.5; *Glu5:* Glu-7.52/37/22.5 and Glu-8.45/22.5.

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Gene transfer and molecular characterization of introgression from wild *Oryza* species into rice

D.S. Brar, R. Dalmacio, R. Elloran, R. Aggarwal, R. Angeles, and G.S. Khush

Following embryo rescue, a series of hybrids, monosomic alien addition lines, and advanced backcross progeny have been produced from crosses of elite breeding lines of rice and various wild species. Genes for brown planthopper and bacterial blight resistance have been transferred from *Oryza minuta* and *O. brachyantha,* respectively, into rice. Meiotic analysis of hybrids of rice with *O. brachyantha* and *O. granulata* showed 0.06 and 0.29 bivalents per cell, respectively, indicating limited homoeology between genomes of rice and wild species. Restriction fragment length polymorphism (RFLP) analysis of 29 derivatives of *O. sativa/O. brachyantha* and 40 derivatives of *O. sativa/O. granulata* using 52 DNA markers and 7 restriction enzymes showed extensive polymorphism between rice and wild species. Of the six chromosomes analyzed, no introgression was detected from chromosomes 7, 9, 10, and 12 of *O. granulata* and chromosomes 10 and 12 of *O. brachyantha.* For each of the remaining chromosomes, one to two RFLP markers showed introgression in some of the derived lines. Although the level of introgression was low, the results show possibilities of introgressing chromosome segments even from distantly related genomes into cultivated rice and thus the feasibility of transferring useful genes from distant *Oryza* species.

Rice is grown under a wide range of agroclimatic conditions and several factors such as diseases, insects, and unfavorable soil and water conditions affect its productivity. The genetic variability for some traits, such as resistance to tungro, sheath blight, yellow stem borer, and tolerance to acid sulfate conditions, is limited in the germplasm of cultivated rice. Under such situations, wild species are a good source of useful variability. However, several incompatibility barriers limit the transfer of such useful alien genes. Among these, limited recombination between the chromosomes of cultivated and wild species is the most important. Recent advances in restriction

fragment length polymorphism (RFLP) and in situ hybridization techniques have enabled precise detection of the introgression of chromosome segments from wild species into rice.

Several useful genes have been transferred from wild species into rice: grassy stunt resistance from *Oryza nivara* (Khush 1977), bacterial blight (BB) resistance *(Xa21)* from *O. longistaminata* (Khush et al 1990), brown planthopper (BPH) and whitebacked planthopper resistance from *O. officinalis* (Jena and Khush 1990), blast and BB resistance from *O. minuta* (Amante et al 1992), and BPH and BB resistance from *O. australiensis* (Multani et al 1994).

Limited information is available on the extent and nature of introgression at the molecular level from distantly related genomes of *Oryza* into cultivated rice. The present study was undertaken to determine the possibility of gene transfer from distantly related species and to detect introgression of chromosome segments using RFLP markers from *O. brachyantha* and *O. granulata* into rice.

Materials and methods

Materials

Hybrids derived from elite breeding lines of *O. sativa* and various wild species, BC_1, monosomic alien addition lines (MAALs), and advanced backcross progeny were used.

Chromosome analysis

Spikelets at a suitable stage were fixed in a 1:3 mixture of acetic acid and 95% ethanol to which traces of ferric chloride had been added. Meiotic chromosome associations were studied at diakinesis/metaphase I in interspecific hybrids following the 1% aceto carmine squash technique.

Alien gene transfer

Advanced backcross progeny from *O. sativa/O. minuta* were screened for resistance to BPH biotype 1 of the Philippines in the greenhouse. Advanced backcross progeny from *O. sativa/O. brachyantha* were inoculated with Philippine races 1, 2, 4, and 5 of BB in the field and race 6 in the greenhouse.

RFLP analysis

DNA was isolated from young leaves of parents (IR56 and elite breeding line IR31917-45-3-2; *O. brachyantha* Acc. 101232; *O. granulata* Acc. 100879), F_1, BC_1, MAALs, and advanced backcross progeny. RFLP analysis was done using mapped markers on chromosomes 6, 7, 9, 10, 11, and 12 (Causse et a1 1994). Fifty-two markers comprising 30 cDNA and 22 genomic probes were used in combination with seven restriction enzymes (*Eco*RI, *Eco*RV, *Dra*I, *Hin*dIII, *Bam*HI, *Xba*I, *Sca*I). The hybridization and washings were carried out at 60 °C at 2x SSC. The probes were labeled with ^{32}P-dCTP using the multiprime kit of Amersham, Inc. following the manufacturer's instructions.

Results and discussion

Chromosome analysis

Meiotic analysis of F_1 hybrid between *O. sativa* and AA genome wild species *O. rufipogon* at diakinesis/metaphase I showed normal chromosome pairing, the average being 11.97 bivalents cell^{-1}, with very low frequency of univalents (0.04 bivalents cell^{-1}). On the other hand, F_1 hybrids of *O. sativa* with *O. brachyantha* and *O. granulata* showed limited chromosome pairing (Table 1), 0.06 and 0.29 bivalents cell^{-1} respectively, indicating very low homoeology between the chromosomes of rice and those of the two wild species. Wuu et al (1963) observed limited pairing (0.03 bivalents cell^{-1}) between *O. sativa* and *O. brachyantha* genomes. Similarly, Katayama and Onizuka (1979) observed 0.19 bivalents cell^{-1} in *O. sativa/O. meyeriana* (a species related to *O. granulata)* hybrids. Meiotic analysis of the BC_1 involving crosses of *O. sativa* with *O. brachyantha* and *O. granulata* showed 2n = 36 chromosomes. These are probably the result of pollination of unreduced female gametes carrying 24 chromosomes with male gametes having 12 chromosomes.

Alien gene transfer

Transfer of BPH resistance from O. minuta. Advanced backcross progeny derived from the cross of IR31917-45-3-2 (an elite breeding line of rice) with *O. minuta* (Acc. 101141) were evaluated for resistance to BPH biotype 1 of the Philippines. The recurrent rice parent is susceptible to all three BPH biotypes whereas *O. minuta* is resistant. Of the 96 backcross F_1 plants (6 BC_2F_1, 50 BC_3F_1, 40 BC_4F_1) screened, 10 (2 in BC_2F_3, 6 in BC_3F_4, and 2 in BC_4F_4) were found to be segregating for resistance to biotype 1.

Transfer of BB resistance from O. brachyantha. Similarly, advanced backcross progeny derived from IR56/*O. brachyantha* (Acc. 101232) were evaluated for resistance to BB races 1, 2, 3, 4, and 6 of the Philippines. IR56 is susceptible to these BB races whereas *O. brachyantha* is resistant. Of the 149 backcross F_1 plants

Table 1. Chromosome associations of diakinesis/metaphase I in interspecific hybrids between elite breeding lines of *O. sativa* and wild *Oryza* species.

Hybrid combination			PMC[a] studied (no.)	Bivalents cell^{-1} (no.)		Univalents cell^{-1} (no.)	
Female parent *(O. sativa)*	Male parent (wild species)	Wild species (accession number)		Range	Mean	Range	Mean
IR64	*O. rufipogon* (AA)	105909	163	10-12	11.97	0-4	0.04
IR31917-45-3-2	*O. minuta*[b] (BBCC)	101141	229	0-8	2.67	20-36	30.48
IR56	*O. brachyantha* (FF)	101232	203	0-2	0.06	20-24	23.85
IR31917-45-32	*O. granulata* (genome unknown)	100879	231	0-2	0.29	20-24	23.40

[a]PMC = pollen mother cell. [b]Also showed 0.04 trivalents cell^{-1}.

(5 BC_2F_1, 74 BC_3F_1, 70 BC_4F_1) analyzed, 27 (0 in BC_2F_3,14 in BC_3F_4, and 13 in BC_4F_4) segregated for resistance to BB races 1, 2, 3, 4, and 6 BC_2 progeny resembled the recurrent *O. sativa* parents, suggesting a limited amount of recombination between the genomes of *O. sativa* and *O. brachyantha.* Gene transfer for resistance to BPH and BB was not accompanied by any undesirable traits of *O. minuta* and *O. brachyantha.* The appearance of a large number of progeny for resistance to BPH and BB may be due to involvement of several loci for resistance.

Molecular characterization of introgression

RFLP analysis was conducted to examine possible introgression of chromosome segments from wild species to cultivated rice. A parental survey for RFLP markers showed extensive polymorphism between rice and *O. brachyantha* and *O. granulata.* Hybridization signal intensities were moderate to low for most of the markers in the wild species (Fig. 1). Strong hybridization signals were detected in cultivated rice as well as *O. rufipogon,* another AA genome species. In comparison, hybridization intensities were much weaker in *O. brachyantha* and *O. granulata.* These observations suggest that the genomes of *O. brachyantha* and *O. granulata* are highly diverged at the molecular level from the AA genome of *O. sativa.*

Despite high divergence, introgression could be detected for few of the RFLP markers in low frequencies in the derived lines in both the crosses *O. sativa/O. brachyantha* and *O. sativa/O. granulata* (Fig. 2, 3, Table 2). Of the six chromosomes analyzed, no introgression was detected from chromosomes 7, 9, 10, and 12 of *O. granulata* and chromosomes 10 and 12 of *O. brachyantha.* For each of the remaining two chromosomes (6 and 11) of *O. granulata* and four (6, 7, 9, and 11) of *O. brachyantha,* one to two RFLP markers were introgressed into the derived lines (Table 2). Nonparental bands were also detected for some of the markers. Although the level of introgression observed was low, these results show the possibility of

Table 2. RFLP analysis of 29 derivatives of *O. sativa/O. brachyantha* and 40 derivatives of *O. sativa/O. granulata* using 52 DNA markers and 7 restriction enzymes.

Chromosome	RFLP markers tested (no.)	RFLP markers showing introgression in derivatives[a]	
		O. sativa/ O. granulata	*O. sativa/ O. brachyantha*
6	9	RG516 (3) RZ884 (2)	RZ588 (1)
7	11	0	RG30 (1)
9	9	0	RG757 (2)
10	6	0	0
11	8	RG118 (1) RG304 (1)	RG304 (2)
12	9	0	0

[a] Figures in parentheses Indicate number of derivatives showing positive introgression for allele(s) of wild species.

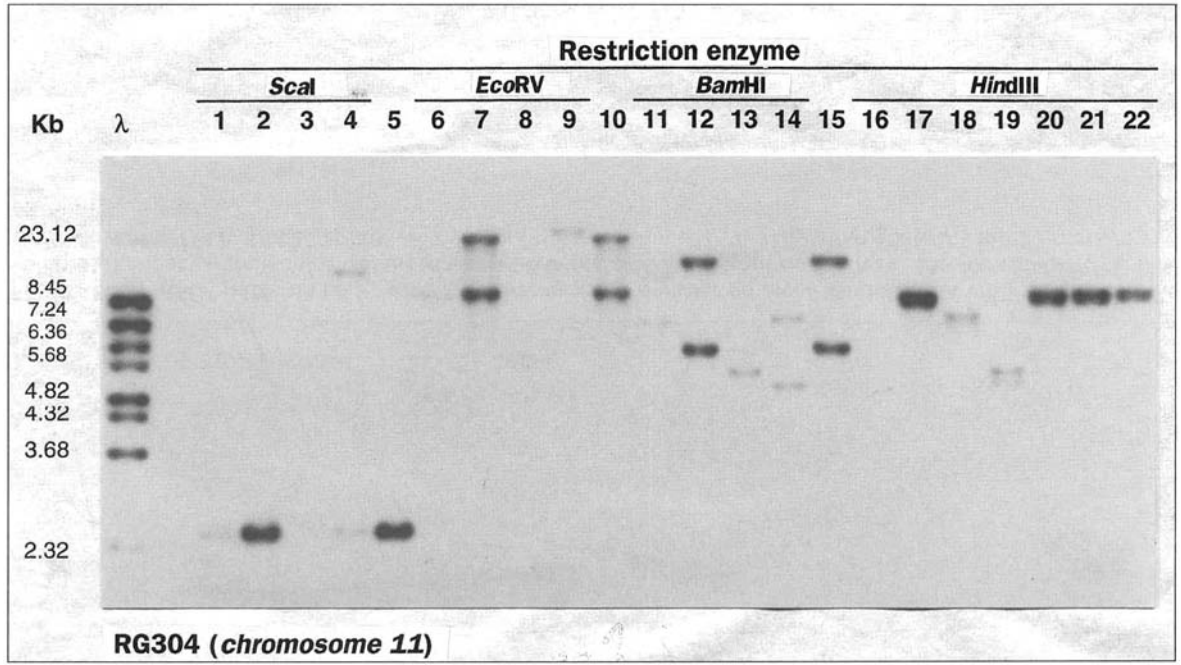

Fig. 1. RFLP patterns of *O. sativa* and four wild species after hybridization with RG304 (located on chromosome 11) in combination with four restriction enzymes, *Sca*I, *Eco*RV, *Bam*HI, and *Hin*dIII. Note the differences in hybridization intensities, strong signals in *O. sativa* (lanes 2, 5, 7, 10, 12, 15, 17, 20, 21) and *O. rufipogon* (AA genome, lane 22) and weaker signals in *O. brachyantha* (lanes 4, 9, 14, 19), *O. granulata* (lanes 3, 8, 13, 18), and *O. australiensis* (lanes 1, 6, 11, 16).

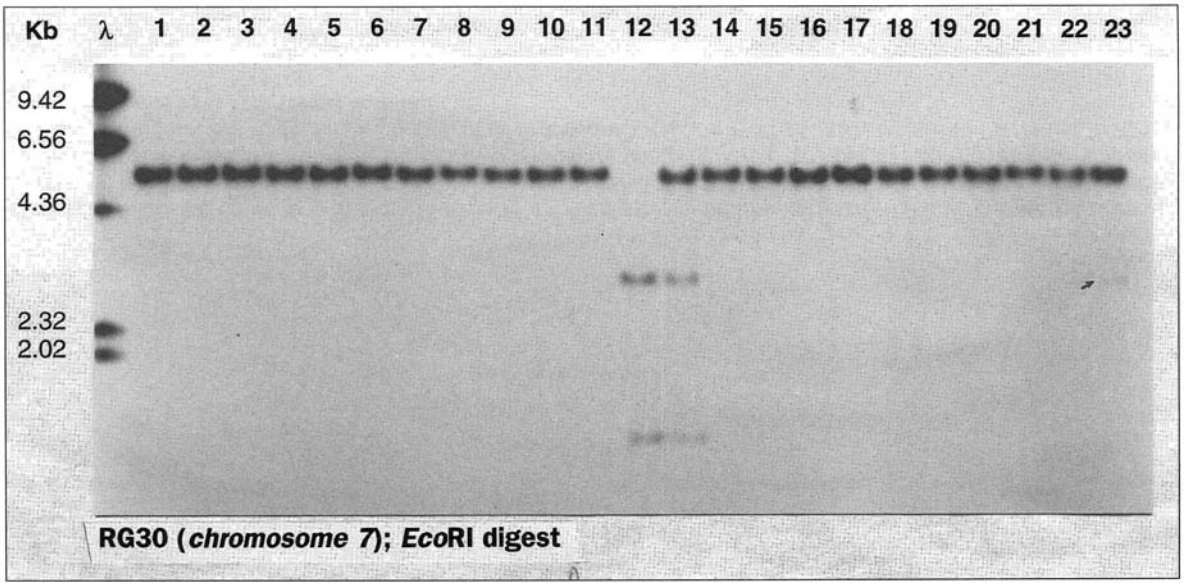

Fig. 2. ***Eco*RI-RFLP patterns of *O. sativa/O. brachyantha* derivatives after hybridization with RG30 (chromosome 7), lanes: λ —molecular weight marker; 1-10 and 14-23 different derivatives; 11—*O. sativa* IR56; 12—*O. brachyantha* (Acc. 101232); 13—F_1 hybrids (introgression of *O. brachyantha* allele in lane 23 marked with arrow).**

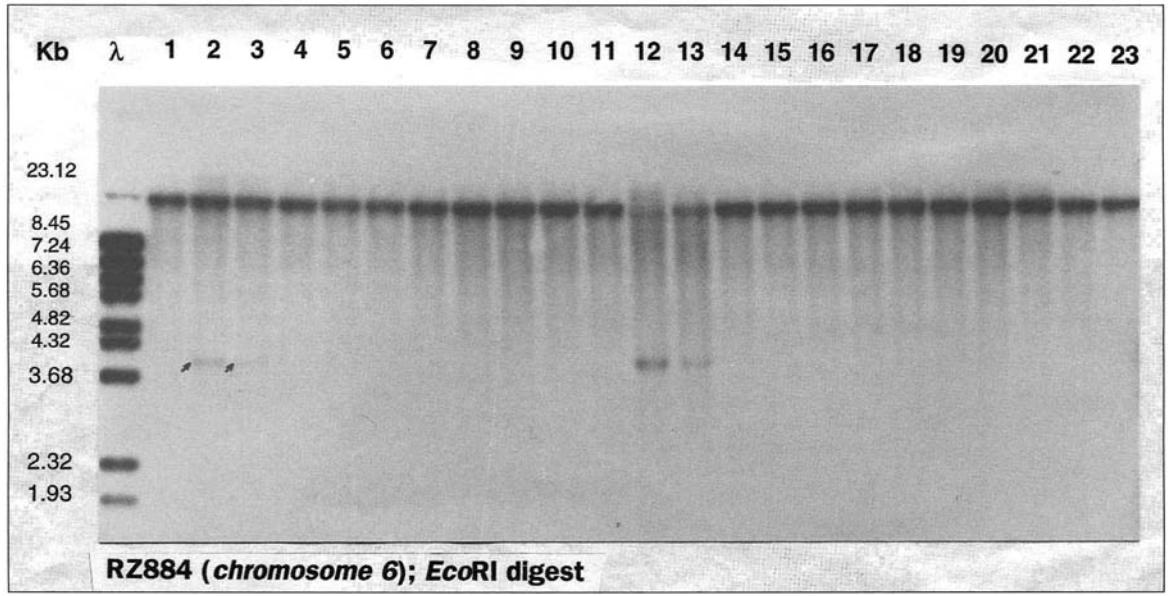

Fig. 3. ***Eco*RI-RFLP patterns *of O. sativa/O. granulata* derivatives after hybridization with R2884 *(chromosome* 6). Lanes: l —molecular weight marker; *1-10* and 14-23 different derivatives; 11—*O. sativa* IR31917-45-3-2; 12—*O. granulata* (Acc. 100879); 13—BC_1 *(introgression of O. granulata allele in lanes 2 and 3 marked with arrow).***

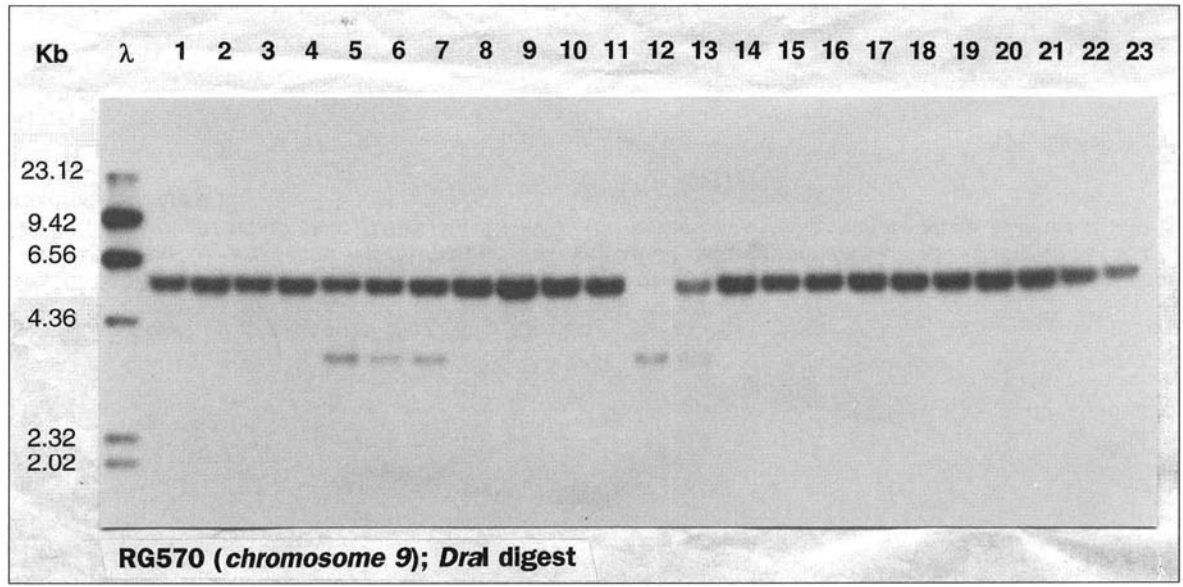

Fig. 4. ***Dra*I-RFLP patterns of *O. sativa/O. granulata* derivatives after hybridization with RG570 (chromosome 9). Lanes: l —molecular weight marker; 1-10 and 14-23—different derivatives; 11—*O. sativa* lR31917-45-3-2; 12—0granulata (Acc. 100879); 13—BC_1, (Note: *O. granulata* allele in MAAL 9, lanes 5, 6, 7).**

introgressing chromosome segments even from distantly related genomes into cultivated rice and thus feasibility of transferring useful genes from distant *Oryza* species. These results also suggest that chromosome pairing at metaphase I may give an underestimate of chromosome homoeology. Therefore, there is a need to reexamine chromosome pairing between *O. sativa* and wild species genomes at earlier stages of meiosis.

Low frequency of introgression and limited chromosome pairing suggest that introgression probably results through crossing over between short homoeologous segments interspersed in otherwise divergent genomes of *O. brachyantha* and *O. granulata.* Replacement of sativa alleles with the corresponding alleles from *officinalis* and *australiensis* in the introgression lines (Jena et al 1992, Ishii et al 1994) further supports the mechanism of alien gene transfer through crossing over rather than the substitution of a complete chromosome or chromosome arm.

RFLP analysis revealed chromosome 9 (Fig. 4) and chromosome 11 as extra chromosomes in some of the unidentified MAAL of *O. granulata.* Putative MAALs of *O. brachyantha* were also characterized using RFLP markers mapped onto the *O. sativa* chromosomes. Analysis of MAALs 6, 7, 9, 10, 11, and 12 showed that one or two alleles of *O. brachyantha* were absent from the additional alien chromosome in respective MAALs: RG516, RG828 (chromosome 6), RG146, RG30 (chromosome 7), RG118 (chromosome 11), and RG901 (chromosome 12). The absence of wild species alleles for the six RFLP markers could be due to introgression or chromosome repatterning of the genomes of wild species.

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Haploid, somaclone, and transformation studies in basmati rice

S.K. Raina, H. Khanna, M.S. Bisht, K. Kumar, U. Kumar, S.T. Irfan, and V.P. Singh

Culture media have been revised and protocols have been standardized for enhancing the anther culture response of indica rices and for improving green plantlet regeneration frequency in primary callus cultures of an elite basmati cultivar, Karnal Local. Using improvised media and techniques, doubled haploids in large numbers could be generated even from hybrids involving fine-grained aromatic rices. Doubled haploid selections, derived from heterotic F_1 hybrids, are presently under national trials. From the fine-grained, aromatic basmati cultivar B370, more than 1,000 somaclones were generated and about 270 were evaluated in detail. Semidwarf and early-maturing selections, evaluated for stability and cooking quality traits in particular, have either been entered in national trials or are in the final stages of evaluation. We have standardized conditions for microprojectile bombardment using precultured mature embryos of Karnal Local. Elaborate investigations for enhancing green plantlet regeneration frequency have resulted in 100% regeneration frequency, and about seven regenerants per embryo-derived primary callus. Using the *bar* gene, imparting resistance to bialophos/phosphinothricin, as the selectable marker, a liquid medium-based selection system has been pursued following bombardment of embryos with Ubi-GUS-BAR plasmid DNA. Recently, regenerants from putative transformed phosphinothricin-resistant calli have been obtained.

In vitro techniques for production of doubled haploids from microspores and regeneration of plantlets from transformed calli following integration of introduced DNA into cells/protoplasts are areas of considerable importance to crop improvement programs. With indica rices, green plant regeneration frequency from anthers/microspores and somatic cells and protoplasts is generally low and highly genotype-dependent. The genotypic factors require that protocols be applicable to a wide range of cultivars.

Techniques for production of doubled haploids provide a means for rapid fixation of homozygosity. In rice, in vitro culture of immature anthers remains the main technique for producing haploids in large numbers. However, for indica rices, poor anther response and genotypic dependence have been major impediments to efficient utilization of anther culture technology by breeding programs. Considering the importance of doubled haploids in the rice improvement programs, especially in India, we have developed culture media and methodologies that are quite effective over a much larger range of genotypes, including basmati cultivars.

This paper deals with the development of methodologies and their utilization for 1) rapid realization of promising recombinant pure lines from potentially desirable rice hybrids, 2) selection of somatic cell variants of an elite but tall and low-yielding basmati cultivar B370, which are semidwarf and high-yielding, and 3) production of transgenic lines of basmati cultivars resistant to insects and pests. Standardizing an efficient plant regeneration system, conditions of microprojectile bombardment, and an efficient in vitro system for selective growth and differentiation of transformants are essential requirements for the biolistic method of transformation, which is the method of choice in the present studies.

Materials and methods

Anther culture: rapid fixation of promising recombinants

F_2s from a basmati hybrid (improved Sabarmati/Khalsa 7) were selected and anthers were plated on basal media (Raina et al 1989). The recent modification to the callus induction medium pertains to the replacement of sucrose with maltose (5%). The plant regeneration medium remained the same (SK-II).

From about 700 pollen-derived plants of heterotic indica/indica F_1 hybrids (Raina et al 1989), doubled haploid selections were made and evaluated under a multilocation trial (New Delhi, Bangalore, Hyderabad) using a completely randomized block design, consisting of three replications.

Selection of promising semidwarf somaclonal variants of cultivar B370

More than 1,000 regenerants were raised from mature embryo-derived calli of B370 and about 270 were field-evaluated. Semidwarfs ranging in height from 65 to 140 cm were identified. Field evaluations and selections for promising semidwarfs were pursued along with elaborate screening for grain quality characteristics, using the procedure of Sood and Siddiqu (1978). The study has been in progress since 1987. During 1992-94, selected semidwarf lines were evaluated for genetic stability and performance, at New Delhi, Bangalore, and Hyderabad. Using a three-replication trial, evaluation for kernal elongation and aroma was done on a single-plant basis, so screening involved several thousand plants from several semidwarf lines.

Standardizing the transformation protocol in a basmati cultivar using a biolistic approach

Enhancing regenerations in embryo-derived calli. Using the basmati cultivar, Karnal Local, mature embryo-derived primary callus was induced on basal SK medium supplemented with various concentrations of KNO_3 (0-40 mM) and $NH_4(SO_4)_2$ (0-6.5 mM). The calli were regenerated subsequently (after 25 d) on MS basal medium and SK-1 medium with the same N supplements as in the medium of callus induction. The regeneration frequency and the number of regenerants produced per callus were counted and compared across the various supplemented media.

Besides, three concentrations of 2.4-D (0.5, 1.0, and 2.0 mg ml^{-1}) were tested for callusing and in particular, regenerability of the derived calli, while keeping the kinetin at 0.5 mg ml^{-1} of NAA, while no NAA was added in the other two cases.

Standardizing the physical and biological parameters for bombardment. A helium gas retrofitted BIOLISTIC^{TM} PDS-1000/He particle delivery system (BIO-RAD) was used. The pAHC27 (Ubi-GUS) and pAct1-Dd (Act-GUS) plasmid constructs used were obtained from Dr. Richard Jefferson (CAMBIA, Australia).

Using the scutellar region of isolated mature embryos as the target site for bombardment, three bombardment distances between the launch assembly and the target were tested. The transient GUS expression (24 h after bombardment) and retention of GUS expression (25 d after bombardment) were used as a measure of the efficiency of bombardment. Four rupture disk pressures (450, 650, 900, and 1,100 psi) were tested. The effect of preculturing of embryos was evaluated by preculturing the embryos for 1-8 d and by comparing it with nonprecultured embryos. The effect of pre- and postosmoticum treatment was tested by varying the duration of osmoticum treatment as well as by using different osmoticum agents in different concentrations. Similarly, influence of promoter sequences (actin and ubiquitin) was assessed on transient and long-term GUS expression.

Transformation studies with pAHC25 plasmid DNA. The pAHC25 (pUbi-GUS-BAR) was received from Prof. H. Uchimiya (Tsukuba. Japan). About 5.000 mature embryos were bombarded with pAHC25 and processed through preculture (6-8 d), preosmoticum (1-2 h), and postosmoticum (16-20 h) treatments. Bombarded calli were either directly transferred, or after a week's proliferation on normal callusing medium, to phosphinothricin (PPT) (4 mg ml^{-1}) selection medium.

Results and discussion

Anther culture

From about 15.000 plated anthers of the F_2s from a basmati hybrid (improved Sabarmati/Khalsa 7). anther callusing response was about 20% and green plant regeneration of the derived calli was about 15%. More than 200 green plant regenerants were obtained. Of the doubled haploid selections made from about 700 pollen-derived plants of heterotic indica/indica F_1 hybrids, two of the selections are presently being evaluated in national trials.

Somaclonal variants

The regenerants and their progeny showed minimal chromosomal changes or genetic abnormalities but a rather high frequency and varying degree of semidwarfness and early maturity. While several promising lines have shown stability over 6-7 generations, a few cases of instability, reversion to taller plant types, and generation of 'bonsai' dwarf (around 40 cm) plants after several generations of stable performance have led to a more detailed evaluation for stability. Such instability was observed only in a few lines that had been grown down south (Aduthurai) during the off-season where the crop reached maturity under comparatively higher temperatures (38/30 °C). Some of the semidwarf (110 cm) and stable lines, showing desired basmati grain characteristics, good yield potential (6 t ha^{-1}), and early maturity (115 d), were evaluated under a replicated trial at New Delhi. One of the selections has been entered in national trials.

Transformation studies

Enhancing the regeneration frequency of the primary callus. Callusing and green plantlet regeneration was significantly affected by N content and form. Total N of 45 mM, having 35 mM KNO_3 + 5 mM $(NH_4)_2SO_4$, elicited the highest regeneration efficiency: highest (100%) number of embryo-calli showing plant regenerations as well as the maximum number (7) of regenerants per embryo-callus. The revised medium, having a total of 45 mM N, was compared with some of the widely used basal media for rice—R-2 (Ohira et al 1973), N_6 (Chu et al 1975), and MS (Murashige and Skoog 1962)—and was found to be significantly better.

The phytohormone combination of 2,4-D (0.5 mg L^{-1}). NAA (2.5 mg L^{-1}), and K (0.5 mg L^{-1}) was the most effective for inducing embryonic calli, as assessed in terms of subsequent plant regenerability.

Physical and biological parameters for bombardment. Bombardment distance (from the launch assembly to target) of 8.7 cm, rupture disk pressure of 650 psi, and embryos precultured for 5-6 d on the callusing medium were found suitable as assessed in terms of transient *gus* gene expression and retention of GUS expression. Ubi-GUS always produced intense blue spots compared with Act-GUS whose expression was relatively low. The results have shown that, although no differences were obvious in comparisons involving short-term expression, differences in long-term retention were distinct. The ubiquitin promoter has been found to be better in several transformation studies involving monocot cell suspensions and immature embryos (Taylor et al 1993).

Ten percent sucrose was found most suitable as an osmoticum and the embryos given 1-2 h preosmoticum and 16-20 h postosmoticum treatment showed the best results. Vain et al (1993) have shown about 7-fold increase in the recovery of stable transformation in maize, following osmotic treatment.

Transformation studies with pAHC25 plasmid DNA. Numerous 'resistant' calli were obtained using the solid selection system. When transferred after 7-10 d, most calli selected using the solid selection system turned necrotic, although a few resistant calli could still be picked up after 5-6 wk. In all such cases, they were GUS-

negative and no regenerants were obtained from any such calli. Using the liquid selection system, it is now possible to pick up isolated cases of fresh callusing embryonic growth in highly necrotic calli following repeated transfers to PPT-supplemented liquid selection medium. Around 200 resistant calli selected using the liquid selection system have been transferred to the regeneration media. Some of these calli have produced regenerants. Molecular and biochemical confirmation of such putative transformants is in progress.

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Genetic variation of a single pollen-derived doubled haploid population in rice

H.-P. Moon, K.-H. Kang, S.-H. Choi, and S.-N. Ahn

Somaclonal variation was observed in the field in doubled haploid plants derived from single pollen culture of the anther-derived japonica variety Hwaseongbyeo. The variations in seven quantitative traits including plant height and one qualitative trait (pubescence) in 436 lines (R_2 generation) were analyzed. R_2 lines that fell outside the 95% confidence interval of the original variety were checked for each trait. The variations in quantitative traits, except days to flowering, appeared to be inferior to Hwaseongbyeo. Variability within the same R_2 line was also seen for quantitative and qualitative traits. Twenty-nine R_2 lines segregated for pubescence and 130 segregated for fertility, suggesting that mutations usually occur before diploidization. Twenty-nine R_2 lines, representing a wide spectrum of variation, were chosen for random amplified polymorphic DNA analysis. The number of lines showing DNA polymorphisms compared with the original variety ranged from 0 to 10, depending on the primer used. This seems to indicate that some primers can span a highly mutable genomic site.

Somaclonal variation induced during the culture of plant cells may provide a useful source of genetic variability for crop improvement and is well documented in many plant species. In rice, somaclonal variation has been previously described in plants regenerated from callus cultures (Oono 1978, Sun et al 1983) and from protoplast cultures (Ogura et al 1989) and included differences in both quantitative and qualitative characteristics including plant height, days to heading, and sterility.

Restriction fragment length polymorphism (RFLP) analysis revealed substantial variation in rice plants regenerated from nonembryogenic tissue cultures (Muller et al 1990). Brown et al (1990) also reported that significant levels of DNA variation were produced as a result of tissue culture, and DNA variation was not significantly different between callus- and protoplast-derived rice plants.

The random amplified polymorphic DNA (RAPD) technique was also employed for analyzing the genetic stability of tissue-cultured plants (Valles et al 1993). Taylor et al (1995) reported that RAPD analysis was suitable for detecting somaclonal variation in sugarcane subjected to prolonged tissue culture. Regenerated plants from pollen culture are well suited as source materials for the study of somaclonal variation because of easy detection of mutated traits and homozygosity in the subsequent generations and single pollen culture is recommended to exclude the problem caused by the intrinsic explant variability. The objectives of this study were to 1) determine the relative frequency of phenotypic variation and 2) monitor DNA polymorphisms using RAPD analysis in plants regenerated from single pollen culture. Regenerated plants derived directly from pollen culture and their following generations have been referred to as R_0, R_1, R_2, etc.

Materials and methods

Hwaseongbyeo, an anther-derived japonica variety, was used in this study. A vacuum anther-collecting apparatus was used to collect anthers from panicles aseptically (Moon et al 1990). For pollen isolation and culture, procedures reported by Cho and Zapata (I 990) were used with modification of media. The preculture medium was modified B5 (B5 + 0.5 ppm BAP + 2 ppm 2,4-D + 0.5 ppm IAA + 5 g glucose L^{-1} + 20 g sucrose L^{-1}); the callus induction medium was modified N6 medium (N6 + 1 mg 2,4-D L^{-1} + 0.2 ppm NAA + 1 mM glutamine + 3 ml coconut water L^{-1} + 30 g sucrose L^{-1}). For establishment of callus clones of single pollen grains, the 2-wk culture was poured on No. 1 Whatman filter papers, and then microcalli were selected under the microscope. These microcalli were multiplied separately on solid callus growth medium (N6 + 2 ppm 2,4-D + 5 g casein hydrolysate L^{-1} + 0.8% agar). Among survived clones, three fast-growing, embryogenic clones were selected and multiplied for 7 mo through regular subcultures. Plants were regenerated from RM3-2 medium (MS + 4 ppm kinetin + 1 ppm NAA + 40 ppm adenine sulfate + 1.6% agar) through 20-d subcultures.

Regenerated plants (R_0 generation) were grown in the glasshouse. Haploid and sterile plants were discarded and seed harvesting was done on fertile diploid plants. The R_1 generation was grown in the ricefield, but progeny testing was not performed because the number of plants was not sufficient due to poor seed set on the R_0 plants. R_2 lines were planted in a single row with 30 plants in the summer of 1993. In 1993, rice plants were damaged during the reproductive stage by the unusually low temperatures and this might have affected the measurements of traits, especially fertility.

The middle 10 plants were used for data collection. Heading date, culm length, panicle length, number of panicles, number of spikelets per panicle, fertility, and flag leaf length were investigated and statistically analyzed.

Twenty-nine R_2 lines showing no variability within a line were selected for RAPD analysis. DNA was extracted from 10 to 15 plants per line. Sixteen Operon primers were used. The 25 µl of polymerase chain reaction (PCR) consisted of 20 ng sample DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin,

0.2 mM of each dNTP, 25 nM of each 10-mer primer, and 0.5 U Taq polymerase. The Perkin Elmer Cetus Thermocycler model 480 was programmed for 45 cycles of 94 °C (1 min), 35 °C (1 min), and 72 °C (2 min). This was followed by a final cycle of 72 ºC for 5 min. PCR products were resolved by electrophoresis in a 2% gel. Two independent runs were performed for each primer and the bands not consistent over the two reactions were not counted.

Results and discussion

Plant regeneration

A total of 2,235 plants were regenerated from three multiplied callus clones and grown in the glasshouse. Their ploidy level was determined and 436 diploid plants were selected based on phenotype.

Considerable variation in morphological traits was observed among R_0 somaclones when compared with Hwaseongbyeo. R_0 plants generally had reduced height, fewer panicles, narrower leaves, and lower seed set. This result is not consistent with previous findings that the regenerants derived directly from tissue culture show little observable variation. This might be partly due to the expression of recessive traits in a homozygous condition because spontaneous doubling of pollen culture-derived haploids was preceded by mutation. The likelihood that these phenotypic variations might be related to differences in chromosome number might be excluded because only diploid plants were selected for further analysis.

Somaclonal variation of R_2 population

Substantial differences between R_2 lines and the original variety were found for all traits examined; a change in leaf pubescence was also observed (Table 1). Compared with the original variety, the mean performance of the R_2 population was inferior, i.e., there were reduced height and panicle length, fewer panicles and spikelets, lower fertility, and shortened flagleaf.

R_2 lines that fell outside the 95% confidence interval of the original variety were checked for each trait (Table 1). Grain fertility showed the highest variation (86%), followed by culm length (78%). Oono (1978) reported that 72% of the rice plants regenerated from callus cultures showed morphological mutations. A similar level of morphological variation was also reported by Sun et al (1983), who found that 76% of 950 T_2 lines showed variation in at least one of six traits measured. The finding in this study, that lines with normal fertility only comprised 14.4%, is not surprising since measurements of variation are prone to error and environmental conditions can affect the stability of these traits.

Another possible reason for the high level of variation is that regenerants were derived from callus cultures maintained for a long period (7 mo). Muller et a1 (1990) reported that the amount and degree of variation are greatly increased in plants derived from callus cultures maintained over a long period.

Table 1. Mean (±SE) and range of agronomic traits of 436 R_2 lines and the number of varied R_2 lines.

Trait[a]	Mean (±SE)	Range		Varied lines[b]	
				no.	%
DH	122±3.5	97-135	(120)[c]	152	35
CL	67±8.6	26-85	(80)	338	78
PL	18±2.6	9-25	(20)	134	31
PN	13±3.5	3-24	(17)	99	23
SN	101±26.3	53-181	(136)	184	42
FE	64±25.9	0-97	(93)	373	86
FL	26±3.9	13-35	(33)	290	67
PB		*Gl,gl*	*(Gl)*	128	29

[a] DH: days to heading, CL: culm length, PL: panicle length; PN: number of panicles per hill, SN: spikelets per hill, FE: fertility, FL: flagleaf length, PB: pubescence.
[b] Line whose mean is outside the 95% confidence interval of Hwaseongbyeo.
[c] Mean of Hwaseongbyeo in parentheses.

It is also possible that the number of lines showing variation in this study might be an underestimation, considering that somaclonal variation of a more subtle or quantitative nature is present (Larkin and Scowcroft 1981).

Variation within lines

Doubled haploid plants are generally homozygous and the progeny from an R_0 plant should be uniform unless any mutation or gene influx happened. Variations within lines (R_2) from regenerated plants derived from long-term cultured callus of haploid cells are often observed to have a high frequency; this cannot be explained by mutations alone during generation advance.

Mutations might occur before or after spontaneous doubling of pollen culture-derived haploids during the culture period. If mutation precedes doubling of the haploid genome, the regenerated plant will be homozygous for the mutated trait and will produce homozygous progeny. However, if mutations occur after doubling of the haploid genome, the regenerant will be heterozygous for the trait mutated and will produce heterozygous progeny.

A large number of R_2 lines showed variation within the same R_2 per trait (Table 2). Twenty-nine and 130 R_2 lines (of 436) showed variation within each of the lines for pubescence and fertility, respectively. Within-line variation was more conspicuous for quantitative traits than for qualitative traits (Table 2).

RAPD analysis of R_2 lines

Twenty-nine R_2 lines shawing variation in plant height, plant type, tillering ability, panicle shape, etc. were selected for RAPD analysis. RAPD analysis, using 16 Operon primers, resolved 55 scorable bands in Hwaseongbyeo (Table 3). Primers produced from two to five amplification products.

The number of R_2 lines showing DNA polymorphism compared with Hwaseongbyeo, depending on the primer, ranged from 0 to 10 (Table 3). PCR prod-

Table 2. Number of R_2 lines that showed variation within the same R_2 line per trait.

Trait[a]	Lines (no.)	Percentage[b]
CL	110	25
PL	42	10
PN	66	15
SN	118	27
FE	130	30
FL	38	9
PB	29	7
Total	136	31

[a] See Table 1. [b] No. of lines divided by a total of 436 lines.

Table 3. Distribution of primers based on how many R_2 line DNA polymorphisms were detected compared with the original variety using that primer.

No. of polymorphic R_2 lines[a]	Operon primer	
	No.	Designation
0	2	OPD-01, OPD-08
1-3	7	OPA-10, OPA-16, OPC-18, OPD-05, OPS-20, OPW-09, OPAA-10
4-6	3	OPC-11, OPE-04, OPU-09
7-9	2	OPD-02, OPS-06
10 and more	2	OPD-09, OPD-11
Total	16	

[a] Twenty-nine R_2 lines were surveyed for DNA polymorphisms.

ucts amplified in 29 R_2 lines by two primers (OPD-01, OPD-08) were identical to those of Hwaseongbyeo, and most likely a DNA polymorphism (base substitutions, short deletions, or insertions) did not occur within the binding sites of those two primers. The other 14 primers revealed a DNA polymorphism in at least one R_2 line compared with Hwaseongbyeo and the number of R_2 lines ranged from 1 to 10 depending on the primer used (Table 3; Fig. 1).

With the data obtained from this analysis, it is difficult to determine why the number of lines varied depending on the primer used and why two primers (OPD-01 and OPD-08; Table 3) failed to reveal the DNA polymorphism. This seems to indicate that some primers can span a highly mutable genomic site, which is consistent with a recent finding of Taylor et al (1995) using sugarcane. Screening of more primers and regenerated lines will be necessary to test this theory.

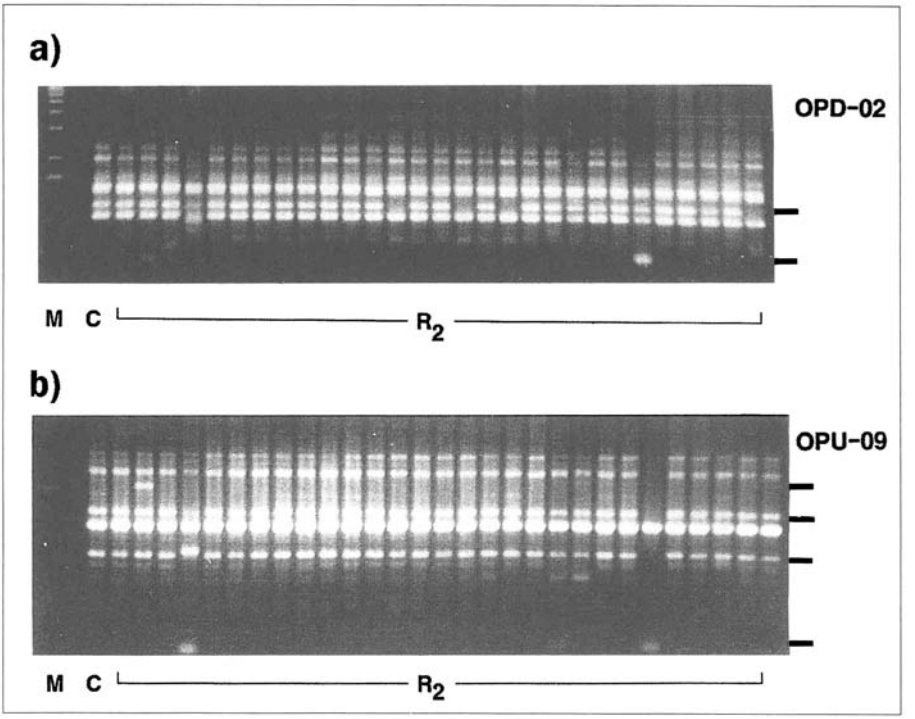

Fig. 1. Bands produced by two 10-bp Operon primers on a subset of 29 R_2 lines chosen from 436 lines. Primers were OPD-02 (a), and OPU-09 (b). Key: M = 1-kb ladder, C: Hwaseongbyeo, R_2: 29 R_2 lines. Bands showing polymorphism between R_2 line and Hwaseongbyeo are shown by lines on the right.

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Variation in some quantitative characters in doubled haploid lines derived from a heterotic rice hybrid

B.B. Bong and M.S. Swaminathan

The hybrid derived from Pusa 743-1-1/IR66 showed significant heterosis for grain yield. Forty-six different doubled haploid (DH) lines were obtained through anther culture of this hybrid. The variation in some quantitative characters within DH lines, F_5 lines derived from the same cross, and conventional (inbred) varieties was examined. The performance of the DH lines was compared with that of the corresponding hybrid, parents, and F_2 plants. The results showed that the coefficient of variation in plant height, panicle number plant^{-1}, panicle length, grain number panicle^{-1}, and grain yield plant^{-1} within the DH lines was similar to or lower than that within the conventional varieties and lower than that within the F_5 lines. The frequency distribution of the DH lines and the F_2 plants showed a similar pattern for plant height, panicle length, and grain weight. But for panicle number plant^{-1} and grain number panicle^{-1}, the DH lines had less variation and lower values than the F_2 plants. Plant height, panicle number plant^{-1}, panicle length, and grain weight of some DH lines were comparable with those of the hybrid. But none of them could reach the heterotic level of the hybrid for grain number panicle^{-1} and only one DH line showed a yield level similar to that of the hybrid and an increased yield by 19% over the better parent.

Doubled haploid (DH) breeding helps shorten the breeding cycle and offers the opportunity to exploit the advantage of superior gametic genotypes. This breeding method uses the anther culture technique, which has been widely applied to rice. More than 100 rice varieties have been developed in China through anther culture breeding (Hu and Huang 1987, Brar and Khush 1993). It was reported that the possibility of obtaining anther-derived rice lines with desirable characters, such as good plant type, salinity tolerance, higher yield, and increased resistance to pests and diseases, from both parents is high (Zapata et al 1991). Anther culture can be used to extract high-yielding purelines from commercial F_1 rice hybrids (Brar et al 1994).

This is helpful in eliminating the cumbersome procedures in hybrid seed production in a strictly self-pollinated crop like rice. However, not much is known about the performance of DH lines as compared with hybrids from which they are derived.

The study presented here examined the interplant variation in some quantitative characters within the DH lines of a heterotic rice hybrid. conventional varieties, and F_5 lines derived from the same cross. It also compared the performance of DH lines with that of the hybrid, parents, and plants of the corresponding F_2 population.

Materials and methods

Selection of the hybrid and development of DH lines

The heterotic hybrid Pusa 743-1-1/IR66 was selected from 15 hybrids developed by diallel crossing of six parents. Anther culture of this hybrid was done at the Biotechnology Center, Indian Agricultural Research Institute (IARI), following established techniques for indica rice. Forty-six DH lines were obtained, representing the first progeny of distinct anther-derived plants.

Field evaluation

The experiment was conducted at the IARI farm in New Delhi during the 1991 wet season. The test entries—46 DH lines, the corresponding hybrid Pusa 743-1-1/IR66, parents (P1=Pusa 743-1-1, P2=IR66), two F_5 lines derived from the same cross, and other conventional (inbred) varieties—were evaluated in a simple lattice design with two replications. Each plot contained five rows with 25 plants row^{-1}. Single seedlings were transplanted per hill at a spacing of 20 × 20 cm. A population of 1,000 F_2 plants derived from the same cross was grown in a nearby plot.

Data collection

Interplant variation within lines. Within each of the six selected DH lines, two F_5 lines and four selected conventional varieties, 50 random individual plants were taken and their plant height, panicle number $plant^{-1}$, panicle length, grain number $panicle^{-1}$, and yield $plant^{-1}$ were determined. The mean and coefficient of variation (CV) within each line were calculated.

Performance of DH lines, the hybrid, parents, and F_2 *plants.* Plant height, panicle number $plant^{-1}$, panicle length, grain number $panicle^{-1}$, 1,000-grain weight, and grain yield of plants in the three middle rows (excluding border plants) were recorded and the mean values were calculated for each DH line, hybrid, and parent. Eighty-five F_2 plants were randomly selected and the characters of the individual plants were observed.

Data analysis

The performance of DH lines, hybrid, and parents was analyzed following the method applied to simple lattice design (Gomez and Gomez 1984). Mean values of the characters of the test entries were compared using the least significant difference (LSD) test.

Results and discussion

The CV range in the DH lines was almost similar to that in conventional varieties, but it was narrower than that of the F_5 lines. For all characters studied, the CV was lowest within DH lines A 10-2, A26-2, and A27-2 and highest within the F_5 line F5-106. The results showed that interplant variation within the DH lines was similar to or lower than that within the conventional varieties. It was also lower than the variation within the F_5 lines. This indicates that the DH lines were phenotypically uniform and it was possible to obtain homozygous lines within two generations. Thus, the anther culture approach is useful for rice improvement, particularly in areas where only one rice crop per year is feasible for conventional breeding.

The pollen grains of hybrid plants bear genotypes different from each other due to various recombination of genes of their parents. Hence, it is necessary to know if the variation between DH lines is adequate for selection and if it is possible to obtain superior purelines resulting from chromosome doubling of desirable gametic genotypes. In this study, attempts have been made to examine the variation of 46 DH lines developed from the hybrid Pusa 743-1-1/IR66 and from the F_2 population derived from the same cross for some quantitative characters, e.g., plant height, panicle number plant^{-1}, panicle length, grain number panicle^{-1}, and 1,000-grain weight (Figs. 1-5). The mean performance of these characters and the grain yield of DH lines were compared with those of the corresponding hybrid and parents.

The DH lines and the F_2 population have similar values for plant height (Fig. l), panicle length (Fig. 3.), and grain weight (Fig. 5). For panicle number plant^{-1} and grain number panicle^{-1} theF_2 plants showed a wider variation than the DH lines (Fig. 2, 4). For all characters except panicle number plant^{-1}, the curves of F_2 plants appeared skewed toward the higher values (Figs. 1-5). This is due to the presence of a significant number of heterozygotes in the F_2 plants and the characters of higher values being dominant. The recessive genes, meanwhile, are revealed directly in the DH lines. Such trends have been previously reported (Liu et al 1983). In the case of panicle

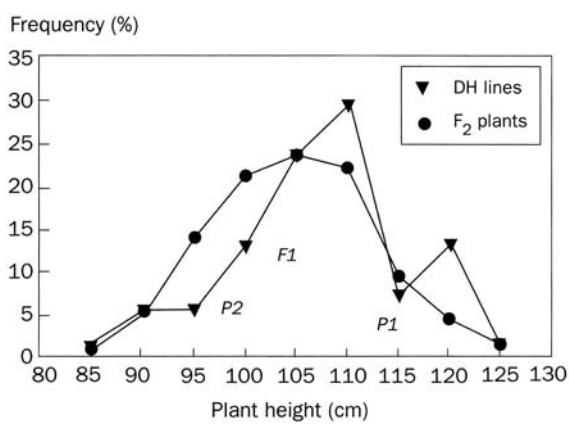

Fig. 1. Distribution of plant height of DH lines and F_2 plants derived from Pusa 743-1-1/IR66.

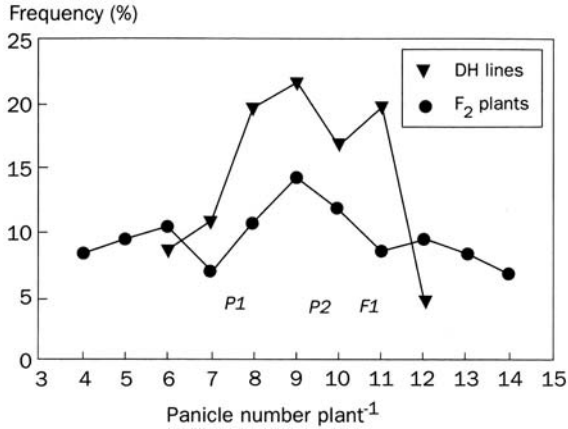

Fig. 2. Distribution of panicle number plant^{-1} of DH lines and F_2 plants derived from Pusa 743-1-1/IR66.

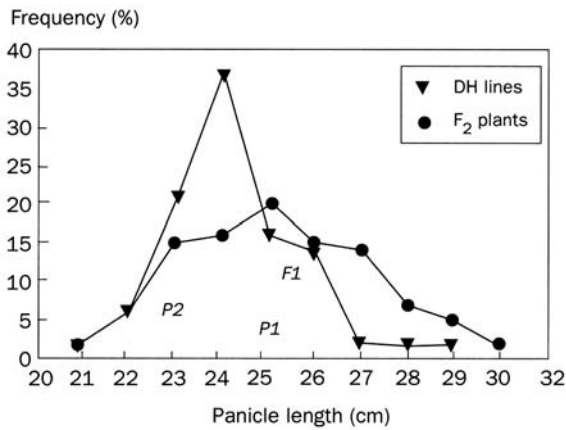

Fig. 3. Distribution of panicle length of DH lines and F_2 plants derived from Pusa 743-1-1/IR66.

number plant^{-1}, the distribution curve of the DH lines was similar to that of the F_2 plants. This trend may be attributed to other factors such as the proportion of dominant alleles to recessive alleles in the parents for this character.

For plant height, the hybrid had an intermediate value between those of the parents. A few DH lines were taller than the tall parent (Fig. 1). For panicle number plant^{-1} and panicle length, there was significant midparent heterosis but nonsignificant better-parent heterosis, and some DH lines were comparable with the hybrid for these characters (Fig. 2, 3). For 1,000-grain weight, better-parent heterosis was significant, and a few DH lines could reach the level of the hybrid (Fig. 4). In contrast, for grain number panicle^{-1}, the magnitude of heterosis in the hybrid was very high, but no DH lines were comparable with the hybrid (Fig. 5). The inheritance of grain number panicle^{-1} is shown to be of overdominant nature, and the heterosis associated with overdominance also may be involved, besides additive and nonadditive genetic effects

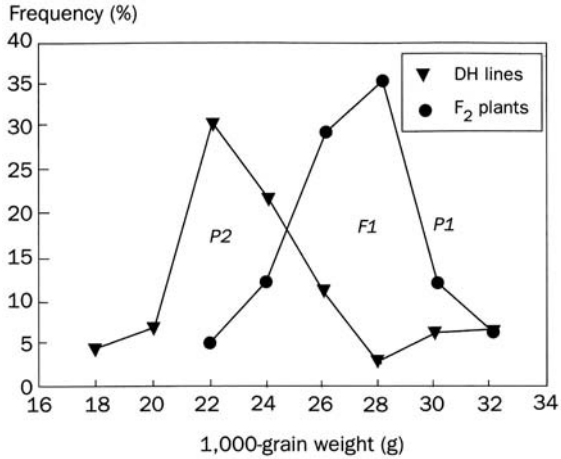

Fig. 4. Distribution of 1,000-grain weight of DH lines and F_2 plants derived from Pusa 743-1-1/IR66.

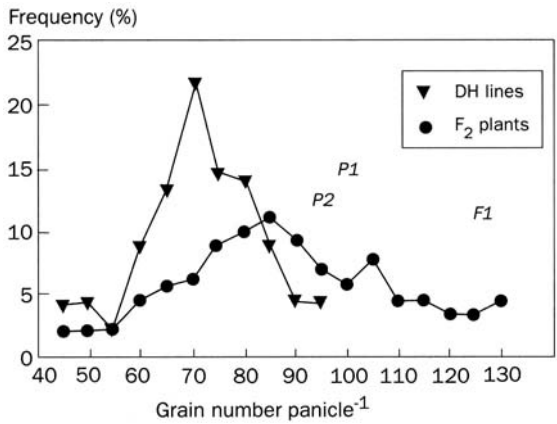

Fig. 5. Distribution of grain number panicle^{-1} of DH lines and F_2 plants derived from Pusa 743-1-1/IR66.

which are not fixed (Bong and Swaminathan 1995). This is the reason all DH lines were inferior to the hybrid for this character.

The grain yield of 46 DH lines ranged from 2.92 to 6.62 t ha^{-1}. The yield was 5.56 t ha^{-1} for P1 (Pusa 743-1-1), 5.25 t ha^{-1} for P2 (IR66), and 7.02 t ha^{-1} for the hybrid (significant better-parent heterosis of 26.2%). Around 10% of the DH lines had higher numerical yields than the parents, but only one DH line showed a significant yield increase of 19% over the better parent. As compared with the hybrid, only one line out of 46 DH lines was found to be not significantly lower yielding than the hybrid (but the yield is numerically lower). The variation of DH lines may depend on its population size. In general, a population of 100 DH lines from a cross is usually sufficient to obtain a superior yielding line (Wenzel et al 1995). However, the results

also depend on the ability to select parent materials. In rice. new DH-derived cultivars have been obtained from fewer than 20 DH lines from a cross (Shouyi and Shouyin 1991). This study suggests that if a heterotic hybrid is used, it is possible to obtain sufficient variation to select high-yielding DH lines from a relatively small population size.

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Gameto- and somaclonal variations in rice cultivars of the Russian Far East

Y.N. Zhuravlev and V.N. Zmeeva

Gameto- and somaclonal lines of two primary rice cultivars were tested for their deviation from parental plants in some agronomic and morphological traits and for their fitness for use in conventional breeding. Both line groups exhibited a wide range of variability in a number of traits, and some changes in agronomic characters were shown to be useful for cultivar improvement. The frequency of changes in characters such as plant height, fertility, and some others was cultivar-specific in both gametoclonal and somaclonal populations. The frequencies for the awned/awnless traits were identical, whereas those for shattering grains differed significantly in the two populations. The patterns of variability in two populations of regenerated plants were only partly overlapping, showing different frequencies and different coefficients of variation (CVs) for changes in many characters. Some changes were stable for generations in the doubled haploid (DH) lines only, indicating their possible appearance before doubling. In a limited number of DH lines (about 5% of the total), the CV values for some characters were significantly greater than those in the starting cultivars. Thus, it is possible that several changes might also appear after doubling.

In vitro regenerated plants, regardless of their origin, often show some traits that are absent in the parents. This may reflect the preexisting genetic heterogeneity of explant cells or may result from the changes induced by the in vitro regeneration procedure per se (Orton 1980, Morrison and Evans 1987). Such changes appear in both somaclones (SCs) and pollen culture regenerants. It was of interest, therefore, to compare the variation patterns specific to gameto- and somaclonal populations of the same cultivar. One can predict that the comparison will be impeded by the processes accompanying meiotic crossing over and chromosome set doubling in microspore-derived doubled haploids (DHs), but this issue is somewhat simplified if two or more cultivars are used for comparison.

Detailed reviews devoted to somaclonal and gametoclonal variations (Morrison and Evans 1987, Morrison et al 1988) do not compare trait deviation frequencies for these two kinds of regenerants in the same plant cultivars. However, this type of comparison can produce new data for understanding the variability that appears in vitro, and also makes it possible to estimate the fitness of different regeneration methods for the specific goals of mixed (in vitro and conventional) selection and breeding. Rice cultivars are highly suited for investigations involving in vitro variability because organogenesis is responsible for plantlet emergence in both regenerant types (Ling et al 1988). Similar cultivation media can be used to minimize the differences in phytohormone treatments.

Methods

Two local japonica rice cultivars were used to obtain 79 somaclonal and 68 DH lines under comparable conditions. Callus proliferation in seed or pollen explants was started on MS media supplemented with 2,4-dichlorophenoxyacetic acid and a-naphthaleneacetic acid. Kinetin and 6-benzylaminopurine were used to promote organogenesis. About 70% of microspore-derived plantlets were haploids, so colchicine treatment was applied to produce DHs.

Field assessment of variation was conducted through two seed generations, with two replications (1 m^2) for each assessment. Ten plants per plot were examined.

Results and discussion

Variation in agronomic characters

All somaclones (SCs) and DH lines of cultivars Dalnevostochny and Novoselsky differed from their respective controls in at least one agronomic character.

In cultivar Dalnevostochny (Fig. 1), all SCs were characterized by shorter plant height, whereas nearly an equal number of DHs were shorter or taller than control plants. In about 20% of SCs, panicle length was shorter and in about 20% of DHs, it was longer than that in the control. Fertility values in SCs were the same or lower than those in the control, but nearly a quarter of DHs exhibited fertility higher than the parent. Nearly the same variation was obtained in SCs and DHs for grain weight per panicle. Weight of 100 seeds was lower than that in control plants in all SCs, while it was greater in about half of the DHs. The heading date for most regenerants was close to that of the control. Among those that varied, the number of lines with earlier or later heading was approximately equal.

In cultivar Novoselsky (Fig. 2), all SCs were of the same height and all DHs were shorter than control plants. This confirms once more that regenerants, especially DHs, usually exhibit shorter height than starting plants (Schaeffer 1982). DHs also exhibited lower values than parent plant characters such as panicle length, fertility, sterility, grain weight per panicle, and 100-seed weight. Conversely, 1/4-1/2 of SCs exceeded the parent cultivar in these characters. Heading date variation, when it occurred, resulted in the appearance of earlier ripening SC and later ripening DH lines.

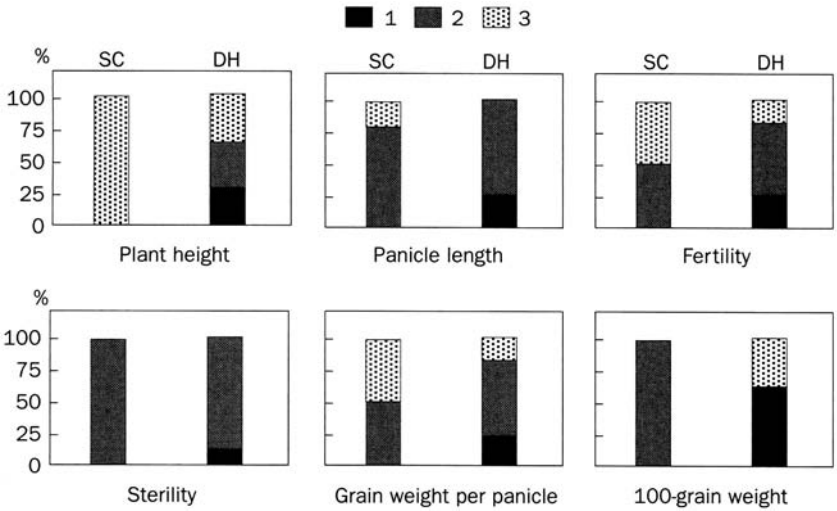

Fig. 1. Frequencies of lines from original cultivars for agronomically important traits in populations of somaclones (SC) and doubled haploids (DH) obtained from tissue culture of cultivar Dalnevostochny. Note: 1) the value of the trait much more than the starting cultivar, 2) the value of the trait equal to the starting cultivar, 3) the value of the trait less than the starting cultivar. P = 0.05.

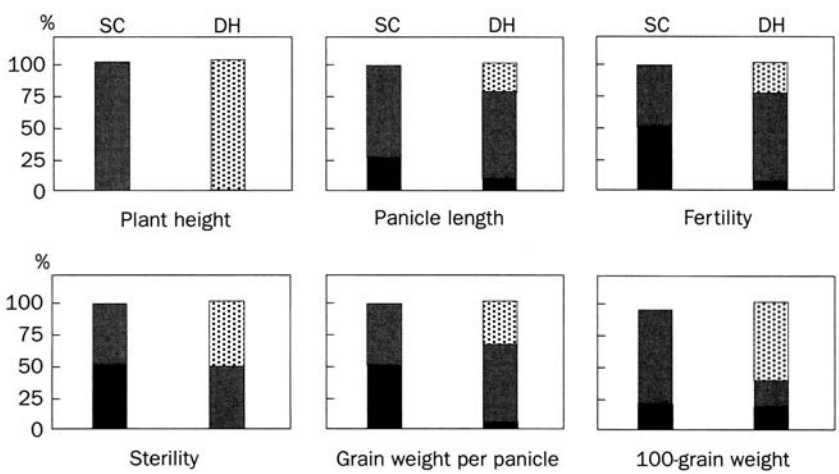

Fig. 2. Frequencies of lines from original cultivars for agronomically important traits in populations of somaclones (SC) and doubled haploids (DH) obtained from tissue culture of cultivar Novoselsky. See note in Figure 1.

Variation patterns differ from each other—Figure 1 from Figure 2 and DH polygons from SC ones. Variation can therefore be considered both cultivar- and method-specific. The genetic nature of the explant affects the pattern of variation frequencies, and tissue culture is an effective means of capturing the preexisting cell heterogeneity in a selective manner. Genetic events, such as mutation, genetic nonhomogeneity, variable gene response, and others, may also be involved.

Coefficients of variation (CVs) for quantitative traits were generally the lowest in DH lines, and this was inheritable in generations. Conversely, SCs often exhibited CVs that exceeded those of the control, and significant but opposite-directed deviations were sometimes revealed in two subsequent generations. This suggests that the gene response changed in the SC regenerants.

However, some traits in some DH lines have CV values significantly higher than those in control plants. For instance, in DH lines of Dalnevostochny, the maximum value of CVs for panicle length reached 13.1%, in contrast to the control's 9.6%. Since CVs for other traits in these lines were less than those in the original cultivar, the large-scale variability seen in panicle length may have originated from changes that appeared after doubling.

Effect of colchicine treatment

The DH lines that exhibited extremely high CVs confirm the hypothesis that colchicine-induced mutations play a definite part in the general variation pattern of a DH population. Because about 30% of our DH lines were spontaneously induced, we can compare the frequency of changes in some traits in DH populations of different origin (Fig. 3). It is clear that the frequency of changes in traits such as plant height, panicle

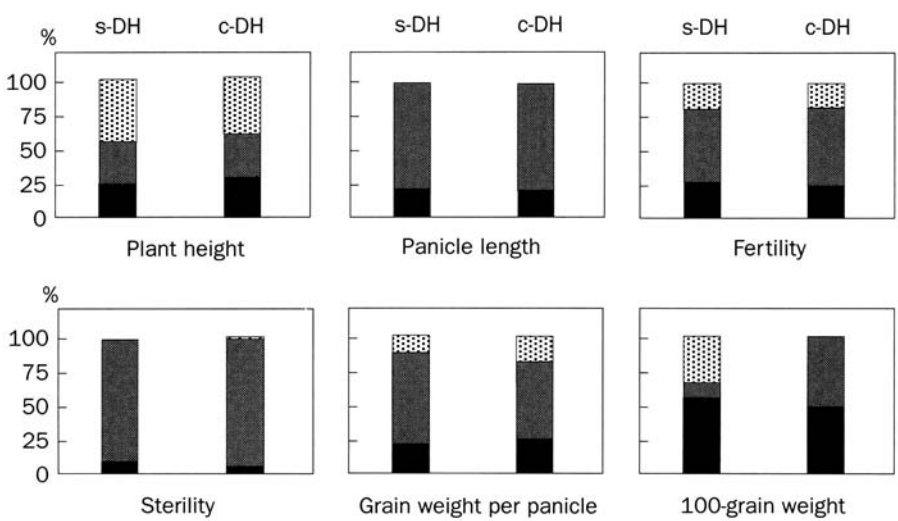

Fig. 3. Spontaneous DH (s-DH) and colchicine-induced DH (c-DH) regenerated from anther culture of cultivar Dalnevostochny. See note in Figure 1.

length, and fertility in colchicine-treated DH lines and that in spontaneously induced ones were the same. Only very limited additional variability in spikelet sterility, grain weight per panicle, and 100-grain weight was observed in colchicine-treated DH lines. Thus, colchicine-induced mutations, if they occur, make no pronounced impact on the distribution of variations among spontaneously induced DH and colchicine-treated DH populations. Their values do not significantly exceed 5% of the total change events, while gametoclonal variability together with the in vitro selection procedure are responsible for the general variation pattern.

Variation in quality characters

Shattering grains is an undesirable character in cultivar Dalnevostochny, hence we were constantly looking for changes that would remedy this problem. Two such plants (out of 1,693) in the second seed generation of SCs and five DH lines (10% of total) were resistant to shattering in the first and in the following generations (seven generations were tested in one SC line). The pronounced difference in the frequency of changes in that trait in DHs and SCs suggests the involvement of recessive genes in its expression. This seems all the more possible since the parent plant does not suffer from shattering grains.

The frequencies of variation in awned/awnless traits and for downy flowers were approximately identical in both populations of regenerated plants. It may be assumed that the same character changes originate in the course of regeneration of both SCs and DHs. However, the genetic nature of the trait changed is important to the value of its expression in different regenerant populations.

Frequency analysis of agronomic character changes in SCs and DHs allows a more fruitful method of cultivar improvement. Dwarf and short forms in cultivar Novoselsky can easily be obtained via gametoclonal variation, whereas the somaclonal method is better for cultivar Dalnevostochny. Desirable panicle characteristics can be more simply incorporated in Dalnevostochny by selection in DHs.

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Anther culture as a rice breeding tool: a profitable investment

L.R. Sanint, C. P.Martínez and Z. Lentini

Although anther culture (AC) of rice proved to be useful in accelerating the introgression of desirable traits into breeding populations, little is known about its economics and viability as a breeding tool. This study examined the relative cost effectiveness of using AC as an alternative for the conventional pedigree method (PM) to accelerate the development of rice germplasm. The response to AC of 604 different rice genotypes (indica, savanna, indica/savanna, and japonica) and the development of two indica cultivars for 7 yr using PM were used as bases for estimating costs. The analysis indicates that AC is a potential alternative tool to complement PM for rice breeding. Cost reductions from using AC instead of the conventional PM method are around 40%. The marginal internal rate of return of AC relative to PM ranges from around 30% yr^{-1} for the indica types to 55% yr^{-1} for japonicas. Although highly profitable, the viability of the investment must also include a broader analysis of the availability of funds for this investment in each institution in the context of other relevant bottlenecks in the research process.

The development of breeding lines adapted to the diverse Latin American ecosystems includes tropical and temperate irrigated conditions, tropical rainfed lowlands, and acid soils. Within these target areas, anther culture (AC) has been proven useful in three ways: 1) accelerating the development of germplasm that are tolerant to low temperatures and with excellent grain quality (long and slender grain type), 2) increasing the recovery of useful recombinants from wide crosses that include resistance to rice blast and rice hoja blanca virus and tolerance for drought, and 3) facilitating the production of materials suitable for restriction fragment length polymorphism and polymerase chain reaction-based gene tagging (Lentini and Martínez 1992).

A relevant feature of AC is that homozygosity is obtained in just one generation, while six generations are usually required through selfing to achieve a similar level of

homozygosity using the conventional pedigree method (PM). The practical implication is that the evaluation and selection from F_2 to F_4. which is required in the conventional PM, can be skipped when AC is used. However. little is known about the economics and viability of AC as a breeding tool. This paper aims to examine this information gap in AC.

Materials and methods

Anther culture

Crosses of the following were evaluated during two periods (1986-88 and 1991-92): 193 indica/indica (tropical irrigated), 113 savanna/savanna (acid soils), 199 indica/savanna (tropical rainfed lowland), and 99 japonica/japonica (temperate irrigated). Analysis was performed using mean response values from processing 39-89 crosses rice type^{-1} on different media (Lentini et al 1995: Table 1).

Efficiency of cultivar selection from AC

Once a doubled haploid (DH) is obtained through AC, it must have certain traits to assure its successful release as a cultivar for a particular target ecosystem. The following assumptions were made in the estimation of the number of DHs required to obtain a given genotype with all the desirable traits through AC: 1) random segregation between genes encoding for AC response and desirable agronomic traits (Chen et al 1991); 2) no significant differences in the population distribution between AC and PM due to segregation distortion (Guiderdoni 1991); 3) qualitative inheritance importance; and 4) preliminary selection prior to AC among F_1 plants produced from three-way or double crosses or among F_2 plants produced from single crosses, for plant type (height, tillering, vigor), grain type, and earliness.

Breeding for each ecosystem requires a certain number of desirable traits for a particular genotype. For this appraisal, the parental genotypes used in the initial crosses were assumed to differ by a minimum of eight loci for indica/indica crosses, nine loci for japonica/japonica crosses. and 12 loci for savanna/savanna and indica/savanna crosses.

Costs of pedigree method

General principles of partial budgeting techniques (Barry et al 1979) were used in a spreadsheet model to determine costs and to conduct a sensitivity analysis. All inputs were valued in 1992 U.S. dollars. Therefore, all cash flows appear at current prices (i.e., they are not affected by future inflation). The resulting stream of current costs was discounted at an annual rate of 5% to compute the present value of costs, which expresses the worth of the future stream of costs in terms of their present value.

The cost of producing a rice cultivar from initial crossing to cultivar release through PM was calculated in terms of activity during the growing season (Table 2). Records for the development of cultivars Oryzica Llanos 4 and Oryzica Llanos 5 (indicas) were used as reference. These cultivars are resistant to blast, rice hoja blanca virus, and planthopper (*Tagosodes oryzicolus*), are early maturing, and have high yield

Table 1. Plant regeneration from anther-derived calli induced on different media.[a]

Rice type	Medium[b]	Crosses (no.)	Anthers cultured (no.)	Calli anther^{-1} (%)		Green plants anther^{-1} (%)		Doubled haploids anther^{-1} (%)	
				Mean	Range	Mean	Range	Mean	Range
Japonica/	1	10	39,100	38.4 (5.2)[c]	22.0-54.8	3.7 (1.2)	0.0-7.5	2.2 (0.32)	1.3-3.1
japonica	2	89	240,750	91.5 (5.6)	38.7-100.0	6.6 (0.2)	4.7-8.5	4.0 (0.12)	3.0-5.0
Savanna/	1	74	778,500	0.1 (0.03)	0.0-0.4	0.1 (0.01)	0.0-0.2	0.06 (0.002)	0.0-0.08
savanna	2	39	165,500	28.7 (3.4)	7.5-49.9	0.9 (0.2)	0.0-2.1	0.6 (0.13)	0.0-1.2
Indica/	1	100	592,500	0.01 (0.002)	0.0-0.03	0.01 (0.005)	0.0-0.06	0.005 (0.001)	0.0-0.03
indica	2	51	275.250	4.3 (0.6)	0.0-8.6	0.2 (0.05)	0.0-0.6	0.1 (0.04)	0.0-0.4
	3	42	145,000	14.3 (5.1)	0.9-39.9	0.9 (0.5)	0.0-4.1	0.2 (0.18)	0.0-1.8
Indica/	1	127	1,009,000	0.2 (0.01)	0.09-0.31	0.5 (0.06)	0.0-1.2	0.2 (0.02)	0.0-0.4
savanna	2	72	397,500	14.4 (1.5)	1.7-27.1	0.7 (0.1)	0.0-1.5	0.5 (0.07)	0.0-1.3

[a] Taken from Lentini et al (1995). [b] Medlum 1 = potato-2 basal, medium 2 = rnodification of N_6 and He_2, medium 3 = medium 2 supplemented with MS micronutrients, 5% maltose, and 10 mg $AgNO_3$ L^{-1}. [c] Number in parentheses is the standard error.

Table 2. Timetable and breeding costs at current prices (US$) to develop one cultivar through anther culture (AC) and pedigree method (PM).

Breeding stage	Breeding via AC[a]				Growing season	Breeding stage	Breeding via PM[b]	
	Indica/ Indica	Savanna/ savanna	Indica/ savanna	Japonica/ japonica			Indica/ indica	Japonica/ japonica
Progenitors	7,564	7,564	7,564	12,935	1	Progenitors	7,564	12,935
Lab operation and maintenance	6,006	10,477	7,912	3,248	2			
Progenitors F_1 and F_2	7,564	7,564	7,564	12,935	2	Progenitors and F_1	8,766	14,991
R_1	9,761	9,761	9,761	16,692	3	F_1	8,559	14,637
R_2	14,551	14,551	14,551	24,882	4	F_2	24,165	41,322
R_3	10,085	10,085	10,085	17,245	5	F_3	39,691	67,872
YT[c]	10,682	10,682	10,682	18,266	6	F_4	23,899	40,867
YT	10,682	10,682	10,682	18,266	7	F_5	14,551	24,882
RT[d]	11,164	11,164	11,164	19,090	8	F_6	10,085	17,245
RT	11,164	11,164	11,164	19,090	9	YT	10,682	18,266
CT[e]	11,409	11,409	11,409	19,510	10	YT	10,682	18,266
CT	11,409	11,409	11,409	19,510	11	RT	11,164	19,090
Release					12	RT	11,164	19,090
					13	CT	11,409	19,510
					14	CT	11.409	19,510
					15	Release		
Total costs	122,041	126,513	123,948	201,670			203,791	348,483
Present value at 5% discount rate	87,435	91,298	89,083	143,450			139,552	238,635

[a]Estimated AC breeding costs. [b]PM breeding costs for one cultivar based on historical methods relative to derivation of cultivars Oryzica Llanos 4 and Oryzica Llanos 5. These are the same as those in Table 3, plus fixed costs. [c] YT = yield trial.[d]RT = regional trial. [e]CT = commercial trial.

potential (Leal-Monsalve et al 1989). They were developed over 7 yr, which is considered the minimum breeding time under tropical (2 crops year^{-1}) irrigated conditions. The cost of developing one indica cultivar for tropical conditions was estimated at US$203,791 with a present value of US$139,552. For temperate zones (japonica), where only one crop year^{-1} is possible, the cost of producing a cultivar is currently US$348,483, with a corresponding present value of $238,635 (Table 2).

Costs of anther culture

The approach for calculating the costs to produce the desirable number of DHs is based on the mathematical expectation of obtaining DHs, and their associated costs, as follows:

$$EV(DH) = P(DH)\,Q_A,$$

where EV(DH) = expected value of regenerated DHs, P(DH) = the probability of obtaining a DH anther^{-1}, and Q_A = the number of anthers cultured.

The lower the P(DH), the higher the number of anthers needed to achieve the same expected value and, consequently, the higher the variable costs for the operation.

A laboratory at the smallest effective operational level (2,150 anthers, 8-h working day) requires an initial investment of about US$30,000, whereas the laboratory we used as the benchmark for calculations (150,000 anthers wk^{-1}) needed approximately US$60,000. The investment requirements for different levels of activity were adjusted by a logistic curve. Four groups of costs associated with operating the laboratory were identified (Table 3).

Optimal level of laboratory activity to maximize savings with AC

A relevant feature of rice AC is that homozygosity is obtained in just one generation. Therefore, DHs do not segregate and breeding time can be substantially reduced. With the use of AC, evaluation and selection from F_2 to F_4 (required in the PM method) can be omitted. Costs incurred in those three generations represent, in current dollars, US$87,755 for indicas and US$150,062 for japonicas (Table 2). The present value of those costs amounts to US$68,813 and US$117,671, respectively.

Anther culture is not cost-free. The net cost savings, after deducting AC laboratory costs, are the relevant figures for making decisions (Table 4). Net cost savings follow a bell-shaped curve with an optimal peak at maximum net savings. With respect to PM, crosses through AC represent a reduction of about 40% in breeding costs (Table 4).

Value of investments in anther culture

To give a measure of the relative attractiveness of adopting AC as a breeding tool in rice breeding programs, we carried out a marginal analysis of the value of additional investments required by AC with respect to PM. It was measured through the marginal internal rate of return (MIRR). The MIRR, calculated over the 20-yr horizon for the useful life of the building, was found to range from 26% for savanna/savanna crosses

Table 3. Costs growing season^{-1} at current prices (US$) to operate a rice anther culture laboratory.

	Costs (US$)				
	Anthers wk^{-1}				
Laboratory cost factor	2,150	60,000	75,000	117,000	150,000
Fixed costs of depreciation[a]	1,017	1,528	1,963	2,011	2,035
Technician wages[b]	54	945	1,512	1,890	2,268
Supervisor wages[c]	713	1,923	2,987	4,385	6,000
Laboratory supplies	18	414	827	1,241	1,654
Unforeseen costs (15%)	270	722	1,094	1,429	1,794
Total cost during growing season	2,073	5,532	8,384	10,955	13,751

[a]Scale factors of infrastructure indivisibility were set at 50, 90, 96, and 100%, respectively. [b]Based on the minimum wage in Colombia. [c]Based on 20% activity to supervise a laboratory handling 150,000 anthers wk^{-1}.

Table 4. Total estimated costs (US$) to develop a variety.

Rice type	Breeding with AC	Breeding with PM	Savings from AC	Optimum anthers wk^{-1} (no.)	Current savings from AC (%)	Net present value savings with AC	MIRR[a] of cash flow AC vs PM (%)	Investment (building & equipment)
Indica	122,041	203,791	81,750	60,000	40.1	52,117	33.7	45,084
Savanna	126,513	203,791	77,278	117,000	37.9	48,254	26.5	60,029
Irrigated/ savanna	123,948	203,791	79,843	75,000	39.2	50,469	28.5	57,919
Japonica	201,670	348,483	146,813	2,150	42.1	95,184	54.6	30,015

[a]MIRR = marginal internal rate of return.

to 55% for japonica/japonica crosses, given the optimal levels of laboratory activity (Table 4). Even if it is assumed that the salaries of professional staff represent "dead costs" because they will be incurred anyway, the MIRR ranges from 16% for savanna/savanna crosses to 37% for japonica/japonica crosses.

Conclusions

Our analysis shows that the savings (in current dollars) from using AC for just one breeding cycle are enough to recover the initial investment (Table 4).

The production of a large number of DHs from AC is labor-intensive and does not require high specialization. This suggests that developing countries may have an economic comparative advantage in the application of this technique. However, this work does not suggest that AC is either more valuable or viable than other investments for rice improvement (i.e., entomology, pathology, physiology, etc.) or that it constitutes a high priority as an investment. The relative allocation of investments for rice breeding with or without AC is a priority-type decision for policymakers.

Given that AC can result in a faster release of a variety, the return on investment for society is much larger than the MIRR presented here. In that case, one would also have to calculate the flow of benefits associated with the earlier release of a new variety. Furthermore, accelerating the development of new varieties is only one of several useful applications of AC in rice research.

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Effects of amino acids, carbohydrates, and water stress on plant regeneration from cell and protoplast cultures of indica and japonica rice varieties

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Studied were the effects of amino acids, carbohydrates, and water stress on regeneration of fertile plants from cell and protoplast cultures of indica (Pusa Basmati 1, IR43) and japonica (Taipei 309) rice varieties. Addition of proline or tryptophan to the regeneration medium was inhibitory to shoot regeneration from protoplast-derived tissues in all three rice varieties. Observations on cell cultures developed on media containing one of eight carbohydrates (cellobiose, fructose, glucose, lactose, maltose, mannitol, sorbitol, and sucrose) at 88 mM concentration indicated maltose to be the preferential carbon source, both for the proliferation embryogenic callus and for shoot regeneration. The water-stress treatments included the use of 1.0% (w/v) agarose instead of 0.5% (w/v) for medium solidification, mannitol (0.2, 0.4 M) in the regeneration medium, or 24-h partial desiccation of calli. In all the rice varieties, best shoot regeneration frequencies (47-97%) from cell suspension, as well as protoplast-derived calli, were obtained when agarose concentration used for medium solidification was increased from 0.5 to 1.0%. Mannitol at both concentrations promoted the growth of embryogenic calli, which formed shoots with greater frequencies when subcultured onto mannitol-free regeneration medium. In Pusa Basmati 1, up to 92% of these calli regenerated shoots. Dehydration of calli for 24 h also enhanced shoot regeneration by 1.2- to 2.9-fold, depending upon the callus source and rice variety.

High-frequency regeneration of fertile plants from cells and protoplast cultures of indica rice varieties, especially those belonging to varietal group 1, remains a limiting factor for scientists undertaking transformation experiments. Little attention has been given to the optimization of carbohydrate and osmotic requirements for somatic embryogenesis in rice. There are now reports that suggest sucrose may not be the optimal sugar for plant morphogenesis in cereals. For example, glucose has been

shown to stimulate embryoid formation in wheat anther cultures (Chu et al 1990), sorbitol improved the growth of embryogenic callus in maize (Swedlund and Locy 1993), and maltose increased the frequency of plant regeneration in indica rice protoplast cultures (Jain et al 1995).

Osmotic stress created by partial desiccation of calli has been reported to increase plant regeneration frequencies in japonica and indica rice varieties (Rance et al 1994). Chowdhury et al (1993) reported increased shoot regeneration from seed cultures of an indica rice, Pusa 169, using proline and/or tryptophan in the regeneration medium. In this paper, we report the effects of amino acids, various sugars, and water stress induced by mannitol, higher agarose concentration. and partial desiccation, on plant regeneration from cell suspension and protoplast-derived cultures of three rice varieties, Pusa Basmati I (indica, varietal group 1, aromatic, semidwarf plant type), IR43 (indica, varietal group l), and Taipei 309 (japonica, varietal group 6).

Materials and methods

Cell suspension cultures of Pusa Basmati 1, IR43, and Taipei 309 were established using the mature seed scutella-derived calli, as described by Jain et al 1995. Cell suspensions were 5-7 mo old for Pusa Basmati 1 and IR43 and 12 mo old for Taipei 309 at the time these regeneration experiments were conducted. Suspension cells (0.5 ml settled cell volume), obtained after 4 d of subculture, were spread over the agarose (0.5% w/v)-solidified Linsmaier and Skoog (1965) medium (LS2.5) in a 9.0-cm petri dish and calli (about 1 mm in diameter) formed after 3 wk. Protoplasts of all three rice varieties were isolated and cultured essentially as described by Jain et al (1995). Protoplasts were cultured in 200 µl aliquots of liquid KPR medium containing 10×10^5 protoplasts, on the surface of cellulose nitrate filter membranes (47 mm diameter, 0.2-µm pore size) overlying KPR-agarose medium with immobilized feeder cells (*Oryza ridleyi* cells for Pusa Basmati 1 and *Lolium multiflorum* cells for IR43 and Taipei 309; Jain et al 1995). The colonies, formed after 4 wk on these membranes, were used for shoot regeneration experiments.

For plant regeneration, calli were randomly transferred, without making any selection based on their embryogenic appearance, to 9.0-cm petri dishes containing 25 ml of a modified MS (Murashige and Skoog 1962) medium supplemented with 2.0 mg L^{-1} of kinetin and 0.5 mg L^{-1} of NAA (designated MSKN). The media were semisolidified with 0.5% (w/v) agarose unless otherwise specified. To study the effect of amino acids, MSKN medium was modified by adding L-proline (5, 10 mM) or L-tryptophan (100, 200 µM). To compare the effects of carbohydrates, one of the following carbohydrates was used at 88 mM in MSKN medium: cellobiose, fructose, glucose, lactose, maltose, mannitol, sorbitol, or sucrose.

To investigate the effect of agarose concentration or mannitol, the agarose concentration of the medium was increased from 0.5 to 1.0% (w/v), or mannitol was added at varying concentrations (0.2, 0.4 M). For partial desiccation treatment, 100-120 calli were transferred to an empty sterile petri dish containing two sterile Whatmann filter paper disks. The dishes were sealed with parafilm and kept in the dark for 24 h.

Cultures were incubated at 26 °C in the dark for 2 wk and then transferred to light (55 µmol m^{-2} s^{-1}, daylight fluorescent tubes, 16-h photoperiod). Plant regeneration frequency was calculated as the percentage of calli that formed shoots after 5-6 wk. Regenerated shoots were transferred to the rooting medium (0.25% w/v phytagel-solidified MS medium with 1.5 mg L^{-1} of NAA) and 4 wk later, they were transferred to pots in the greenhouse.

Results

Effect of amino acids on shoot regeneration

Addition of proline or tryptophan to the regeneration medium decreased or completely inhibited shoot regeneration in protoplast-derived calli of Pusa Basmati 1, IR43, and Taipei 309 (Table 1).

Effect of carbohydrate source on plant regeneration

Observations, which were made on callus morphology and the shoot regeneration response of the protoplast-derived tissues developed on media with different carbohydrates, showed maltose to be the preferential carbon source both for the proliferation of embryogenic callus and for shoot regeneration (Table 2). After 2 wk, calli growing on maltose-containing regeneration medium were white to cream in color and almost entirely embryogenic. Subsequently, these cultures exhibited the highest shoot regeneration frequencies (Table 2, last column). The maltose-containing medium induced shoot formation in 30-42% of the protoplast-derived calli, depending upon the rice variety, compared with 10-12% shoot regeneration in sucrose-supplemented medium.

Effect of water stress on plant regeneration

Table 3 shows the effects of water stress created by using a higher (1.0%) agarose concentration for medium solidification and inclusion of mannitol (0.2, 0.4 M) into the medium or partial desiccation on frequency of shoot regeneration from rice calli.

Table 1. Effects of proline and tryptophan on shoot regeneration from protoplast-derived tissues of Pusa Basmati 1, IR43, and Taipei 309.

Rice variety	Percentage (± SE) of protoplast-derived tissues forming shoots[a]				
	Control	Proline (mM)		Tryptophan (µM)	
		5	10	100	200
Pusa Basmati 1	14.2±2.2	5.8±1.7	3.3±0.8	1.3±0.8	0
IR43	8.3±2.2	5.0±1.7	1.7±0.8	0	0
Taipei 309	17.3±1.8	4.0±0.6	0.7±0.7	0	0

[a]Data represent the mean ±SE shoot regeneration response of at least 200 calli (40 calli per petri dish) after 5 wk of culture. The regeneration (MSKN) medium contained 3.0% (w/v) sucrose as the sole carbohydrate source and was semisolidified using 0.5% agarose.

Table 2. Effect of carbohydrate source on callus growth and plant regeneration from protoplast-derived tissues of Pusa Basmati 1, IR43, and Taipei 309.[a]

Rice variety	Carbohydrate source (88 mM)	Percent (± SE) increase in fresh weight	Percentage (± SE) of calli regenerating shoots
Pusa Basmati 1	Cellobiose	193±15	2.7±2.8
	Fructose	180±12	22.9±6.8
	Glucose	133±9	0
	Lactose	48±12	0
	Maltose	250±35	37.3±3.4
	Mannitol	NG[b]	0
	Sorbitol	NG	0
	Sucrose	313±20	10.3±1.2
IR43	Cellobiose	82±39	0
	Fructose	NG	0
	Glucose	430±103	19.7±3.5
	Lactose	65±16	0
	Maltose	435±64	42.3±6.6
	Mannitol	NG	0
	Sorbitol	NG	0
	Sucrose	560±79	11.7±1.5
Taipei 309	Cellobiose	215±20	2.4±0.9
	Fructose	NG	0
	Glucose	363±39	24.0±3.0
	Lactose	4553	0
	Maltose	530±47	30.1±4.3
	Mannitol	NG	0
	Sorbitol	100±15	0
	Sucrose	892±150	11.8±0.9

[a]Data represent the mean ± SE shoot generation response of at least 200 calli (40 calli per petri dish) after 5 wk of culture. [b]NG = no growth.

In all the rice varieties, the best shoot regeneration frequencies were obtained when the agarose concentration used for the solidification of medium was increased from 0.5 to 1.0%. In Pusa Basmati 1 and IR43, the shoot regeneration from cell suspension-derived and protoplast-derived calli increased by 2- to 7-fold, to 60-97%. Most of these calli regenerated more than 10 shoots each. Mannitol, at both concentrations, promoted the growth of embryogenic calli, which formed shoots with greater frequencies when subcultured onto mannitol-free MSKN medium. In Pusa Basmati 1, up to 92% of these calli regenerated shoots. Dehydration of callus for 24 h enhanced shoot regeneration by 1.2- to 2.9-fold, depending upon the callus source and rice variety. Plants obtained using these water-stress treatments were transferred to the greenhouse, and appeared phenotypically normal, flowered, and set seeds within 125-160 d of transplanting.

In summary, we have shown that the carbohydrate source and osmotic stress are important factors that affect the process of embryogenesis and plant regeneration from cell and protoplast cultures of indica and japonica rice varieties. High shoot regenera-

Table 3. Effects of mannitol, agarose concentration, and partial desiccation on shoot regeneration from cell suspension derived and protoplastderived calli of Pusa Basmati 1, IR43, and Taipei 309.

Rice variety	Agarose concentration (% w/v)	Mannitol concentration/ pretreatment	Percentage (±SE) of calli regenerating shoots[a]	
			Cell suspension-derived calli	Protoplast-derived calli
Pusa Basmati 1	0.5	None	25.3±3.6	32.5±3.8
	1.0	None	96.7±2.5	77.5±12.5
	0.5	Mannitol 0.2 M	91.7±7.1	54.2±10.2
	0.5	Mannitol 0.4 M	67.2±2.4	NT[b]
	0.5	Desiccation 24 h	72.5±14.2	43.3±11.7
IR43	0.5	None	10.1±1.7	30.8±3.3
	1.0	None	72.0±12.7	60.0±2.9
	0.5	Mannitol 0.2 M	32.5±8.0	38.3±3.0
	0.5	Mannitol 0.4 M	39.0±2.8	NT
	0.5	Desiccation 24 h	18.3±8.7	NT
Taipei 309	0.5	None	15.0±2.2	34.2±5.5
	1.0	None	47.3±4.3	69.2±6.8
	0.5	Mannitol 0.2 M	20.8±5.5	50.0±9.0
	0.5	Mannitol 0.4 M	31.4±1.5	NT
	0.5	Desiccation 24 h	18.0±8.5	44.2±4.4

[a] Data represent the mean ± SE shoot regeneration response of at least 200 calli (40 calli per petri dish) after 6 wk of culture; first 4 wk on MSKN medium with agarose concentration and mannitol concentration/desiccation treatment described in columns 2 and 3 and then for 2 wk on mannitol-free, 0.5% agarose-solidified MSKN medium. All the media contained 3.0% w/v maltose as the sole carbohydrate source. [b]NT = not tested.

tion frequencies (60-97%) were obtained for IR43 and Pusa Basmati 1, simply by increasing the agarose concentration from 0.5 to 1.0% and using maltose as the carbohydrate source in regeneration medium. These shoot regeneration frequencies are comparable with the best regeneration frequencies reported to date in tissue culture-responsive group 6 japonica rice varieties.

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Changes of endogenous phytohormones during plant regeneration from rice callus

L.-F. Liu and C.-H. Lee

The plant regeneration ability of rice callus was studied. It was found that the regeneration frequency of calli induced from immature embryo of ANT39 was about 65%. On the other hand, the regeneration frequency of TN5 calli was nearly zero. However, this can be increased to 80%, if the calli of TN5 have been treated with 0.6 M sorbitol. High-performance liquid chromatography analysis showed that the highly regenerable calli contain a high level of abscisic acid (ABA) and indole-3-acetic acid (IAA), but a low level of cytokinin during the callus induction period. Then, IAA suddenly decreased and cytokinin dramatically increased during the plant regeneration period. By contrast, unregenerable calli contain a low level of ABA, IAA, and cytokinin during the callus-induction period. Then, IAA slightly increased and both ABA and cytokinin were not changed during the regeneration period. A strong correlation between the localization of IAA as well as zeatin and the differentiation of tracheary cells during regeneration can be found by immunohistochemistry. This suggests that the osmotic stress may regulate the endogenous phytohormone content in rice callus and then affect cell growth and differentiation.

Plant cells possess totipotency, which means whole plants can be regenerated from a single cell by modulating culture conditions. This phenomenon is widely used in plant propagation and crop improvement through genetic engineering. However, the mechanisms of totipotency have been so far poorly understood, and have been discussed mainly in the concentration and ratio of exogenous phytohormones (Amirato 1983, Flick et al 1983). From our previous studies, it was discovered that plant regeneration ability of cultured rice cells can be promoted significantly by osmotic stress treatment (Lai and Liu 1988, Liu and Lai 1991). It provides an alternative research avenue, that the growth and differentiation of cells could be modulated by cellular physiological water status itself.

In whole plants, the concentration and compartmentation of endogenous phytohormones are significantly influenced by osmotic stress (Skriver and Mundy 1990). In this experiment, therefore, the changes of endogenous phytohormones in osmotic-stressed rice callus, and its roles in plant regeneration were further studied. Three kinds of phytohormones, including indole-3-acetic acid (IAA), zeatin, and abscisic acid (ABA), were quantified by high-performance liquid chromatography (HPLC) and visualized in cells by immunohistochemical localization.

Materials and methods

Callus induction and plant regeneration

Two rice cultivars *(Oryza sativa* L. cv Tainan 5: TN5, and cv Ai-nan-tsao 39: ANT39) were used in this experiment. Primary calli were induced from 10- to 12-d-old immature embryos on MSD10 medium (Murashige and Skoog) basic medium plus 10 µM 2,4-D (2,4-dichlorophenoxy acetic acid)), or MSD 10S6 medium (MS basic medium plus 10 µM 2,4-D and 0.6 M sorbitol). Sorbitol was treated as osmotic agent. After 2 wk, calli were transferred to MSK20Nl0 medium (MS basic medium plus 20 µM kinetin and 10 µM NAA) for plant regeneration. The regeneration frequency was calculated as (calli number with shoot/total calli number) × 100.

Quantification of endogenous phytohormones

After being transferred to the regeneration medium, the calli were collected and fixed by liquid N_2 at 0, 2, 3, 4, and 6 d. A biotin-avidin-based enzyme immunoassay to quantify the endogenous phytohormones was mainly modified from Maldilney et al (1986). Fixed calli were homogenized and extracted with 80:20(v/v) methanol:distilled water containing 40 mg BHT (butylhydroxytoluol) L^{-1}. The IAA, ABA, and cytokinin were first separated by C-18 column, and then purified by HPLC. The concentrations of phytohormones were calculated as n mole g^{-1} fresh weight of callus.

Cellular localization of endogenous phytohormones

After being transferred to regeneration medium, calli were collected at 0, 3, 6, 9, and 12 d. For visualization of IAA and ABA, the calli were prefixed in 2% EDC (l-ethyl-3[3-dimethyl-amino propyl] carbodimide hydrochloride) for 24 h, and then post-fixed in PFAG solution (1% glutaldehyde and 4% paraformaldehyde in phosphate buffered saline [PBS] pH 7.2) for 12 h (Skene et al 1987, Ohmiya et al 1990, Bertrand and Benhamou 1992). For visualization of zeatin, the calli were prefixed in 20 mM sodium metaperiodate and 50 mM carbonate-bicarbonate buffer, pH 9.6, for 12 h, and then fixed in 2 mM sodium borohydride, Tris-HCl buffer, pH 7.6, for 12 h, and finally post-fixed in PFAG solution for 12 hrs (Zavala and Brandon 1983, Eberle et al 1987). The tissues were then dehydrated in t-butanol, transferred, and embedded into paraffin; 10-µm sections were cut with microtome and mounted on glass slide. The sections were immunolabeled by using the method modified from Brandtzaeg (1982), Grzanna (1982), and Knox (1982). This, in brief, is the series of treatments: primary antibody of IAA, zeatin, or ABA (from mouse), secondary antibody (biotin-conju-

gated anti-mouse goat IgG), streptavidin conjugated alkaline phosphatase, and finally stained by NBT (4-nitroblue tetrazolium chloride) and BCIP (bromo-4-chloro-3-indolyl-phosphate). The sections were also stained by Schiff's reagent for observation of starch. All slides were then dehydrated in a series of ethanol and xylene, and sealed in Entellen.

Results and discussion

The plant regeneration abilities of calli from the two rice cultivars used in this experiment were significantly different (Fig. 1). For ANT39, the calli showed high plant regeneration frequency (65%). Calli of TN5 induced on MSD10 medium showed extremely low plant regeneration (nearly 0%). However, the frequency can be greatly increased to 80%, if the callus-inducing medium is MSD10S6. The above phenomena provide a very good system to study the physiology and biochemistry during plant regeneration.

The concentration changes of endogenous IAA, zeatin, and ABA were analyzed by HPLC. It was found that both the highly regenerable calli, from ANT39 and TN5 (using MSD10S6), contain a high level of ABA and IAA, but a low level of cytokinin during the callus induction period. Then, IAA suddenly decreased and cytokinin dramatically increased during the plant regeneration period. By contrast, unregenerable calli of TN5 induced on MSD10 medium contain low levels of ABA, IAA and cytokinin during the callus induction period. Then, IAA slightly increased and both ABA and cytokinin were not changed during the regeneration period (Fig. 2).

The distribution of endogenous phytohormones in callus cells were visualized using the immunohistochemical method. In general, IAA and ABA were found in some specialized cells (Fig. 3b) and tracheary cells (Fig. 3c); zeatin was found in

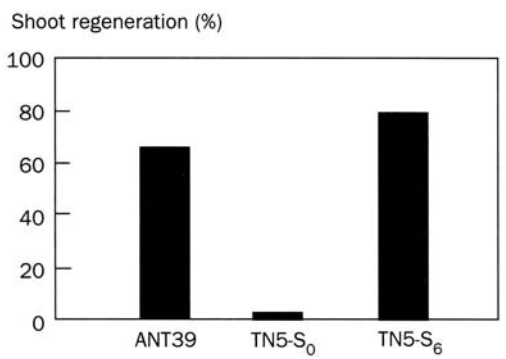

Fig. 1. Shoot regeneration frequency of rice callus induced from immature embryo. ANT39: callus of Ai-nan-tsao 39 induced on MSD10 medium; TN5-S_0: callus of Tainan 5 induced on MSD10 medium; TN5-S_6: callus of Tainan 5 induced on MSD10S6 medium.

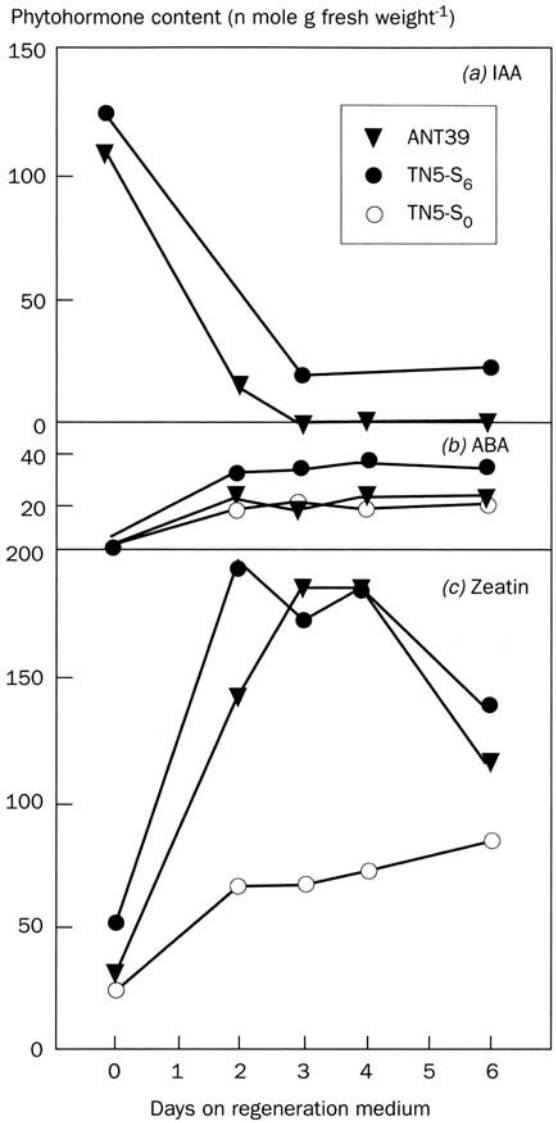

Fig. 2. Changes in concentration of endogenous phytohormones in rice callus during the early stage of plant regeneration: a) IAA; b) ABA; c) zeatin.

cytoplasmic dense cells and tracheary cells. In highly regenerable calli, the tracheary cells differentiated quickly after the calli were transferred to the regeneration medium. During this early stage, the IAA and zeatin existed prominently in the tracheary cells (Fig. 3d,e,f). The unregenerable calli showed much less differentiation of tracheary

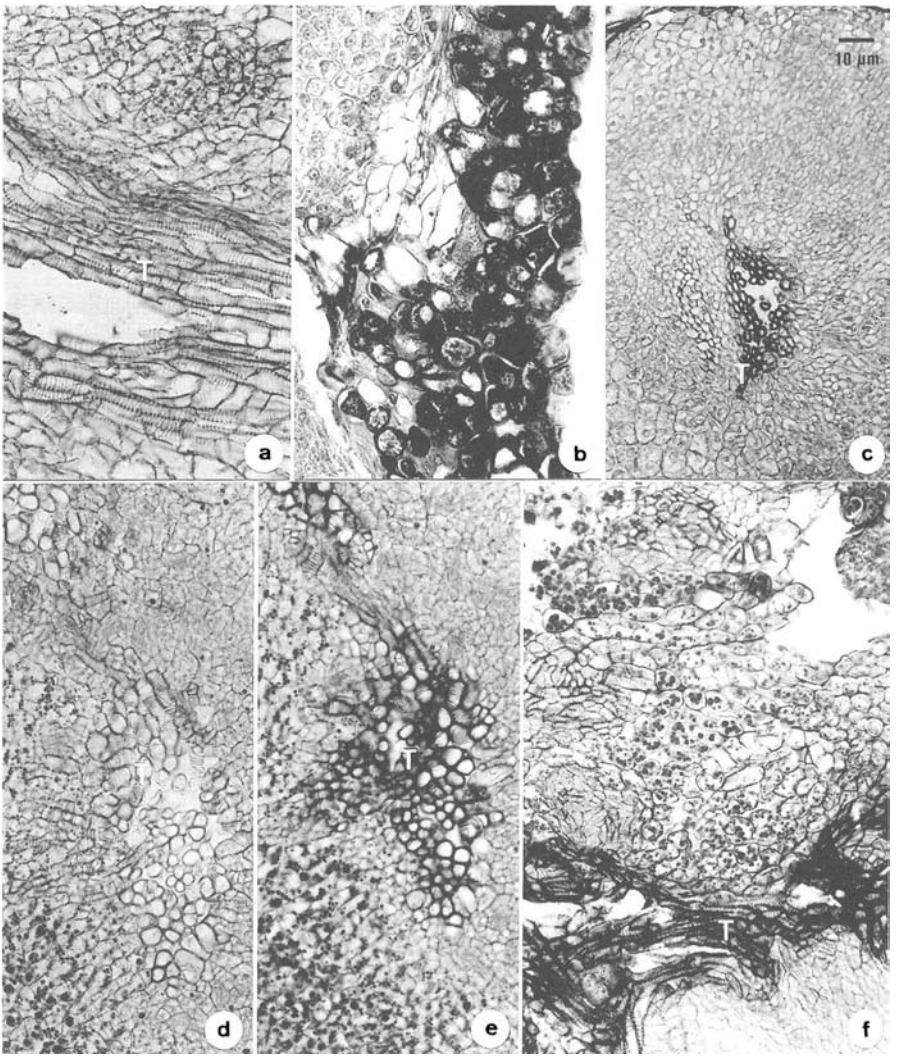

Fig. 3. Cellular localization of endogenous IAA in rice callus: a) callus of TN5 induced on MSD10 medium cannot be immunolabeled by IAA and zeatin antibodies; b) callus of ANT39, the specialized cells can be immunolabeled by IAA antibody; c) callus of ANT39, the tracheary cells(T) are immunolabeled by IAA antibody; d) callus of ANT39 being transferred to regeneration medium for 3 d—the callus treated without primary antibody shows no immunological reaction; e) callus of ANT39 being transferred to regeneration medium for 3 d—the tracheary cells are immunolabeled by IAA antibody; f) callus of ANT39 being transferred to regeneration medium for 6 d—the tracheary cells are well-differentiated and prominently immunolabeled by IAA. The localization of zeatin in rice callus is similar to that of IAA.

cells and much lower detectable IAA and zeatin in cells (Fig. 3a). This suggests that IAA and zeatin have important roles in the differentiation of tracheary cells, which may support the further formation of shoots and roots.

In the past, it was usually emphasized that the ratio of exogenous phytohormones affects plant regeneration. Since the new analysis technique was developed, the importance of endogenous phytohormones has been noticed. In this experiment, we established good systems for analysis of three kinds of endogenous phytohormones in the same tissue simultaneously. It helps to understand the possible functions and also the interactions among different phytohormones in the same cells.

The results of this experiment show the dramatic changes in the level of IAA. Similar findings have been reported only in carrot cells. During cell growth stage, carrot cells contained high levels of IAA, which synthesized through the tryptophan-mediated pathway. After 2,4-D was removed from medium, carrot cells initiates somatic embryos in which the IAA content was decreased, and the synthesis was switched to the nontryptophan pathway (Michalczuk et al 1992a,b). Further studies are being conducted to examine whether rice callus cells possess different IAA synthesis pathways during cell growth and differentiation.

The effects of osmotic status of shoot formation or somatic embryogenesis had been reported in tobacco (Brown et al 1979), wild carrot (Wetherell 1984), *Hevea brusiliensis* (Etienne et al 1991), and white spruce (Misra et al 1993). However, there is no explanation for this osmotic effect. From our studies, it is promising that the osmotic stress may regulate the endogenous phytohormone content and distribution in rice calli and then affect cell growth and differentiation.

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Protoplast culture and genetic transformation of indica and basmati rice

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Plant regeneration from cell suspension-derived protoplasts of two semidwarf, commercially cultivated rice varieties—PR4141 and Pusa Basmati 1—has been accomplished. Cell suspension cultures were initiated from mature seed scutellum-derived embryogenic calli in modified R2 medium supplemented with proline, maltose, and 2,4-D and were maintained in R2 and AA2 media. The best protoplast yield was obtained using cellulase and pectolyase and incubation for 4 h. Protoplasts of Pusa Basmati 1, cultured in Kao and Michayaluk's 8P medium and using the agarose embedding procedure and membrane nurse culture method with *Lolium multiflorum* cells as the feeder, exhibited microcolony development in both instances. However, plating efficiency and colony growth were higher using the nurse culture method. Transfer of developing colonies after 3 wk to Linsmaier and Skoog medium supplemented with 2,4-D enhanced the colony growth in both varieties. The two-step regeneration procedure led to shoot regeneration up to 14% in Pusa Basmati 1 and 10% in PR4141. Thus, complete shoots were obtained from cultured protoplasts in 6 wk. Regenerated shoots were rooted on MS medium supplemented with naphthalene acetic acid. Protoplast-derived plants of Pusa Basmati 1 were fertile and were grown to full maturity. Genetic transformation has been attempted in both varieties using methods of polyethylene glycol-mediated DNA uptake by protoplasts and particle gun and some selectable marker and reporter genes such as *bar, npt11,* and *gus.* Transformed cells/tissues have been grown on inhibitory concentrations of hygromycin and glufosinate and complete plants have been regenerated.

A combination of protoplast culture and direct DNA delivery techniques has been successfully used to produce fertile transgenic rice (Shimamoto et al 1989, Hayashimoto et al 1990, Peng et al 1992). In association with such transformation systems, better plating efficiency of protoplasts and efficient plant regeneration are necessary. But protoplast culture and plant regeneration have been found to be genotype-dependent with indica rice varieties being least responsive (Lee et al 1989, Datta et al 1990, Jain et al 1995). Successful establishment of embryogenic cell suspension cultures, using R2 and AA2 media and nurse culture methods (Kyozuka et al 1987, Wen et al 1991, Jain et al 1995), has been shown to improve the protoplast culture of rice. Therefore, this paper deals with the development of a protoplast-to-plant system in two commercial indica rice varieties and standardization of transformation procedures.

Materials and methods

Embryogenic callus cultures were established from mature seed scutellum of Pusa Basmati and PR4141 on agar (0.8% Difco Bacto) solidified Murashige and Skoog (1962) medium supplemented with 2 mg 2,4-D L^{-1} and 0.5 mg kinetin L^{-1}. Cell suspension cultures were raised in modified R2 medium (Ohira et al 1973) supplemented with L-proline (560 mg L^{-1}), maltose (30 g L^{-1}), and 2,4-D (1.0 mg L^{-1}). Cell suspension cultures were maintained in AA2 medium (Muller and Grafe 1978) through weekly subculturing. Protoplasts were isolated from actively growing cell suspension cultures using enzyme mixture (cellulase, l.0% and pectolyase, 0.1%) for 4 h at 28°C in the dark. Protoplasts were cultured at densities ranging from 5×10^5 to 10×10^5 protoplast ml^{-1} in Kao and Michayluk's medium using membrane nurse culture method with *Lolium multiflorum* cells (provided by Prof. E.C. Cocking, University of Nottingham, UK) as the feeder. Genetic transformation was attempted using protoplast and polyethylene glycol-mediated DNA uptake and embryogenic calli and the particle gun method. Plasmid pUBA (Toki et al 1992) was used for the transformation.

Results and discussion

Best yield of protoplasts (8.5×10^6 g fresh $weight^{-1}$) was obtained from 4- to 8-mo-old cell suspensions after enzyme (cellulase 1.0% and pectolyase 0.1%) incubation for 4 h at 28 °C. This enzyme mixture has been widely used for protoplast isolation in rice. Protoplasts of the varieties were heterogeneous and size ranged from 16-32 to 13-38 µm, respectively. The absence of a cell wall was confirmed by the lack of fluorescence after staining with Calcoflour White. Fluorescence diacetic acid (FDA) test revealed that protoplasts were viable in both varieties. Protoplasts of Pusa Basmati 1, cultured in Kao and Michayluk's 8P medium using the agarose (0.2% Sigma) embedding procedure and membrane nurse culture method with *Lolium multiflorum* cells as the feeder, exhibited microcolony development in both instances. The plating efficiency of Pusa Basmati 1 in terms of viable colony formation after 4 wk was 0.1% using agarose embedding, which was increased to 0.4% using nurse cells (Table 1).

Table 1. Protoplast plating efficiencies and plant regeneration in Pusa Basmati 1 and PR4141.

Protoplast culture method/variety	Plating efficiency (%)	Shoot regeneration (%)
Agarose embedding		
Pusa Basmati 1	0.10	10.5
PR4141	0	0
Nurse culture		
Pusa Basmati 1	0.40	14.0
PR4141	0.22	10.0

Whereas, embedded protoplasts of PR4141 did not sustain divisions, it was essential to use nurse cells of *Lolium* for obtaining microcolonies. Using nurse cells, the plating efficiency of Pusa Basmati 1 (0.4%) was better than that of PR4141 (0.22%). Both homologous as well as heterologous feeder cells have been found to improve plating efficiencies of protoplasts in both japonica as well as indica rices (Kyozuka et al 1987, Lee et al 1989, Jain et al 1995). Nurse cultures help in maintaining a critical density of active cells, producing sufficient levels of growth-promoting factors for sustained cell divisions in cultured protoplasts. Transfer of developing colonies after 3 wk to Linsmaier and Skoog (1965) agarose-solidified medium supplemented with 2,4-D (2.5 mg L^{-1}) enhanced the colony growth in both the varieties.

The two-step regeneration procedure, i.e., transferring 4- to 5-wk-old colonies to MS medium supplemented with naphthalene acetic acid (NAA, 0.5 mg L^{-1}) and kinetin (2 mg L^{-1}) solidified with agarose (1.0% Sigma) for 2 wk in total dark followed by transfer of colonies to fresh medium having same composition but reduced agarose (0.4%), led to shoot regeneration up to 14% in Pusa Basmati 1 and 10% in PR4141. Simple dehydration treatment has been found to promote plantlet regeneration from rice calli (Tsukahara and Hirosawa 1992). Transfer of colonies to the MS medium with elevation of agarose (1.0%) during step 1 also induces dehydration in the protoplast colonies. Using this procedure, complete shoots were obtained from cultured protoplasts in 6 wk. Regenerated shoots were rooted on MS medium supplemented with NAA (1.5 mg L^{-1}). Protoplast-derived plants of Pusa Basmati 1 have been grown to full maturity in the pots and their next progeny in the field. Embryogenic calli, bombarded with plasmid DNA after proliferation under nonselective conditions for 2 wk, were exposed to a selection pressure of bialaphos (5 mg L^{-1}). Likewise, 2-wk-old protoplast-derived colonies growing on membranes were also transferred to LS medium containing bialaphos (5 mg L^{-1}). Bialaphos was very effective as a selecting agent as more than 85 calli exhibited browning in both the varieties. Besides, the use of *nptII* and *gus* have also given positive results. Complete plants have been regenerated from transformed cells/tissues grown on inhibitory concentrations of hygromycin and bialaphos.

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Protoplast regeneration and variation in protoclones of indica rice

N.M. Ramaswamy, S. Sukumar, D. Tamil Selvi, A. Selvi, and S.R. Sree Rangasamy

Stable variability of agronomic traits in the protoclones of two locally adapted indica rice cultivars, IR50 and CO 45, was observed across three generations. Protoplast regeneration was achieved using seed calli of CO 45 and cell suspension cultures of IR50. Regeneration of proliferated microcalli occurred in MS basal medium in IR50 and in MS + kinetin in CO 45. The Pt_1, Pt_2, and Pt_3 generations of the protoplast-derived plants were evaluated in the field for plant height, tiller number, productive tiller number, panicle length, spikelet fertility, and grain weight per plant. The extent of the stable variability for these traits expressed in the protoclones during the field tests indicates useful genetic diversity and the utility of the protoclones in rice breeding programs.

In rice, protoplast culture and regeneration have been achieved in japonica and indica cultivars (Lee et al 1989) and wild species (Baset et al 1991, Zhu Deyao et al 1995) using suspension calli derived from different explant sources—pollen, hypocotyl, root, immature embryo, and seed. Protoplast regeneration has also been achieved using the leaf sheath (Gupta and Pattanayak 1993, Gupta et a1 1995, Gupta and Gupta 1995).

Only a few reports are available on the field performance of rice protoclones (Ogura et al 1989, Ramaswamy et al 1995). In the study reported here, protoplast regeneration was achieved using two locally adapted indica rices (IR50 and CO 45). The quantitative characters of the protoclones were evaluated in the Pt_1-Pt_3 generations.

Protoplast isolation and culture

Protoplast isolation was accomplished using seed calli of CO 45 and cell suspension cultures of IR50. Rice suspension cells were initiated from embryo-derived calli of IR50. To induce callus, seeds were sterilized in 0.1 c/c mercuric chloride for 5 min and placed in agar-solidified MS medium supplemented with 2,4-D (2.0 mg L^{-1}), kinetin (0.5 mg L^{-1}), and sucrose (3%).

The embryogenic calli were then transferred to liquid AA medium to establish embryogenic cell suspension cultures. Suspension cells were cultured in a 150-ml erlenmeyer flask containing 30 ml of medium and kept at 25 °C in an 80-rpm gyratory shaker. All cells were subcultured twice a week. Cells were collected on days 7 and 9 and treated overnight with enzyme solution (CPW + cellulase RS 2.0%. macroenzyme R10 1.5%) for protoplast isolation. Isolated protoplasts were purified by washing and centrifugating in CPW solution. The yields of the protoplasts were 3.0-5.6 × 10^6 ml^{-1} and 0.15-1.5 × 10^6 ml^{-1} for IR50 and CO 45, respectively.

IR50 protoplasts were cultured by agarose embedding (0.8%). The agarose block was cut into pieces. Nurse cells with 1.0 ml of liquid K3 medium supplemented with NAA (1.5 mg L^{-1}) + BAP (0.5 mg L^{-1}) were then added to the plates. A plating efficiency of 8.2% was observed. CO 45 protoplasts were plated into MS medium supplemented with BAP (1 mg L^{-1}), NAA (1.5 mg L^{-1}), and sucrose. A plating efficiency of 7.0% was observed. IR50 microcalli proliferated and regenerated in MS basal medium and CO 45 microcalli proliferated in MS + kinetin (1 mg L^{-1}) + NAA (1 mg L^{-1}).

Field evaluation of protoclones

The protoplast-derived plants in the Pt_1, Pt_2, and Pt_3 generations were observed in the field for their quantitative characters. Seeds collected from Pt_0 plants were raised as single-plant progeny in the three generations with 15- × 10-cm spacing and evaluated for six characters—plant height, tiller number. productive tiller number, panicle length, spikelet fertility, and grain weight per plant.

In the Pt_1, panicle length and number of productive tillers were higher in the IR50 protoclones than in the control. However. the number of productive tillers. spikelet fertility, and grain weight per plant were higher in the CO 45 protoclones than in the control. In the Pt_2, grain weight per plant was high both in the IR50 and CO 45 protoclones. Correspondingly, an increase in the number of productive tillers was observed in IR50 but not in the CO 45 protoclones. The range of variation for all characters across the three generations was wider in the IR50 protoclones than in the control. In the CO 45 protoclones, the ranges of variation for plant height (63.2-95.5 cm) and the number of productive tillers (10-24) were less than those of the control (60.5-120.5 and 12-30 cm).

Variation in plant height, tiller number. spikelet fertility, and grain weight per plant was higher in CO 45 than in the IR50 protoclones. In the Pt_3 CO 45 protoclones, there was a reduction in the mean number of productive tillers, and the range was wider (4.7-17.5) compared with the control (14-19). This showed increased variation in productive tillers among the protoclones. However, the range of variation of the IR50 protoclones was wider (4.0-38.0) compared with the CO 45 protoclones.

Moreover, a reduction in mean panicle length was observed in the CO 45 protoclones whereas an increase was noted in the IR50 protoclones. Panicle length variation was wider in the CO 45 (15.6-25.9 cm) than with the IR50 protoclones (16.99-20.16 cm). In terms of spikelet fertility, an increase was seen in the CO 45 protoclones whereas a slight reduction was seen in the IR50 protoclones. Grain weight per plant was slightly less in the CO 45 protoclones than in the control—the increase

was more than twice that in the IR50 protoclones. The range of variation for plant height, panicle length, spikelet fertility, and grain weight per plant was higher in the CO 45 than in the IR50 protoclones.

IR50 protoclones maintained more or less uniform plant height across the three generations. The mean panicle length and number of productive tillers in IR50 protoclones were higher across the three generations than in the respective controls. The mean grain weight per plant was more or less uniform in the Pt_1 generation than it was in the controls. An increase in mean grain weight was noticed in the Pt_2 and Pt_3 generations.

CO 45 protoclones showed a drastic reduction in plant height when compared with the controls across the three generations. The mean number of productive tillers was lower than the controls in the Pt_2 and Pt_3 generations but higher than the control in the Pt_1. A decrease in mean panicle length was observed across the three generations. Mean spikelet fertility was low in the Pt_0, but it increased in the Pt_1-Pt_3 generations. Higher mean grain weights were observed in the Pt_1 and Pt_2 generations than in the controls.

Discussion and conclusions

Stable variability of agronomic traits in the protoclones of both IR50 and CO 45 was observed across the three generations. Ogura et al (1989) made similar observations in japonica types.

IR50 protoclones showed uniformity in plant height across the three generations. The increase in grain weight per plant could be attributed to the corresponding increase in the productive tillers and panicle length. Similar observations were noted in japonica protoclones by Ogura et al (1989) and Ogura and Shimamoto (1991) and in a review by Finch et al (1991).

The selfed Pt_0 progenies of CO 45 significantly showed a uniform reduction in plant height across the three generations, which indicated an additive expression of dwarfness and a stable inheritance of this trait.

Early flowering (85-90 d compared with 110 d in the controls) was also observed in the CO 45 protoclones. Such induced variation is not only attributed to media effects, but also to gamma irradiation as reported by Cai et al (1987) and Qi et al (1988).

In the present study, the significant variations observed in the protoclones for plant height (dwarfism) and early flowering in the selfed Pt_0 progenies of CO 45 may be due to the effect of endogenous or exogenous hormonal regulations and the genotypic differences. The extent of the stable variability for these traits expressed in the protoclones during the field tests indicates useful genetic diversity and their utility in rice breeding programs.

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Transfer of cytoplasmic male sterility in rice using the donor-recipient method

T. Kinoshita and K.-I. Mori

Male sterile cytoplasm in rice can be transferred by using the "donor-recipient method", which involves asymmetric cell fusion. In this experiment, protoplasts were isolated from gamma-irradiated suspension cells of the A-58CMS line (containing the cytoplasm of Chinsurah Boro II) and electrofused with those from Japanese rice variety Fujiminori after metabolic inhibition with iodoacetoamide. In R_0 plants, considerable variation in seed fertility was produced. Completely sterile plants were most likely caused by the combination of Fujiminori's nuclear genome and Chinsurah Boro II [*cms-bo*] cytoplasm. Transfer of cytoplasmic male sterility from A-58CMS was also achieved using the promising strain, 'Kochihibiki'. The donor-recipient method may have a practical use in shortening the time it takes to produce male-sterile strains.

Transfer of male sterile cytoplasm in rice can be accomplished by using the "donor-recipient method," which involves asymmetric cell fusion (Aviv et al 1984). Protoplasts isolated from gamma-irradiated suspension cells of A-58CMS were electrofused with those from the maintainer line Fujiminori after metabolic inhibition with iodoacetoamide. In R_0 plants regenerated from the fused products, considerable variation in seed fertility was observed, i.e., from completely sterile to nearly normal.

It was also recognized that a single completely sterile plant was propagated asexually to several clones and some of them showed a segregation of fertile and sterile panicles in the R_0 generation. Further, the stable transmission of cytoplasmic male sterility (CMS) was confirmed in the progeny when crossed with Fujiminori. When male fertile R_0 plants were crossed to A-58CMS, no pollen restoration occurred in their progeny. Therefore, it is estimated that the nuclear genotype of male fertile plants is the same with Fujiminori (*rf1rf1*). Thus, it was postulated that the appearance of male fertile plants is caused by the sorting out of sterile elements from heteroplasmon after cytoplasmic hybridization.

The nature of the *atp6* gene corresponded fairly well with expression of CMS in cybrid plants. However, two R_0 plants having a novel pattern were produced by the outcome of recombination in mitochondrial genomes. Further, it was found that the 10.4-kb *Xho*I fragment containing *rps1* in the mitochondrial DNA (mt DNA) was deeply related to CMS together with the chimeric *atp6* gene (*urf-rmc*).

Transfer of CMS from A-58CMS was also achieved in the promising strain, Kochihibiki and it has practical applications for breeding hybrid rice.

Heterosis breeding is widely practiced to raise the yield potential in rice. CMS can be used for seed production of hybrid rice on a large scale.

Kinoshita and Mori (1992) demonstrated that CMS can be transferred in rice using the donor-recipient method. Following this, stable transmission and variability of fertility were examined through generations and mitochondrial alteration in cybrid plants were investigated by restriction fragment length polymorphism (RFLP) analysis with the probes of known genes and cosmid clones.

Materials and methods

The A-58CMS line has the cytoplasm of Chinsurah Boro II [*cms-bo*] and the nuclear background of variety A-58 Kokushokuto-2. Fujiminori is a recommended rice vanety in Japan.

Protoplasts were isolated from gamma-irradiated suspension cells of A-58CMS and electrofused with those from Fujiminori after metabolic inhibition with iodoacetoamide. R_0 plants were regenerated from the fused products using nurse culture. Crossing experiments were conducted between R_0 plants and testers, and transmission of pollen and seed fertilities were examined. Isolation of total DNA and hybridization analysis of *atp6* (ATPase subunit 6) followed conventional procedures. mt DNA was also prepared from cultured cells of parental and R_0 plants, and cosmid clones covering physical map of mt DNA and several gene probes were used for RFLP analysis to detect the structural difference of mt DNA between male-sterile and fertile cybrids.

Results and discussion

Transmission of CMS

In R_0 plants, considerable variation in seed fertility was produced. It is estimated that completely sterile plants were caused by the combination of Fujiminori's nuclear genome and *cms-bo* cytoplasm. However, it is noted that one of the completely sterile plants produced both male-fertile and male-sterile clones when propagated asexually, suggesting the existence of a heteroplasmic state in the original cybrid (Izhar et al 1983).

In the progeny of the crossings between male-sterile R_0 plants and Fujiminori (nuclear genotype: *rf1rf1*), a stable transmission of CMS was demonstrated in most of the R_0 plants except in two lines (Cy-35 and Cy-56). Later, it was proven that both lines are derived from R_0 plants having a single copy of *atp6* equivalent to those of

Fujiminori. In male-fertile R_0 plants, the crossing experiments showed there is no alteration of nuclear genotype (*rf1rf1*) from Fujiminori. Therefore, it was implied that the sorting out of sterile elements from the first heteroplasmic state produced various degrees of fertility restoration in the R_0 plants. It was recognized that seed fertility of male-fertile R_0 plants increased prominently through the generations and restored the stability in R_1 lines (Table 1).

Mitochondrial structure in cybrids

Kadowaki et al (1990) demonstrated that the extra *atp6* gene in *cms-bo* cytoplasm plays an important role in CMS expression, and the chimeric atp6 gene was identified as *urf-rmc*. As shown in Table 2, total DNA of 32 male-sterile R_0 plants possessed two copies of the *atp6* equivalent with A-58CMS. Further, two plants from male-sterile R_0 plants contained an extra signal of atp6 besides the two confirmed in A-58CMS. There is a possibility that a new constitution of mt DNA was caused by a recombination of the parental mt DNAs. On the other hand, male-fertile R_0 plants mostly consisted of the pattern equivalent of that in Fujiminori. Since the progeny of an exceptional plant showing the [*cms-bo*] pattern were only male-fertile, it was estimated that the rapid sorting out of sterile elements occurred in the R_0 plant. Thus, the expression of pollen and seed fertilities was also supported from the behavior of the atp6 gene, depending on the postulation of Kadowaki et al (1990).

To detect the mitochondrial structure related to CMS, the mt DNAs from cultured cells of cybrids were prepared and Southern hybridization analysis was

Table 1. Transmission of pollen and seed fertility from male-fertile R_0 plants to R_1 lines after self-fertilization.

	Seed fertility (R_0)(%)		Mean fertility (R_1)(%)	
Line[a]	Early	Late[b]	Pollen	Seed
Cy-141	3	10.3	97.0	72.6
Cy-3A	5	14.1		63.6
Cy-3B	5	14.1		75.2
Cy-6B	5	9.2	69.3	78.7
Cy-4H	37	34.1	-	86.0
Cy-4M	25	34.1	-	68.3
Cy-16A	22	35.7	79.3	-
Cy-16B	27	35.7	95.3	75.7
Cy-16C	21	35.7	93.3	73.8
Cy-14L	-	78.7	86.0	80.4
Cy-14M	40	78.7	-	69.0
Cy-44H	75	78.6	93.3	78.6
Cy-44L	22	78.6	82.0	88.2
Cy-44M	50	78.6	88.7	85.8
Cy-48	43	75.9	85.3	78.0
cy-35	53	83.4	87.3	88.8
cy-102	56	70.6	96.7	71.1
Cy-62	66	76.0	84.7	58.3

[a]A letter in the line no. means that the clone propagated asexually. [b]Surveyed 1 yr after regeneration.

Table 2. Hybridization analysis of the *atp6* gene in the R_0 cybrids and their parents.[a]

Line	Total	Hybridization pattern		
		Fujimonori	*cms-bo*	Novel
Fujiminori	1	1	-	-
A-58CMS	1	-	1	-
Sterile cybrids (R_0)	36	2	32	2
Fertile cybrids (R_0)	12	11	1	0
Total	48	13	33	2

[a] Total DNAs were prepared from leaves and roots of intact plants.

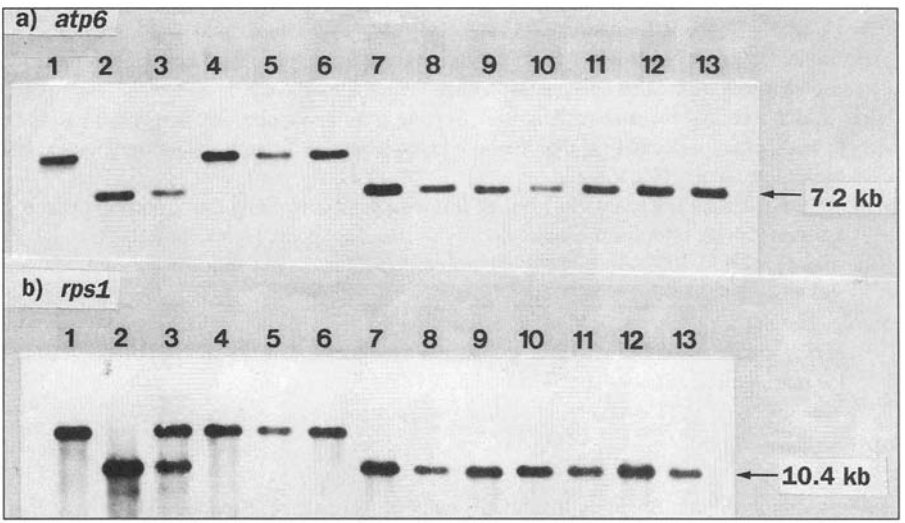

Fig. 1. Hybridization of gene probes *(atp6* and *rps1)* to the total mt DNA digests from the cultured cells of cybrids and their parents. a) mt DNA was digested with *Hin*dlll and probed with *atp6* (2.0-kb *Eco*R1 fragment); b) mt DNA was digested with *Xhol* and probed with *rps1* (4.4-kb *Sal*l fragment). Lanes: 1, Fujiminori; 2, A-58CMS; 3, I-44 Bhutmuri 36 (boro type); 46, male-fertile cybrids; 7-13, male-sterile cybrids.

conducted with cosmid clones covering the physical map and several known gene probes. First, RFLPs were found among the mt DNAs from the parental lines and R_0 cybrids with the cosmid clones containing the *atp6* gene. The 7.2-kb *Hin*dIII fragment exists only in both A-58CMS and seven male-sterile cybrids (Fig. 1 a). To enrich the physical map of mt DNA constructed by Yamato et al (1992), nine mitochondria1 genes (*rps4, rps11, atp9, rps1, rps7, rps3, rp116, nad3,* and *nad12*) were newly located on the map and used for RFLP analysis related to CMS. One of them, *rps1* (ribosomal

protein small subunit 1) was involved in a cosmid clone of HB94. RFLPs were detected between male-sterile and male-fertile cybrids corresponding to those in the parental lines when digested with *Xho*I and probed with HB94. Further, it was demonstrated that the 10.4-kb *Xho*I fragment was specifically found in A-58CMS and male-sterile cybrids by the hybridization analysis of *rps1* (Fig. 1b).

In addition to confirming the chimeric *atp6*, it was found that the regions around *rps1* are also related to the mitochondrial difference between male-sterile and male-fertile cybrids. It is plausible that a new chimeric gene related to *rps1* is responsible for CMS in conjunction with the nuclear genotype, *rf1rf1*.

Transfer of CMS from A-58CMS was also achieved using the promising strain, 'Kochihibiki', through the donor-recipient method. It may have a practical use in shortening the time it takes to produce male-sterile strains.

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Interaction of rhizobia with rice for symbiotic nitrogen fixation

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Until recently, the likelihood of cereals and other nonlegume crops symbiotically fixing nitrogen seemed remote, but recent advances in knowledge of the genetics of rhizobia and the interaction of rhizobia with their roots have increased greatly the chance of success. We have induced interaction between oxygen-tolerant *Azorhizobium caulinodans* ORS571 (isolated from stem nodules of the tropical legume *Sesbania rostrata*) and the root systems of rice and wheat and conducted an assay for nitrogen fixation. Interaction with rice resulted in the development of plants with short, thickened lateral roots containing intercellular and intracellular rhizobia in the cortex, interspersed among normally developing lateral roots. These plants possessed low levels of nitrogen-fixing activity as determined using the acetylene reduction assay. No acetylene reduction activity was detected after interaction with *nif* mutants of *Az. caulinodans.* Interaction of *Az. caulinodans* with wheat resulted in plants with very significant nitrogen fixation activity and evidence of symbiotic nitrogen fixation.

A major concern is how to supply, in the future, sufficient fixed nitrogen to our major crops—rice, wheat, and maize—none of which are presently nitrogen self-sufficient. The objective of increasing the yield plateau of rice grown under tropical conditions from 10 to 15 t ha^{-1} requires sustainable methods of nitrogen supply (Bennett and Ladha 1992). The challenge is to increase productivity while reducing inputs and controlling pollution.

One possibility involves causing cereals and rhizobia to engage in symbiosis. Newton (1994) suggested that if such a symbiosis could be established, it would overcome the 'leaky' nature of associative symbioses. In a critical evaluation of the prospects for nitrogen fixation in nonlegumes, Quispel (1991) concluded that successful and fully efficient use of atmospheric nitrogen for plant growth can only be expected in endosymbiotic systems. Only in such systems can the prerequisites for

effective nitrogen fixation be fulfilled, i.e., a reliable supply of metabolic substrates from host photosynthesis providing sufficient energy and reducing conditions, protection against high oxygen concentrations, transport of the nitrogen fixation products to the plant host, development of membrane systems for bidirectional transport between host and endosymbiont, and protection against antagonistic bacteria in the environment. Encouragingly, all these suggested requirements are met in the effective endosymbiotic nitrogen-fixing root nodule system resulting from the interaction of rhizobia with the nonlegume *Parasponia,* a tropical member of the elm tree family (Ulmaceae) (Webster et al 1995). Our strategy in investigating the interaction of rhizobia with rice and other nonlegume crops for symbiotic nitrogen fixation has involved trying to establish a similar endosymbiotic interaction.

Crack entry of rhizobia

More than a century ago, Schneider (1893) undertook experiments to force an interaction between rhizobia and the roots of maize and other nonlegume crops. He observed extensive infection by rhizobia in parenchymatous cells, at the crack entry point, near the vicinity of the emergence of maize lateral roots. Unfortunately, further invasion did not occur. *Azorhizobium caulinodans* ORS571 is known to form stem and root nodules on the tropical legume *Sesbania rostrata.* These nodules are of lateral root origin; the invasion pathway is by crack entry. *Bradyrhizobium* ORS310 also forms stem nodules on another tropical legume, *Aeschynomene indica,* which are also of lateral root origin. Again, the invasion pathway is by crack entry. We have shown that such rhizobia, isolated from stem nodules—when interacted with the roots of seedlings of maize, rice, and wheat—are also able to invade these nonlegume crops via crack entry where the lateral roots emerge through the root epidermis (Cocking et al 1994). We suggested that these particular rhizobia have invasive properties, probably associated with the secretion of rhizobial cellulase and pectinase, which enable them to penetrate between the cells of the primordia of emerging lateral roots and, subsequently, into cells of the cortex.

Nitrogen fixation and oxygen

It is known that the nitrogenase of *Az. caulinodans,* unlike that of most other rhizobia, is tolerant of up to 3% of oxygen in the gas phase when cells are grown in nitrogen-free liquid medium (O'Gara and Shanmugan 1976). *Bradyrhizobium* ORS310 is also known to have a nitrogenase that is tolerant of up to 0.5% oxygen under similar conditions (Alazard 1990). Because it was anticipated that nitrogen fixation in the interactions involved in the crack entry of rhizobia into emerging lateral roots of nonlegume crops might be inhibited by the oxygen levels present. nitrogen fixation studies were undertaken using *Az. caulinodans* and *Bradyrhizobium* ORS310.

Development of mutualistic endosymbiosis

Our identification of oxygen-tolerant, nitrogen-fixing, stem-nodulating rhizobia, which invade by crack entry, provided us with a direct experimental approach in our attempt to establish nonlegume crop nitrogen-fixing endosymbiosis. The expectation was that these rhizobia might find an inter- or intracellular niche in invaded lateral roots with sufficiently reducing conditions and with the whole structure integrated in the long-distance transport system of the plant. As pointed out by Quispel (1991), it may not be necessary for the highly evolved system of peribacteroid membranes as found in the rhizobia1 bacteroid system (with their peribacteroid membranes). Also in some Leguminosae, in *Parasponia,* and in the *Frankia* actinorhizae, efficient nitrogen fixation is possible in an apparently less evolved system.

Our experimental approach has utilized both rice and wheat. We have investigated the extent to which the interaction of the existing biodiversity of rhizobia and plant varieties can result in a mutualistic endosymbiosis.

Rice seeds were surface-sterilized, grown in pots in a mixture of vermiculite and perlite and 7-d old plants were inoculated with rhizobia under controlled growth conditions. After 2 and 4 wk, plants were assayed for nitrogen fixation (Cocking 1995). In these pot experiments, the highest nitrogen fixation activity was obtained using *Az. caulinodans* (Acc. IRBG314), kindly provided by Dr. J.K. Ladha, IRRI; with the rice variety Lemont (maximum ethylene production 154 nanomoles $plant^{-1}d^{-1}$). In these pot experiments, it was also observed that the onset of nitrogen fixation was associated with the formation of short, thickened lateral roots (STLRs) invaded by rhizobia, and that STLRs were absent in rice plants that were not fixing nitrogen. A study of these STLRs to examine the efficiency of crack inoculation has been initiated.

In experiments with wheat, 7-d-old plants (variety Canon), growing in pots in vermiculite and perlite, were inoculated with *Az. caulinodans.* The control treatments, with no ethylene production, included inoculation with *nif Az. caulinodans,* and no inoculation. Very significant levels of nitrogen fixation were detected with *Az. caulinodans* (IRBG314) (mean ethylene production 3683 nanomoles $plant^{-1}d^{-1}$). These very significant levels of nitrogen fixation in wheat were correlated with a progressive increase in the number of STLRs, with rhizobia present both between and within cells (Cocking 1995).

Following these findings, an experiment was set up directly in pots to evaluate nitrogen fixation (ARA assays), dry weight, and total nitrogen in two inoculated wheat varieties, and in controls with no inoculation, and with no inoculation but with the supply of fixed nitrogen as NH_4NO_3. The results have clearly demonstrated that inoculation with *Az. caulinodans* is beneficial to plant growth, resulting in a significant increase in the dry weight and nitrogen content of plants compared with uninoculated plants. Overall, these results indicate the likelihood of the early development of the use of inoculation with *Az. caulinodans,* and similar rhizobia, under field conditions for the formation of a mutualistic endosymbiosis in wheat (S.R.S. Sabry, S.A. Saleh, C.A. Batchelor, J. Jones, J. Jotham, G. Webster, S.L. Kothari, M.R. Davey, E.C. Cocking, 1995, unpubl. data).

Outlook for effective rice endosymbiosis

This recent success in establishing an effective nitrogen-fixing endosymbiotic interaction of *Az. caulinodans* with wheat (Sabry et al 1995) raises the question as to whether this will also now be achievable with rice. Clearly. we require a better understanding of the interaction of azorhizobia with rice. In this respect, it is encouraging that IRRI has recently established a Working Group for its Frontier Project on Nitrogen Fixation in Rice. Success hopefully should not be far away for symbiotic nitrogen fixation in rice; wheat has already provided the essential clues.

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Notes

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Development of microsatellite markers and characterization of simple sequence length polymorphism in rice (*Oryza sativa* L.)

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Microsatellite markers containing simple sequence repeats are a valuable tool for genetic analysis. Our objective is to augment the existing restriction fragment length polymorphism map of rice with simple sequence length polymorphisms (SSLP). In this study, we describe 20 new microsatellite markers that have been assigned to positions along the rice chromosomes, characterized for their allelic diversity in cultivated and wild rice, and tested for amplification in related *Oryza* spp. Our results indicate that the genomic distribution of microsatellites in rice appears to be random, with no obvious bias or clustering to particular regions, that mapping results are identical in interspecific and intersubspecific populations, and that amplification in wild relatives of *O. sativa* is reliable in species most closely related to cultivated rice but falls off as the genetic distance increases. Two microsatellite markers amplified multiple loci that were mapped onto independent rice chromosomes, suggesting duplicated regions within the rice genome. The availability of increasing numbers of mapped SSLP markers can be expected to increase the power and resolution of genome analysis in rice.

New marker technologies and mapping strategies make it increasingly easy to generate high-resolution molecular maps for a wide array of crop and animal species. Microsatellites, or simple sequence repeats (SSR), detect simple sequence length polymorphisms (SSLP) and are an important source of genetic markers because they offer a way of obtaining single-copy, codominant markers from regions of repeated DNA, they tend to be highly variable, and they are technically simple to use. They are easily and economically assayed using the polymerase chain reaction (PCR) and gel electrophoresis, and can be efficiently distributed throughout the world by publishing the sequence of the PCR primers used to amplify the markers. Microsatellites have been successfully used as tools in fingerprinting and variety identification, germplasm

evaluation, gene diagnostics, and gene localization in a variety of crops (Yang et al 1994, Devos et al 1995, Rongwen et al 1995, Blair and McCouch 1996).

To date, very few microsatellite markers are available for rice (Wu and Tanksley, Zhang and Kochert 1992), but there is a clear need for markers that reveal a high level of allelic diversity so that closely related germplasm can be distinguished and crosses involving members of the same subspecies can be effectively analyzed.

The aim of our project is to expand the existing restriction fragment length polymorphism (RFLP) map with microsatellite markers. In this report we present primer sequences and amplified SSR motifs, map location, estimates of allelic variation and stability in a range of rice germplasm, and an estimate of the polymorphism information content of 20 new markers.

Materials and methods

Isolation and sequencing of microsatellite-containing clones

A 300-bp insert genomic library of rice cv IR36 was constructed in the lambda-Zap II vector using the DNase partial-blunt-adaptor-gel isolate method (Stratagene Co., La Jolla, CA, USA). This library was screened by plaque and colony hybridization for clones containing poly$(GA)_n$, poly$(ATC)_n$, and poly$(ATT)_n$ microsatellites. Minipreps of positive clones were sequenced by the Cornell sequencing facility using an Applied Biosystem 373A machine.

Primer design and PCR evaluation

PCR primer sequences consisting of 18-24 bp on either side of the microsatellite repeats were selected using the PRIMER program (Eric Lander, Whitehead Institute). Primers were synthesized by Research Genetics (2130 Memoral Parkway SW, Huntsville, AL 35801; e-mail address: krushing@resgen.com) and can be obtained by requesting them directly from Research Genetics. All primer pairs were tested for PCR amplification using *O. sativa* cv IR36 DNA (100 ng) as the template. PCR was performed in 50 µl reactions containing 0.2 µM of each primer, 200 µM deoxyribonucleotides, 50 mM KCl, 10 mM Tris-Cl, pH 8.3, 1.5 mM $MgCl_2$, 0.01% gelatin, and 1 unit of Taq polymerase. The PCR profile was: 94 °C for 5 min (denaturation), followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min, and finally by 5 min at 72 °C for final extension. The PCR reaction was performed in PTC100 96U thermocycler (MJ Research Inc., Watertown, MA).

Mapping of SSLP

Primers showing amplification of a single band on a 2% agarose gel were evaluated for polymorphism on denaturing polyacrylamide gels using DNA from the parents of two mapping populations. The first population was an interspecific backcross population derived from a cross between *O. sativa* (cv BS 125) and *O. longistaminata* (Acc. WL02) (the "SL pop"), previously used for the development of a high-density RFLP map (Causse et al 1994). The parental survey for this population was made with DNA from BS 125 and its F_1 hybrid (BS125/WL02). The second population was a doubled haploid (DH) population of 135 individuals derived from an intersubspecific

cross between rice varieties IR64 (indica) and Azucena (tropical japonica) (the "DH pop") (Guiderdoni et al 1992, Huang et al 1994). If both parental pairs showed polymorphism, markers were preferentially mapped onto the backcross population, for which the map is the most saturated. All the individuals of the mapping population were genotyped using the same conditions as those described for the parental survey. Up to five polymorphic markers were multiplexed on a single polyacylamide gel for scoring of segregation: each marker was loaded separately, starting with the one with the lowest molecular weight and run 5 min to allow DNA to penetrate in the gel before subsequent loading.

Nonradioactive detection

PCR products were run on 4% polyacrylamide gels containing 7 M of urea, with a sequencing gel apparatus (Owl Scientific Inc., Woburn, MA). Bands were revealed using the silver staining procedure described by the manufacturer (Promega Corp). The plate on which the gel was bound was treated for 20 min with 10% acetic acid with gentle shaking, then washed three times for 2 min with distilled water. After incubating for 30 min in staining solution (0.2% w/v silver nitrate, 0.05% formaldehyde) the plate was immersed in distilled water for 10 s and transferred to developing solution (6% w/v Sodium carbonate, 0.05% formaldehyde, and 0.0002% sodium thiosulfate).

Rice germplasm and pedigree analysis

Seed from 20 rice cultivars and four wild rice species (Table 1) was provided by the International Rice Genebank Collection at IRRI and the USDA Small Grains Germplasm Collection at Aberdeen, Idaho. DNA was extracted from young leaves of plants grown in the Guterman Greenhouse at Cornell University and used as template for amplification of microsatellite markers.

The stability of microsatellite marker alleles was assayed by following their inheritance within the pedigree of rice variety IR8. The first cross in the pedigree was made in the early 1930s (Partharsarathy 1972) and IR8 was released in 1966 (IRRI 1966). Seed of IR8 has been amplified regularly in the hybridization nursery and as a check in yield trials (IRRI 1994), providing a test of the genetic stability of microsatellite loci that spans 50-60 yr.

Results

SSR motifs

Of primer pairs showing clear amplification signals on agarose and polyacrylamide gels, 18 bracketing a poly $(GA)_n$ microsatellite, one bracketing a poly $(ATC)_{10}$, and one a poly$(ATT)_{14}$ microsatellite are reported here (Table 2). The number of SSR repeat motifs in these 20 markers varied from 10 to 27, with an average of 17.4. Most of the sequences also revealed degenerate di- or trinucleotide repeats near the major SSR and variation in these adjunct repeats may contribute to the allelic variation detected at these loci.

Table 1. Species evaluated for SSLP using 25 microsatellite primer pairs.

Species	Variety	Acc. no.
Homo sapiens	Tera-2 cell line	–[a]
Mus musculus	Strain C57, Black 6	–
Lycopersicon esculentum	TA209	
Triticum aestivum	Chinese spring	–
Pennisetum glaucum	Thiotande	–
Zea mays	TX202	–
Oryza officinalis	–	IRGC 101150
O. rufipogon	–	IRGC W 1185
O. nivara	–	IRGC 103838
O. glaberrima	–	IRGC 100127
O. sativa	Rexoro	PI 389995
O. sativa	Sinawpagh	CI 5094
O. sativa	Bluerose	IRGC 151
O. sativa	Supreme Bluerose	CI 5793
O. sativa	Texas Patna	CI 8321
O. sativa	Kitchii-samba	IRGC 49796
O. sativa	GEB24	IRGC 4898
O. sativa	Latisail	IRGC 8340
O. sativa	Cina	PI 220755
O. sativa	Slo-17	IRGC 637
O. sativa	CP231	CI 8993
O. sativa	Bluebonnet	CI 8322
O. sativa	Benong	IRGC 13530
O. sativa	DGWG	IRGC 123
O. sativa	Peta	PI 233289
O. sativa	Sigadis	IRGC 611
O. sativa	CP-SLO	CI 9535
O. sativa	IR8	IRGC 10320
O. sativa	IR127	IRGC 11374
O. sativa	IR24	IRGC 19907
O. sativa	IR64	–
O. sativa	Azucena	IRGC 328

[a] Indicates no data.

Allelic diversity

Table 3 summarizes the number of alleles detected in cultivated and wild *Oryza* germplasm for each of the 20 new microsatellite primer pairs. All 20 markers amplified clearly on 20 varieties of *O. sativa* (Asian cultivated rice) listed in Table 1, on *O. glaberrima* (African cultivated rice), and on the wild species most closely related to *O. sativa (O. rufipogon* and *O. nivara,* having the AA genome), while all but four of the markers also amplified on the more distantly related *O. officinalis* (a perennial CC genome species). The number of alleles detected in 20 Asian cultivars (indica and japonica subspecies) varied from two to nine with an average of five and the average increased to eight alleles when additional AA genome species were included. When new alleles were detected in species outside of the indica-japonica germplasm group, they were detected at 65% of the loci in the AA genome species, *O. rufipogon, O. glaberrima,* and *O. nivara,* and at 81% of the loci in *O. officinalis* (Table 3). Therefore, crosses between any of these AA genome species with indica or japonica varieties

Table 2. Microsatellite information.

Locus	Clone	Chromo-some	Size (bp) in IR36	Primer sequence (forward)	Primer sequence (reverse)	Simple sequence repeat motif
RM1	GA12	1	113	GCGAAAACACAATGCAAAAA	GCGTTGGTTGGACCTGAC	ACAA(AG)26CCAC
RM2	GA122	7	150	ACGTGTCACCGCTTCCTC	ATGTCCGGGATCTCATCG	GAGG(GA)2A(GA)13ATGG
RM3	GA21	6	145	ACACTGTAGCGGCCACTG	CCTCCACTGCTCCACATCTT	TGCC(GA)2GG(GA)25GGGA
RM4A	GA25	11	159	TGACGAGGTCAGCACTGAC	AGGGTGTATCCGACTCATCG	TCTC(AG)16TTTG
RM4B	GA25	11	159	TTGACGAGGTCAGCACTGAC	AGGGTGTATCCGACTCATCG	TCTC(AG)16TTTG
RM4C	GA25	12	159	TTGACGAGGTCAGCACTGAC	AGGGTGTATCCGACTCATCG	TCTC(AG)16TTTG
RM5	GA273	1	113	TGCAACTTCTAGCTGCTCGA	GCATCCGATCTTGATGGG	TTAA(GA)14GGCT
RM6	GA285	2	163	GTCCCCTCCACCCAATTC	TCGTCTACTGTTGGCTGCAC	AGGA(AG)16CACA
RM7	GA304	3	180	TTCGCCATGAAGTCTCTCG	CCTCCCATCATTTCGTTGTT	CCTT(GA)19CCGA
RM8	GA317	2	252	CACGTGGCGTAAATACACGT	GGCCAAACCCTAACCCTG	TGAA(GA)14C(GA)2CGCC
RM9	GA330	1	136	GGTGCCATTGTCGTCCTC	ACGGCCCTCATCACCTTC	ATGG(GA)15GT(GA)2GGAG
RM10	GA337	7	159	TTGTCAAGAGGAGGCATCG	CAGAATGGGAAATGGGTCC	GAGC(G4)15GGAG
RM11	GA397	7	140	TCTCCTCTTCCCCCGATC	ATAGCGGGCGAGGCTTAG	GACA(GA)17GAAA
RM13	GA478	5	141	TCCAACATGGCAAGAGAGAG	GGTGGCATTCGATTCCAG	ATTA(GA)6TA(GA)TA(GA)16TGG
RM14	GA506	1	191	CCGAGGAGAGGAGTTCGAC	GTGCCAATTTCCTCGAAAAA	CGAC(GA)18AAGA
RM16	GA53	3	181	CGCTAGGGCAGCATCTAAAA	AACACAGCAGGTACGCGC	CAAT(GTC)5G(GA)16ATTA
RM17	GA56	12	184	TGCCCTGTTATTTTCTTCTCTC	GGTGATCCTTTCCCATTTCA	CACA(GA)21AGAA
RM18	GA97	7	157	TTCCCTCTCATGAGCTCCAT	GAGTGCCTGGCGCTGTAC	ACGT(GA)4AA(GA)(AG)16TGAA
RM19	ATC3	12	226	CAAAAACAGAGCAGATGAC	CTCAAGATGGACGCCAAGA	CTGT(ATC)10GTCA
RM20A	AT59	12	234	ATCTTGTCCCTGCAGGTCAT	GAAACAGAGGCACATTTCATTG	CCTT(ATT)14ATAA
RM20B	A759	11	234	ATCTTGTCCCTGCAGGTCAT	GAAACCAGAGGCACATTTCATTG	CCTT(ATT)14ATAA
RM21	GA275	11	157	ACAGTATTCCGTAGGCACGG	GCTCCATTGAGGGTGGTAGAG	GGTA(GA)18GGAG
RM22	GA580	3	194	GGTTTGGGAGCCCATAATCT	CTGGGCTTCTTTACTCGTC	GAGC(GA)22GTCC

Table 3. Allelic variation in cultivated and wild relatives of rice.

Marker locus	Alleles in 20 cultivars (no.)[a]	Total alleles in 20 cultivars and 4 wild species (no.)[b]
RM 1	7	11
RM 2	4	6
RM 3	5	9
RM 4A,B,&C	7	11
RM 5	6	8
RM 6	4	8
RM 7	5	7
RM 8	3	6
RM 9	9	13
RM 10	5	8
RM 11	5	9
RM 13	6	9
RM 14	5	7
RM 16	5	7
RM 17	2	5
RM 18	4	7
RM 19	9	10
RM 20A	7	11
RM 20B	4	8
RM 21	8	9
RM 22	6	7
RM 122	6	7
RM 148	3	4
RM 164	4	6
RM 167	6	9
RM 168	5	8
Average	5.27±0.33	8.06+

[a]Cultivars are listed in Table 1. [b]Wild species are listed in Table 1.

appear equally likely to transmit new alleles, and as expected, crosses with more genetically distant material are likely to contribute a greater amount of allelic diversity. No correlation was observed between the number of different alleles detected in this set of germplasm and the number of SSR repeats in the original IR36 clone.

Mapping of SSLP markers

The 20 polymorphic markers identified in this study revealed 23 independent loci. Eleven of the loci were mapped using only the interspecific backcross population and seven were mapped only with the IR64/Azucena DH population. Four loci (RM11, RM13, RM19, and RM20B) were mapped on both populations to determine if there was evidence of chromosomal rearrangements of these highly variable loci in the interspecific cross. All four mapped to identical positions on both populations. The 23 mapped SSLPs are located on 8 of the 12 rice chromosomes and show good distribution throughout the genome (Fig. 1).

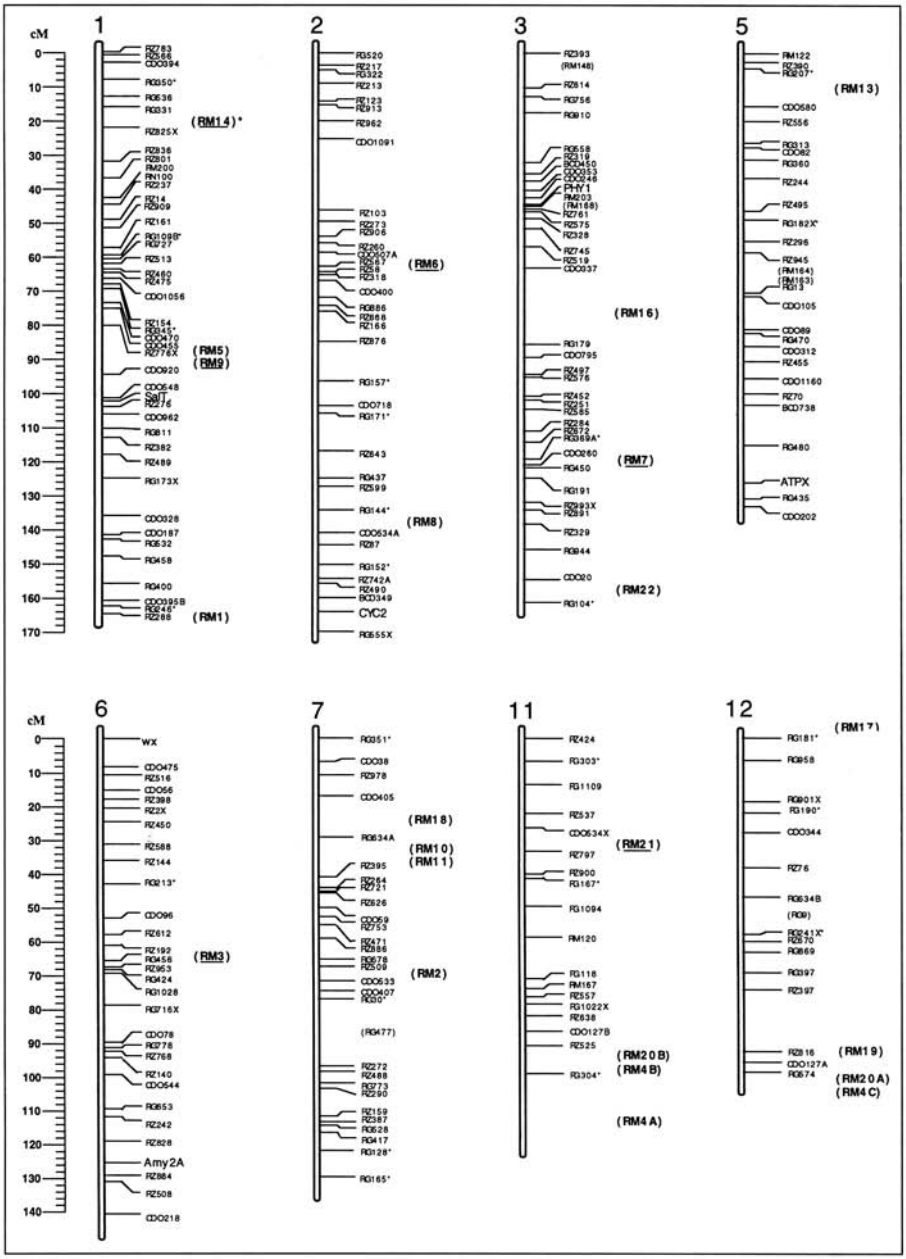

Fig. 1. Framework RFLP map (Causse et al 1994) of eight rice chromosomes showing the map positions of 20 new microsatellite loci (shown in bold to right of previously mapped markers). Underlined markers were mapped using a doubled haploid (DH) population (Huang et al 1994); others were mapped onto the interspecific backcross (SL) population (Causse et al 1994).

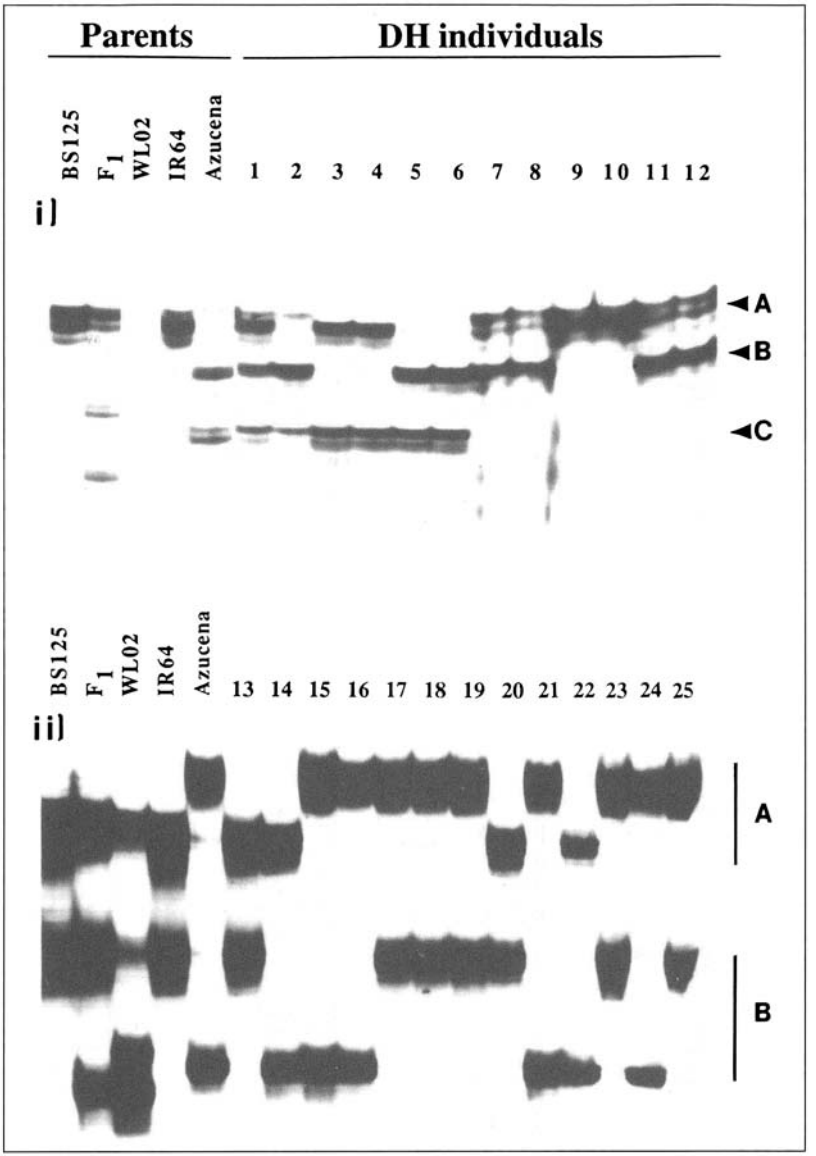

Fig. 2. Multiple-locus microsatellite markers; i = RM4 and ii = RM20. Both panels show banding patterns observed in mapping parents from both the SL (BS125, F_1, WL02) and DH (IR64, Azucena) populations, and independent segregation of loci (A, B, and C) in a subset of DH lines derived from the DH population (Guiderdoni et al 1992). RM4A, RM4B, and RM2OB map to chromosome 11, while RM4C and RM2OA map to chromosome 12.

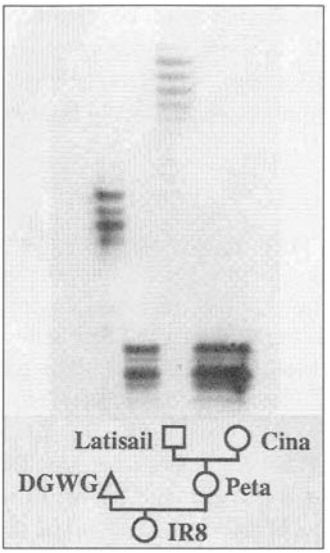

Fig. 3. Allelic diversity at RM21 in the pedigree of rice cultivar IR8.

The markers RM4 and RM20, containing a poly $(GA)_{15}$ and a poly $(ATT)_{14}$ motif, respectively, each amplified multiple loci with a single pair of primers (Fig. 2). Three discrete bands were observed when RM4 was amplified on both the DH and SL mapping populations. One band was present in both indica parents (IR64 and BS125) and two bands were observed in the japonica parent (Azucena) and in the *O. longistaminata* parent (WL02). These bands segregated independently when RM4 was amplified on the DH population (Fig. 2i). Two of the bands mapped to chromosome 11 (RM4A & B) and one to chromosome 12 (RM4C). When RM20 primers were used to amplify DNA, four strong bands were visible in inbred rice varieties and two of the bands cosegregated at each locus (Fig. 2ii). RM20B was mapped to the distal portion of chromosome 11, and RM20A was mapped distally on chromosome 12, closely linked to RM4C (Fig. 1). Interestingly, the two primer pairs that each generated multiple bands (RM4 and RM20) show linkage in both locations and map to distal regions of chromosomes 11 and 12. RFLP data (CDO 127 A & B, Causse et al 1994. Shimano et al 1995) provided previous evidence of sequence homology in these two regions of the rice genome.

Pedigree analysis

Using the IR8 pedigree (Fig. 3), we were able to unequivocally trace the parental origin of IR8 alleles at 19 of the 26 microsatellite loci evaluated. The parental origin of seven loci could not be determined because the Peta and DGWG alleles were identical, suggesting that these two indicas may have an allele that is identical by descent at these loci. The allelic stability of microsatellite markers in this three-

generation rice pedigree suggests that it will be possible to exploit SSLPs to trace the parental origin of specific chromosomal segments through pedigrees used in breeding. This is of particular interest where microsatellite markers map close to genes or quantitative trait loci conditioning agronomically important traits.

Summary

The 20 SSLP markers developed in this study are inexpensive to use and are highly informative. The silver staining method for the detection of SSLP markers is a valuable detection alternative to the radioactive procedure. Multiplexing of SSLP markers on polyacrylamide gels increases the efficiency of SSLP analysis when many plant samples are being analyzed with multiple markers. In addition, microsatellite marker analysis can be automated (Reed et al 1994), which is attractive for large-scale marker-assisted selection programs.

Previous work provided evidence that microsatellites are abundant in the rice genome. Mapping of SSLP markers indicated that they were well distributed throughout the rice genome. The fact that SSLP markers could be mapped to the same locations in both an intra- and an interspecific population demonstrates that they are in conserved, evolutionarily stable regions of the genome and may be used with confidence for analysis of germplasm as distantly related as *O. longistaminata.* The usefulness of microsatellite markers in rice genome analysis can be expected to increase dramatically as saturated genetic SSLP maps become available.

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Genetic mapping of resistance to rice tungro spherical virus (RTSV) and green leafhopper (GLH) in ARC11554

L.S. Sebastian, R. Ikeda, N. Huang, T. Imbe, W.R. Coffman, M. Yano, T. Sasaki, and S.R. McCouch

The green leafhopper (GLH), *Nephottetix virescens* (Distant), is a vector for rice tungro spherical virus (RTSV) and rice tungro bacilliform virus (RTBV), which cause tungro disease in rice. Results of this study demonstrated that a gene or genes, conferring resistance to GLH and RTSV, is located toward the end of chromosome 4. Genetic analysis of 240 F_2 plants derived from a cross between ARC11554 (resistant to both RTSV and GLH) and TN1 (susceptible to RTSV, RTBV, and GLH) indicated that GLH resistance is controlled by a single dominant gene. RTSV resistance was also observed to be simply inherited based on an independent evaluation of 111 F_2 plants from the same cross. RTSV resistance was analyzed using enzyme-linked immunosorbent assay and GLH resistance was evaluated based on antibiosis experiments. A BC_5F_1 (ARC11554 /6*TN1) near-isogenic line (NIL) was used to identify the putative location of the resistance characters and it was crossed with TN1 to construct a segregating population for linkage analysis. One random amplified polymorphic DNA and eight restriction fragment length polymorphism markers on a single chromosomal segment at the end of chromosome 4 were tightly linked with both GLH and RTSV resistance in the NILs. Confirmation of recombinant phenotypes is being pursued to isolate lines that are susceptible to GLH and resistant to RTSV and vice versa.

Screening for resistance to either of the tungro viruses is a very challenging task because early generation screening is destructive. When screening for either RTBV or RTSV resistance, the materials may be lost due to infection caused by either of the viruses. Vector resistance can also affect the results by causing antibiosis or antixenosis and lowering the transmission of RTSV.

Genetic mapping of tungro resistance genes through linkage to molecular markers is one of the biotechnological approaches being used to assist in the development of

tungro-resistant varieties. Identifying markers linked to the resistance genes would make selection for the desired gene(s) more efficient (Tanksley et al 1989). In rice, extensive efforts have resulted in the construction of rice molecular maps (Causse et al 1994, Kurata et al 1994). These maps are now being used for mapping genes of agronomic importance. The objectives of this study are to determine the inheritance of resistance genes to GLH and RTSV in ARC11554 and to map these genes using molecular markers.

F_2 segregation for GLH and RTSV resistance

Two hundred and forty F_2 plants derived from a cross between ARC 11554 and TN1 were scored for GLH antibiosis. The reaction of the F_2 plants indicated that a single dominant gene was most likely involved in conferring GLH resistance in ARC 11554 (Fig. 1). Nymph survival scores were assigned as "resistant" (0–81% nymph survival or 0–65 using arcsin-transformed data) and "susceptible" (>81– 100% nymph survival or 65–90 using arcsin-transformed data). Sixty-four (26%) of the plants were classified as susceptible and the remainder were classified as resistant. The chi square test confirmed the 3:1 ratio ($c^2 = 0.27$, $0.5<P<0.75$).

For RTSV infection, 111 F_2 plants were seeded in individual pots and inoculated separately with ten viruliferous adult GLH 10-d-old seedling^{-1}. The forced inoculation of ten viruliferous adult GLH seedling^{-1} was to ensure that GLH would feed on each seedling thus avoiding escapes. Sampling for enzyme-linked immunosorbent assay (ELISA) was done twice, 3 and 4 wk after inoculation. Plants with ELISA reading lower than 0.11 were considered as resistant (Bajet et al 1985). The segregation pattern observed in the 111 F_2 plants indicated a single dominant gene for RTSV resistance (3:1 $c^2 = 0.42$, $0.5<P<0.75$).

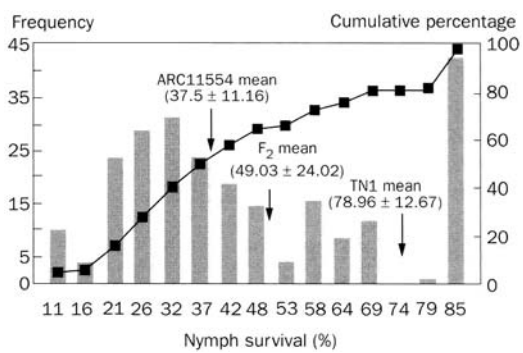

Fig. 1. Bar graph showing nymph survival among F_2 plants derived from ARC11554/TN1 (nymph survival is arcsin-transformed value of percent nymph survival). Line graph shows cumulative nymph survival. Numbers in parentheses are the means ± standard deviation.

Linkage of molecular markers with GLH and RTSV resistance genes

Linkage analysis was used to test for cosegregation of molecular markers with RTSV and GLH resistance. A BC_5F_1 (ARC11554 /6*TN1) near-isogenic line (NIL) was used to identify the putative location of the resistance characters and it was crossed with TN1 to construct a segregating population for linkage analysis. One hundred and eight BC_6F_2 lines were used for restriction fragment length polymorphism (RFLP) analysis. All BC_6F_3 families from each of the BC_6F_2 lines were phenotyped for RTSV resistance (ELISA) while 67 families were used for GLH resistance (test tube antibiosis method).

For RFLP analysis, 197 markers were used to survey for polymorphism between ARC11554 and TN1. A total of 114 (57.8%) markers detected polymorphism, 17 of these markers had bands that were shared by ARCl1554 and the BC_6F_2 bulk but not by TN1. When the 17 putative positive markers were used as probes in the mapping population, eight markers on chromosome 4 showed cosegregation with RTSV resistance and GLH resistance.

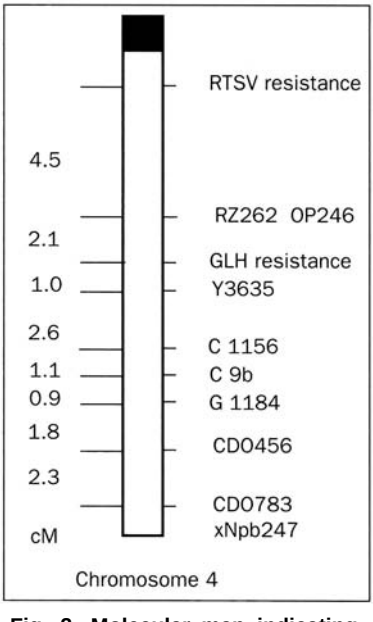

Fig. 2. Molecular map indicating the arrangement of GLH and RTSV resistance genes relative to the molecular markers on chromosome 4. OP246 is an RAPD marker; CDO and RZ are Cornell markers; Y, C, and G are Rice Genome Program of Japan markers.

The approximate position of the markers and the two traits are shown in Figure 2. Genetic distances were derived using MAPMAKER 3.0 at LOD 3.0 (Lander et al 1987, Lincoln et al 1992). The RTSV resistance gene maps above RZ262 while GLH resistance gene is between RZ262 and Y3635. However, because of the complex nature of phenotyping and the possibility of errors, the scores may not accurately represent the genotype. Therefore, RTSV and GLH resistance genes are most probably within the vicinity of RZ262.

A random amplified polymorphic DNA (RAPD) marker (OP246) which cosegregated with the ARC11554 (resistant) allele of RZ262 was also identified using DNA bulks of BC_6F_2 lines. The bulks used were homozygous (ARC11554 or TN1 allele) for RZ262 and CDO456 but showed recombination between these two markers.

The correlation of nymph survival with percentage RTSV infection using 67 BC_6F_3 families was highly significant (r=0.75). The linkage between GLH and RTSV resistance genes was not expected although ARC11554 has been known to have resistance to both RTSV and GLH (Hibino et al 1990). The percentage infection to RTBV among the BC_6F_3 families was consistently high (87.5-100%) which indicated effective GLH feeding. To clarify the linkage of RTSV and GLH resistance, one or more of the recombinant families could be genotyped on a per plant basis and then phenotyped in the F_4. Agroinoculation could also be used in introducing RTSV into the rice plant. This will omit the effect of the GLH resistance during inoculation. This scheme can clarify if linkage and not pleiotropy actually exists. This will also point out where the recombination is occurring between the two genes as well as between the markers and the genes. Lines resistant to RTSV alone and to GLH alone could be isolated through this scheme. This should be possible since recombinants exist.

Conclusion

Resistance to both RTSV and GLH has been mapped to a common region of chromosome 4. This is the first report of the map location of any gene related to rice tungro disease (RTD) resistance in rice germplasm. GLH antibiosis and RTSV resistance were identified based on independent phenotyping of F_2 populations derived from ARC11554. The use of molecular markers will hasten the transfer of the gene(s) to cultivated varieties and eventually their deployment for RTD management. When these markers are converted to a user-friendly form (e.g., polymerase chain reaction-based markers), they will provide an invaluable complement to the tedious and highly variable screening method currently used to evaluate RTD resistance. Molecular markers should provide a more accurate way of determining whether disease and insect resistance genes are present in an individual.

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Identification of DNA markers tightly linked to blast resistance genes in rice

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The identification of DNA markers closely linked to a blast *(Pyricularia oryzae* Cav.) resistance gene and the establishment of indirect selection method for the blast resistance gene based on linked DNA markers were reported. A pair of near-isogenic lines (K80R and K79S) was developed using local indica rice cultivar Hong-jiao-zhan as resistance donor and IR24 as the recurrent parent. Ten putatively linked markers were identified by screening 177 mapped restriction fragment length polymorphism (RFLP) markers. Using 143 plants from the F_2 population of K80R/K79S, three RFLP markers (RG81, RG869, and RZ397) on chromosome 12 of rice were verified to be linked to the blast resistance gene. Resistant genotypes of each F_2 individual were determined by inoculation of F_3 lines. RG869 was found to be linked to the resistance gene with a genetic distance of 5.1 cM. To fine-map this gene with more DNA markers, bulk segregation analysis was employed to identify random amplified polymorphic DNA (RAPD) markers linked to the resistance gene. Six arbitrary primers out of 199 were able to produce positive RAPD bands. Tight linkage between the resistance gene and three RAPD bands each from a different primer was confirmed after amplification of DNAs of all F_2 individuals. The linked DNA fragments were cloned and partially sequenced, and corresponding primers were synthesized. Results of the specific polymerase chain reaction amplification were in agreement with that of RAPD analysis. Half-seed RAPD analysis procedure for blast resistance detection was established. The amplified DNA patterns of extract from the endosperm half of the mature seeds were identical to those of the total DNA from leaves.

Rice blast, caused by *Pyricularia oryzae* Cav., is generally considered the most important disease of rice. Blast resistance in newly developed rice varieties would be lost soon after large-scale cultivation because of the development of new races or pathotypes of the pathogen. Breeding varieties with multiple resistance genes has been suggested as a solution to such problems (Ou 1985). However, conventional ways of host resistance identification would not always be helpful for rice breeders to determine whether different resistance genes have been integrated into a breeding line.

On the other hand, indirect selection based on tightly linked genetic markers seems to be more promising in pyramiding the resistance genes. This relies on the exploitation of tightly linked markers and the establishment of convenient and cost-effective detection procedure. The development of DNA restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) techniques has provided powerful tools for mapping genes of interest (Tanksley et al 1989, Williams 1990). The conversion of RFLP and RAPD markers to polymerase chain reaction (PCR)-based markers makes marker-assisted selection convenient and cost-effective. We report here our progress in the identification of DNA markers closely linked to a blast resistance gene and the establishment of indirect selection method for blast resistance genes.

Materials and methods

Plant materials

A pair of near-isogenic lines (NILs), K80R (resistant to rice blast) and K79S (susceptible), was used. The resistance donor of the pair is a Chinese local indica cultivar Hong-jiao-zhan, well known for its wide-spectrum and durable resistance to blast. The recurrent parent is an indica cultivar, IR24. The pair of NILs is the product of three backcrosses followed by nine generations of selfing.

An F_2 population of K80R/K79S consisting of 150 plants was developed and all the plants of the F_2 and F_3 populations were inoculated by injecting conidial suspension of race ZB1 of *P. oryzae* into the emerging leaf of the plant at tillering. The DNA of each F_2 individual and the four parental lines were extracted.

RFLP probing

Total DNA extraction, restriction endonuclease digestion, electrophoresis, and Southern blot analysis were performed as described previously (Zheng et al 1990, Lu and Zheng 1992). Seven restriction endonucleases were used to digest the total DNA. After cosegregation of RG81 and blast resistance was discovered, an additional 14 enzymes were used for other markers located near RG81 on chromosome 12.

RFLP probes were first selected from the Cornell map (McCouch et al 1988, McCouch and Tanksley 1991) at approximately 15-cM intervals. Later, additional markers were used to fill in gaps in the coverage. Altogether, 177 probes were used.

Isogenic DNA pool composition and RAPD analysis

Equal quantities of DNAs from 10 homozygous resistant F_2 individuals were mixed to construct a resistant pool (R pool), while those from 10 homozygous susceptible F_2 individuals were mixed to construct a susceptible pool (S pool). They were then diluted to 10 ng μL^{-1}. The RAPD analysis was performed as described previously (Lu et al 1994). Primers generating polymorphic RAPD bands between two pools were used to analyze their cosegregation with blast resistance in the F_2 population.

Specific amplification

RAPD fragments that cosegregated with blast resistance were cloned and sequenced. A pair of primers was designed for specific amplification. Compared with the RAPD analysis described above, the temperature profile was changed to the following: 94 °C for 30s, 53 °C for 30s, 72 °C for 1 min, and 35 cycles of the three steps were performed.

RAPD analysis using half-seed DNA

The half-seed DNA was prepared following the method of Chunwongse et al (1993) with minor modification. Each seed was cut in half, the endosperm half was immersed into 50 µL PCR buffer, and 5 µL proteinase K (10 mg mL^{-1}) was added. The tubes with half-seed were incubated at 50 °C for 1.5 h. Centrifuged briefly, they were boiled at 100 °C for 5 min and were again centrifuged. Each 5-µL extract was taken out for PCR.

Linkage analysis

The computer program MAPMAKER (Lander et al 1987) was used for linkage analysis. Distances between markers were presented in centimorgans (cM) derived using the Kosambi function (Kosambi 1994).

Results

Screen of putatively linked RFLP markers

Of the 177 probes tested, 75 were polymorphic between the donor and recurrent parents. They were then used to search for polymorphisms between the two NILs. The majority produced identical restriction fragment patterns for the recurrent parent and both of the NILs.

The probes produced an identical pattern for the donor parent and the resistant NIL and another identical pattern for the recurrent parent and the susceptible NIL, which were potentially linked to resistance genes and called positive clones. Initially, eight positive clones were found. After additional flanking probes and more enzymes were used, two more positive clones were found, bringing the total to 10.

Verification of linkage and estimation of map distance

Most of the F_2 plants showed extreme responses to inoculation. One hundred and ten plants were scored 0 or 1 (highly resistant) and 33 plants scored 7 or 9 (highly

susceptible) to race ZB1, fitting a 3:1 ratio as expected when resistance was controlled by a single dominant gene(x^2 = 0.33, P = 0.50-0.75).

RG81 was the first clone found to be linked to blast resistance in this study, but all the DNA markers near RG81 were monomorphic between the NILs with seven restriction enzymes. After an additional 14 enzymes were used, RG869 and RZ397 were found to be polymorphic between the NILs and showed close linkage to the resistance gene. RG81 and RZ397 were completely cosegregated.

The resistant genotypes of 85 F_2 individuals were identified using F_3 lines. Map distances were estimated. The gene for resistance to race ZB1 of *P. oryzae* Cav. was tentatively named *Pil2* (t). RG869 and RG81 (RZ357) were on the same side of *Pil2* (t). The genetic distance between RG869 and *Pil2* (t) was 5.1 cM.

Identification of tightly linked RAPD markers

Of the 199 10-mer arbitrary primers screened, linkage between the resistance gene and three RAPD bands (1.3-kb fragment produced by primer P622,0.56-kb fragment by primer 0265, and 0.35-kb fragment by primer P286) was confirmed. Complete cosegregration of RAPD markers and blast resistance was detected against all the 143 F_2 individuals, indicating tight linkage of these three markers and the blast resistance gene.

Amplification of DNA from half-seed of the two isolines and a subset of F_2 individuals also has been made. The band patterns from halt-seed DNA were identical to that from leaf DNA.

Conversion of RAPD markers to specific PCR markers

The three RAPD fragments were cloned and used as probes to analyze RFLPs of the F_2 individuals. The 1.3-kb and 560-bp fragments were found to contain repeat sequences. The 350-bp fragment was found to contain single-copy sequence and detect null allele in susceptible individuals. The result of RFLP analysis using the clone of the 350-bp fragment was in agreement with that of RAPD analysis.

Clones of the 560- and 350-bp fragments were sequenced and primers were synthesized. Specific amplifications of DNA from the two isolines and all the 143 F_2 individuals had been made for the 560-bp fragment. A 560-bp fragment was observed for all the resistant individuals but none for the susceptible ones. Moreover, the difference between the resistant and susceptible individuals can be much more easily scored in specific amplification.

Discussion

Fine-mapping of different blast resistance genes favors the pyramiding of resistance genes and developing varieties with durable resistance. Availability of PCR-based markers makes marker-assisted selection practically feasible for plant breeders.

Several blast resistance genes (*Pi4*(t), *BR14, BR26*) were tagged to DNA markers on chromosome 12, in the same region where *Pil2*(t) was located. Whether these genes are the same single gene or from a resistance cluster is unknown. Molecular markers will be useful to elucidate this issue.

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Molecular genetic analysis and sequence characterized amplified region-assisted selection of blast resistance in rice

N.I. Naqvi and B.B. Chattoo

This study was conducted to identify and tag single genes for complete resistance to the rice blast fungus, *Magnaporthe grisea,* with DNA markers, and to devise a marker-assisted indirect selection method for these individual dominant genes. A single dominant gene, *Pi10,* which confers complete resistance to *M. grisea* isolate JMB840610 was identified in the race Moroberekan. Cosegregation linkage analysis using two random amplified polymorphic DNA tags, RRF6 and RRH18, showed that they were tightly linked to the *Pi10* locus (RRF6, 2.8±0.9 cM; RRH18, 3.9±1.2 cM). Nucleotide sequencing data for the two ends of RRF6 was used to design specific primers for use in sequence characterized amplified region (SCAR)-assisted indirect selection of *Pi10.* Another major gene, *Pi157,* conferring complete resistance to an Indian isolate (B157) of the blast fungus, was also found in Moroberekan. Cosegregration linkage analysis performed for polymorphic clones showed that *Pi157* was tightly linked (3.0±0.6 cM) to the single-copy DNA clone RG341 on chromosome 12. We are in the process of developing a SCAR-based indirect selection method for *Pi157.*

Rice blast, caused by the fungus ***Magnaporthe grisea,*** has been a major challenge to rice breeders and pathologists since the beginning of recorded rice cultivation (Ou 1972). It is perhaps the most widely distributed plant disease, occurring in about 85 countries worldwide. In spite of a great deal of research on the pathogen and the disease, blast still remains a serious constraint to rice production in both irrigated and upland environments.

Major genes have been successfully used for developing cultivars resistant to rice blast (Khush 1989). Many such resistant cultivars have been released by rice breeders and are being used by farmers. Major resistance genes can be deployed either to prevent populations of the blast fungus from easily adapting or to minimize the selection pressure on blast pathogen populations. These objectives can be accomplished by pyramiding conventional blast resistance genes to generate improved cultivars with durable blast resistance. Phenotypic selection cannot be used to pyramid resistance because the presence of one major gene obscures the effect of other genes. Molecular markers linked to major blast resistance genes offer a powerful tool for marker-aided indirect selection of these resistance loci in gene pyramiding strategies.

Materials and methods

Blast isolates and inoculations

The isolate JMB840610 (international race IB46) of *M. grisea* is a stable isolate routinely used at IRRI. Isolate B157 (international race IC9) is from Maruteru, Andhra Pradesh, India. Inoculum preparation, inoculation by the spray method, and disease evaluation were performed as described by Bonman et al (1986). CO39 is susceptible to both isolates and was subsequently used in crosses as a recipient parent for developing the segregating populations for the individual resistance genes against isolates JMB840610 and B157.

Rice varieties and segregating populations

The BC_3F_2 population used to identify random amplified polymorphic DNA (RAPD) markers RRF6 and RRH18 linked to the *Pi10* gene was based on that described by Naqvi et al (1995). *Pi10* was found to segregate in a recombinant inbred line (RIL) population derived from the cross CO39/Moroberekan (Wang et al 1994). RILs 61, 97, 171, and 215 are homozygous resistant, whereas RILs 21, 132, 151, and 226 are susceptible to isolate JMB840610 of the blast fungus. These eight RILs were used for developing a sequence characterized amplified region (SCAR)-based indirect selection of the *Pi10* locus based on RRF6 (Naqvi and Chattoo 1995).

A set of 209 RILs was further tested for their reaction to isolate B157, and individual lines resistant and susceptible to this isolate were identified. These lines were used for the cosegregation linkage analysis using restriction fragment length polymorphism (RFLP) markers provided by S. Tanksley (Cornell University).

RFLP analysis

Procedures followed for genomic DNA extraction, restriction enzyme digestion, and gel electrophoresis were as described earlier (Naqvi et al 1995). The restriction enzymes *Dra*I, *Eco*RI, *Eco*RV. *Hin*dIII, *Xba*I, and *Sca*I were used. Agarose gels were treated for 10 min with 0.25 M HC1 and blotted to Hybond N+ nylon membranes (Amersham), using 0.4 N NaOH. Standard protocols were used for Southern hybridization, stringency washes, and signal detection.

Linkage analysis

The MAPMAKER computer program (Lander et al 1987) was used to place markers on the linkage map. Calculation of standard errors, estimation of confidence intervals, and ordering of markers were carried out as described by Naqvi et a1 (1995) and Naqvi and Chattoo (1995).

Cloning and sequencing of *Pi10*-linked RAPD marker

RAPD analysis was carried out as described by Naqvi et al (1995). The RAPD fragments to be cloned were isolated from 1.8% agarose gels following the method described by Errington et a1 (1990) using sterile pieccs of Whatman 3MM chromatographic paper. Ragged ends (protruding 3' overhang generated by the Taq DNA polymerase) of the gel-purified RAPD fragments were end-filled with the Klenow enzyme. Reactions were carried out in volumes of 20 µl containing 10 mM Tris-HCl, pH 7.5, 5 mM $MgC1_2$, 7.5 mM DTT, 800 ng of RAPD fragment, 50 µM each of the 4 dNTPs, and a unit ofthe Klenow enzyme (Pharmacia). The reaction mix was incubated at 25 °C for 15 min and was stopped by heating at 75 °C for 10 min. The DNA was gel-purified, and blunt-end ligated into the *Eco*RV site of the phagemid pBluescript KS+ vector (Stratagene). The identity of the cloned fragment was verified by Southern hybridization of the cloned product to blots of BC_3F_2 individuals that segregated to the *Pi10* locus. Nucleotide sequencing was performed with M 13 forward and reverse primers by the dideoxy-chain termination method using thc Sequenase™ version 2.0 DNA sequencing kit (USB Inc., Cleveland).

Primers for SCAR amplification and analysis

Two pairs of oligonucleotide primers (24-mers, forward and reverse) were designed on the basis of the sequence of the cloned RAPD product RRF6, for use in SCAR amplification. One set of primers included the original 10 bases of the RAPD primer in addition to the next 14 internal bases from the end. SCAR amplification of genomic DNA was performed in a standard polymerase chain reaction (PCR) consisting of 35 cycles of 1 min at 94 °C, 1 min at 58 °C, and 2 min at 72 °C. For each PCR, 100 ng of genomic DNA were used. Amplified products were resolved electrophoretically in 2.2% agarose gels, stained with ethidium bromide and visualized under UV light.

Results and discussion

Based on the data from segregating populations (F_2 and RILs derived from the cross C039/Moroberekan) screened for blast reaction against isolate JMB840610, it was confirmed that Moroberekan contains the dominant blast resistance gene *Pi10.* The RAPD tags RRF6 and RRH 18 for *Pi10* were therefore tested in a cosegregation linkage analysis to confirm this observation. These two RAPDs were found to be tightly linked (RRF6, 2.8±0.9 cM; RRH18, 3.9±1.2 cM). Figure 1 shows the RFLP analysis of representative RILs using probe RRF6. Figure 2 presents the molecular map derived from the cosegregation linkage analysis performed with RRF6, RRH18, and other RFLP markers present on chromosome 5.

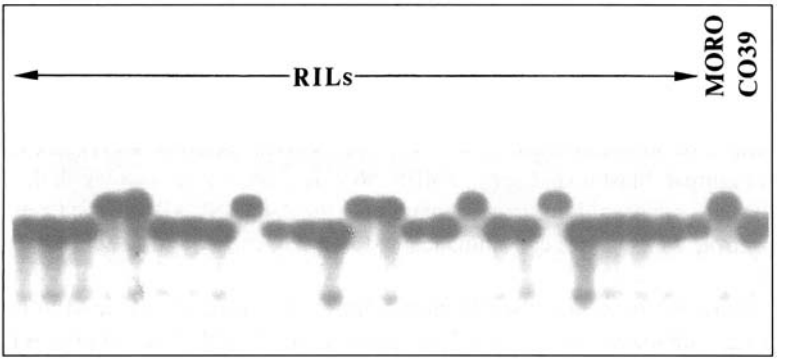

Fig. 1. Southern blot showing a survey of the *Pi10*-linked RAPD marker RRF6 with *Sca*I digests of genomic DNAs from Moroberekan, CO39, and F_8 RILs derived from the two parental cultivars. A total of 248 RILs of known blast reaction were surveyed for the cosegregation linkage analysis using RRF6.

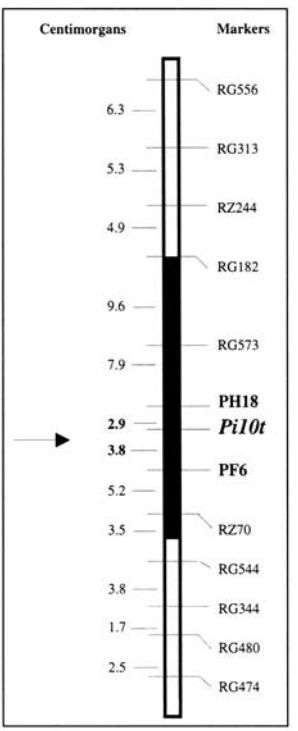

Fig. 2. RFLP map of the *Pi10* genomic region on rice chromosome 5. This integrated map shows the position of *Pi10* and the linked DNA markers deduced from recombinational linkage analysis. Map distances were derived by using the Kosambi function. Shadowed region marks the introgressed segment from Moroberekan present in the resistant RILs. All markers were ordered with log of odds (LOD) > 4.

Figure 3 outlines a schematic representation of the strategy used to convert the RRF6 amplicon into a codominant SCAR marker from Moroberekan. SCARs for RRF6 were amplified using specific primers based on the nucleotide sequence of *Pi10*-linked RAPD. The length-variant codominance seen for RRF6 in Tongil and CO39 was also observed in the RRF6 SCARs from the cross Moroberekan/CO39. As seen in Figure 4, blast resistance to JMB840610 is always associated with the 2.7-kb SCAR whereas susceptibility cosegregates with the 2.4-kb SCAR derived from RRF6. As a control, we have the codominance of this SCAR in Tongil (2.7 kb) and CO39 (2.4 kb).

We have also been successful in identifying a major gene, *Pi157,* in Moroberekan, conferring complete resistance to an Indian isolate, B157, of the blast fungus. A number of polymorphic DNA clones were tested to RIL survey filters (209 RILs derived from the cross CO39/Moroberekan). Cosegregation linkage analysis performed for polymorphic clones showed that RG341 was tightly linked (3.0±0.6 cM) to the *Pi157* locus. We are in the process of clarifying the position of *Pi157* relative to RG341 by screening additional markers in this region on chromosome 12 (Fig. 5). A SCAR-based indirect selection method for *Pi157* is also being developed based on its tight linkage to RG341.

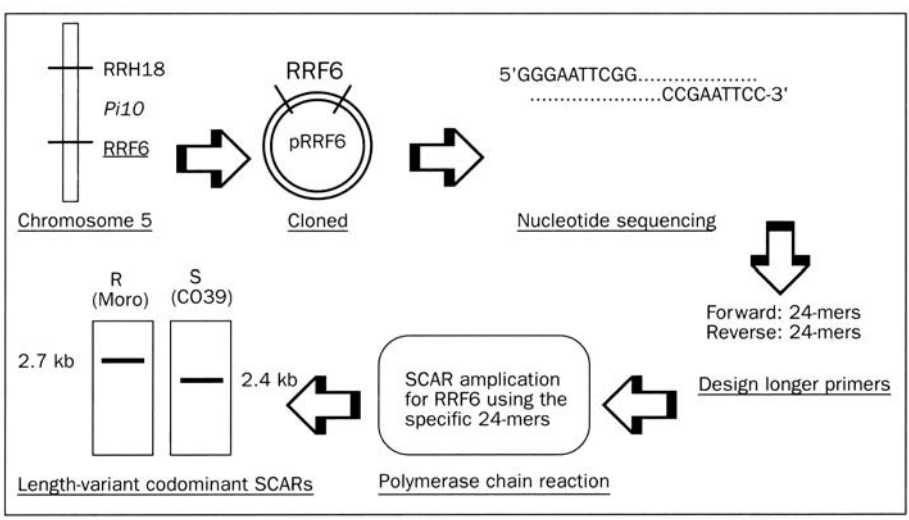

Fig. 3. A schematic representation of the strategy used for developing a SCAR-based indirect selection method for the blast resistance locus *Pi10* from Moroberekan.

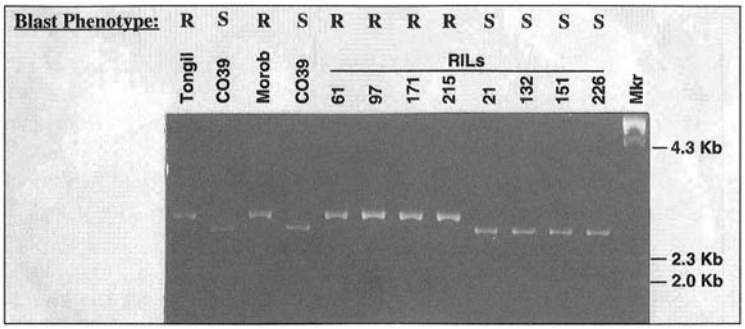

Fig. 4. Ethidium bromide-stained electrophoretic profile of the SCARs derived from RRF6. Blast phenotype refers to the interaction of the rice cultivars/isolines with isolate JMB840610 of the blast fungus. R (*Pi10*+) indicates an incompatible interaction or resistance, whereas S (*Pi10*) refers to a compatible interaction or susceptibility. Tongil, Moroberekan, RILs 61, 97, 171, and 215 are resistant to isolate JMB840610, and show the 2.7-kb SCAR, whereas CO39, RILs 21, 132, 151, and 226 are blast-sensitive and show the 2.4kb allele of the SCAR derived from RAPD marker RRF6 linked to *Pi10.*

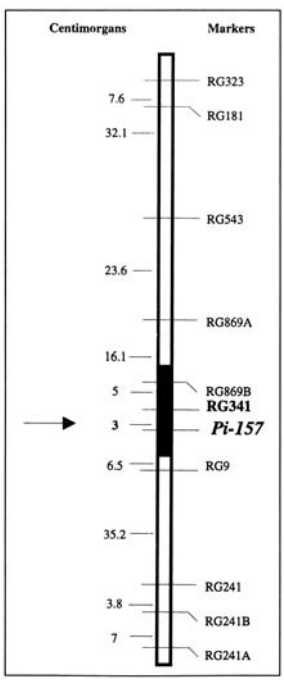

Fig. 5. Recombinational linkage map of the *Pi157* region on rice chromosome 12. All distances are given as cM and were derived by using the Kosambi function. The segment introgressed from Moroberekan in the resistant RILs is represented by a shadowed region. The exact location of the *Pi157* locus with respect to the flanking markers is being studied.

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Notes

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Analysis and pyramiding of bacterial blight resistance genes in rice by using DNAmarkers

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Quantitative resistance to bacterial blight of rice involved in Japanese variety Asominori was analyzed using molecular markers—random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP)—and recombinant inbred lines derived from the Asominori/IR24 cross. Resistance to two Japanese and two Philippine strains was commonly controlled by the region around *Xa1.* The region of minor factors affecting quantitative resistance was also inferred. A major gene for resistance to this disease, *Xa11,* was newly tagged with RAPD markers. Based on this finding and previous results on *Xa*-gene tagging, RFLP and RAPD markers were used to combine resistance genes. Fourteen pyramid lines carrying *Xa1* and *Xa4, Xa1* and *xa5, Xa1* and *Xa7, Xa1* and *Xa10, Xa1* and *Xa11, Xa3* and *Xa7, Xa3* and *Xa10, Xa4* and *Xa7, Xa4* and *Xa11, xa5* and *Xa7, xa5* and *Xa10, xa5* and *Xa11, Xa7* and *Xa10,* and *Xa10* and *Xa11* were newly selected and their disease reactions to several bacterial strains of Japan and the Philippines were evaluated.

Bacterial blight of rice caused by *Xanthomonas oryzae* pv. *oryzae* is a major rice disease, now widely distributed in most rice-growing countries. Although bactericides are applied to control bacterial blight in Japan, none are highly effective or economical. Therefore, the deployment of resistant varieties appears to be the most appropriate control method. Use of horizontal resistance and accumulation of a large number of resistance genes in a single variety are two kinds of breeding strategies employed to cope with the breakdown of varietal resistance. To implement these breeding strategies, careful genetic analysis of horizontal resistance and development of the pyramiding materials are necessary.

Rice linkage maps based on DNA markers and DNA markers themselves (McCouch et al 1988, Saito et al 1991, Kurata et al 1994) are promising tools in deciding which breeding strategy should be adopted.

With this point of view, this study analyzed quantitative resistance conveyed by Japanese variety Asominori using DNA markers and developed pyramids through marker-aided selection.

Genetic analysis of resistance conveyed by Asominori

Asominori shows not only qualitative resistance to Japanese race 1 but also quantitative resistance to other Japanese races and Philippine isolates. Qualitative resistance and quantitative resistance were analyzed.

Seventy-one recombinant-inbred (RI) lines used in this study were the permanent mapping population of DNA markers and 386 RFLP loci were mapped using them (Tsunematsu, Yoshimura, Yano, Kurata, Harushima, Sasaki, and Iwata, 1995, Kyushu University and Rice Genome Research Program, Japan, unpubl. data). The RI lines were inoculated twice in 1992 (F_6) and 1995 (F_9) with Japanese races 1 (T7174) and 3 (T7133) and inoculated once in 1993 with Philippine races 4 (PXO71) and 6 (PXO 99). Lesion length was measured 2-3 wk after inoculation. For analysis of resistance to T7174, linkage mapping was done since the RI lines were easily classified into resistant and susceptible groups. To infer the regions affecting resistance to T7133, PXO 71, and PXO 99, the lines were divided into two groups based on the parental genotypes (Asominori and IR24) of each RFLP locus, and a mean comparison between the two genotypic groups was performed by *t*-test. The putative regions affecting resistance were the loci where there was a significant difference at the 1% level.

The segregation of resistance to T7174 in the RI lines indicated that resistance to T7174 from Asominori was governed by a single gene. The gene was considered to be *Xa1*. For the reaction to T7133, PXO 71, and PXO 99, the chromosomal region near *Xa1* commonly contributed to the quantitative resistance conveyed by Asominori. Since the *Xa1* region much affected the resistance to the three races, the RI lines carrying *Xa1* were excluded to infer the minor-affecting regions. The minor-affecting factors, which are commonly effective for the three races, were not found. From the replicated experiments for T7133 in 1992 and 1995, the minor-affecting loci to T7133 were found on chromosomes 1 and 6. The minor-affecting loci to PXO 71 and PXO 99 were inferred at several regions in different chromosomes (Table 1).

Some of the regions inferred as minor-affecting regions in this study will be proven by the development of isogenic lines. The development of near-isogenic lines (NILs) carrying the minor-affecting regions is under way.

Pyramiding

The availability of NILs carrying single resistance genes (Ogawa et al 1991) and the use of DNA markers tightly linked to resistance genes enable us to select plants with multiple resistance genes without inoculation.

In our pyramiding work, seven resistance genes, *Xa1, Xa3, Xa4, xa5, Xa7, Xa10,* and *Xa11* were chosen as the genes of interest. Since the RFLP and RAPD markers that were tightly linked to the above resistance genes (except for *Xa7* and *Xa11*) were

identified using NILs with IR24 genetic background (Yoshimura et al 1992, 1995a,b), the DNA markers tightly linked to *Xa7* and *Xa11* were first surveyed. Two-gene pyramids were then developed and resistance was evaluated.

RFLP and RAPD tagging in IR-BB7 and IR-BB11

Kaji and Ogawa (1995) reported linkage between *Xa7* and G1091 on chromosome 6. Therefore, the RFLP markers near G1091 on chromosome 6 were used to find the polymorphism between IR-BB7 and IR24. When probed with Npb329, R276, and R1559, RFLPs were detected between IR-BB7 and IR24, indicating that these RFLP markers can be used for pyramiding work.

To identify RAPD markers linked to *Xa11*, IR-BB11 and IR24 were screened using 500 arbitrary primers. The primer L19 (5'-GAGTGGTGAC-3') yielded amplified fragment from IR-BB11 but not from IR24. Segregation of the L19 fragment in F_2 of IR24/IR-BB11 fitted well to a 3:1 ratio and linkage between *Xa11* and $L19_{1200}$ was detected at a distance of 1.1 cM.

Two-gene pyramiding

In addition to the previous development of two pyramids having a pair of resistance genes, *Xa4* and *xa5*, and *Xa4* and *Xa10* (Yoshimura et al 1995a), 14 more lines were selected from the progeny derived from the crosses between NILs, using DNA markers and inoculation test.

Table 1. Minor-affecting loci for resistance to Japanese race 3 and Philippine races 4 and 6 found in the RI lines from Asominori/IR24 cross.

Strain	Marker	Chromosome	*t*-value	Probability	Effect[a]
T7133('92)	XNpb92	1	3.14	0.003	-3.20
	R1021-1	1	2.78	0.009	-2.40
	G1314B	6	3.22	0.003	-2.82
	C472	9	2.88	0.007	-2.45
	C3029B	12	2.94	0.006	-2.62
T7133('95)	XNpb92	1	3.66	<0.001	-5.33
	C560	2	2.82	0.008	-3.69
	C621B	2	2.74	0.009	-3.53
	XNpb170	6	3.05	0.005	-4.24
	C1361	10	2.86	0.007	-2.73
PXO 71	G1015	3	3.69	<0.001	4.31
	XNpb41	8	3.60	0.001	-4.39
	R728	11	3.10	0.004	-3.87
	R367	12	4.05	<0.001	4.44
PXO 99	C112-1	1	2.85	0.007	3.26
	XNpb249	3	2.90	0.007	3.72
	C263-5	5	2.79	0.009	-3.44
	XNpb13	9	3.03	0.005	3.19

[a]Difference of lesion length (cm) obtained by subtracting the mean from genotype of IR24 from that of Asominori at each marker.

Table 2. Lesion length (cm) caused by bacterial blight strains in two-gene pyramids for resistance gene.[a]

		Philippine races					Japanese races		
Line	Genes	Race 1 PXO 61	Race 2 PXO 86	Race 3 PXO 79	Race 4 PXO 71	Race 6 PXO 99	Race 1 T7174	Race 3 T7133	Race 4 H75373
IRBB 1/4	*Xa1 + Xa4*	1.0	19.5	24.4	7.3	18.6	0.4	2.6	1.6
IRBB 1/5	*Xa1 + xa5*	1.6	2.5	2.8	10.5	19.0	0.1	0.6	0.8
IRBB 1/7	*Xa1 + Xa7*	1.4	1.1	0.8	24.7	21.7	0.0	0.1	0.6
IRBB 1/10	*Xa1 + Xa10*	10.2	1.9	18.6	23.1	26.3	0.1	12.6	13.9
IRBB 1/11	*Xa1 + Xa11*	-[a]	-	-	-	-	0.2	1.1	13.9
IRBB 3/7	*Xa3 + Xa7*	1.7	0.9	0.7	12.9	22.2	0.0	0.3	0.5
IRBB 3/10	*Xa3 + Xa10*	9.0	0.9	1.8	13.1	20.7	2.1	3.2	16.1
IRBB 4/5	*Xa4 + xa5*	0.7	1.1	2.1	2.2	7.3	0.1	0.5	0.5
IRBB 4/7	*Xa4 + Xa 7*	0.6	1.0	0.5	3.8	16.9	0.0	0.2	0.5
IRBB 4/10	*Xa4 + Xa10*	1.7	1.5	17.0	7.0	15.8	2.7	3.3	2.4
IRBB 4/11	*Xa4 + Xa11*	1.5	17.7	14.5	6.2	14.8	2.6	2.0	3.5
IRBB 5/7	*xa5 + Xa 7*	1.0	0.7	0.6	12.6	16.4	0.0	0.4	0.5
IRBB 5/10	*xa5 + Xa10*	-	-	-	-	-	1.9	1.2	0.7
IRBB 5/11	*xa5 + Xa11*	-	-	-	-	-	1.5	0.1	1.0
IRBB 7/10	*Xa7 + Xa10*	2.6	0.6	0.7	17.5	19.3	0.1	0.6	0.7
IRBB 10/11	*Xa10 + Xa11*	12.5	0.9	24.2	24.4	27.9	11.1	1.7	15.0
IRBB 1	*Xa1*	9.2	18.1	20.9	26.0	23.9	0.1	11.3	14.5
IRBB 3	*Xa3*	2.7	10.3	4.5	13.4	24.9	1.3	4.7	18.4
IRBB 4	*Xa4*	2.1	19.2	22.6	8.5	26.2	2.4	2.9	2.6
IRBB 5	*xa5*	2.2	2.5	2.8	14.1	23.3	1.1	1.1	0.5
IRBB 7	*Xa 7*	2.4	1.3	0.6	23.7	19.3	0.1	0.8	0.5
IRBB 10	*Xa10*	9.5	0.7	16.1	23.3	23.9	11.8	13.4	19.1
IRBB 11	*Xa11*	11.5	20.7	20.0	24.1	23.8	9.3	2.0	16.3
IR 24	None	7.4	22.6	22.4	25.5	27.0	13.2	13.7	15.8
DV85	*xa5, Xa7*	0.9	1.0	1.5	3.8	17.8	0.7	0.6	0.5
Asominori		4.4	5.5	5.9	12.6	14.6	0.1	2.0	1.1

[a]– = not tested.

The resistance of most of the lines was evaluated using five Philippine races and three Japanese races (Table 2). All of the lines showed possible reactions with broader spectra when inoculated with Philippine and Japanese bacterial strains. The lines carrying *xa5* and *Xa11* were more resistant to Japanese strain T7133. The same phenomenon was observed in the line with *Xa4* and *xa5* and it was termed quantitative complementation (Yoshimura et al 1995a). The line with *xa5* and *Xa7* showed different reaction to PXO 71 and PXO 99 from the donor variety DV85. Therefore, DV85 could have additional gene(s) for resistance to PXO 71 and PXO 99. The analysis of RI lines from the cross Kinmaze/DV85 is now in progress.

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Development of diagnostic markers for the bacterial blight resistance gene, *xa5*

M.W. Blair and S.R. McCouch

Microsatellite markers tightly linked to the bacterial leaf blight (BLB) resistance gene, *xa5,* were identified in this study. A molecular genetic survey was conducted to find microsatellite markers that detected polymorphisms between a near-isogenic line (IRBB5) carrying *xa5,* the donor parent and the susceptible recurrent parent (IR24). Microsatellite markers were used to show that the isoline contained one large introgression around *xa5* and three spurious introgressions on other chromosomes. Two F_2 populations, from the crosses IR24/IRBB5 and Chinsurah Boro II/IR64, were evaluated for disease reaction phenotype and for marker genotype, and used to estimate linkage. Chinsurah Boro II is an alternative source for the *xa5* gene. Disease reaction was scored by clip-inoculating leaves at maximum tillering stage with two Philippine races of *Xanthomonas oryzae* pv. *oryzae.* Both restriction fragment length polymorphism and microsatellite markers were used to select individual F_2 plants from the IR24/IRBB5 population that were more near-isogenic to the recurrent parent. Two microsatellite markers were located at approximately 2 and 18 cM from the *xa5* locus. A third microsatellite was found to be linked to the known location of the *Xa21* gene. Polymerase chain reaction-based markers are economical and easy to use and would have immediate applicability in efforts to pyramid the recessive *xa5* gene with other BLB resistance genes.

Several of the major resistance genes to the bacterial leaf blight (BLB) pathogen, *Xanthomonas oryzae* pv. *oryzae,* have been tagged with restriction fragment length polymorphism (RFLP) or randomly amplified polymorphic DNA (RAPD) sequence markers (McCouch et al 1992, Yoshimura et al 1992, Yoshimura et al 1995). However, both of these types of markers have limitations when used to follow the inheritance of resistance genes in the narrow crosses typical of a breeding program. Recently, a group of rice microsatellite markers has been developed and mapped (Wu

and Tanksley 1993; O. Panaud, X. Chen, and S.R. McCouch, 1995, unpubl. data). Microsatellite markers have advantages for applied plant breeding goals because they are codominant, are based on polymerase chain reaction (PCR) amplification, represent single-loci, and detect high levels of polymorphism. A new generation of microsatellite markers, which is closely linked to target genes, could be very useful for efficient selection and combination of multiple BLB resistance genes.

Near-isogenic lines (NILs) with single BLB resistance genes have been developed from repeated backcrossing and selection for the target gene in the background of the recurrent parent variety IR24 (Ogawa et al 1988). NILs have been used to characterize the resistance spectrum of each gene and to determine their genomic locations (McCouch et al 1991). The chromosomal locations of tagged BLB resistance genes are not randomly distributed, but rather show clear evidence of clustering. Several major resistance genes to the blast pathogen, *Magnaporthe grisea,* are also found near BLB resistance genes (Causse et al 1994). In contrast, the *xa5* resistance gene, which is located at the end of chromosome 5 is not linked to any previously described resistance genes (McCouch et al 1991).

The objectives of the present study were to map microsatellite markers in relation to the *xa5* resistance gene and to evaluate their utility in situations typical of disease resistance breeding programs. Another goal was to select a more nearly isogenic isoline, containing *xa5,* that did not include the spurious introgressions detected in the currently used NIL.

Materials and methods

Plant material

A microsatellite polymorphism survey was conducted for the resistant NIL containing *xa5* (IRBBS), the susceptible recurrent parent (IR24), and the donor parent that provided this resistance (IR1545-339). Two populations were phenotypically screened for *xa5* resistance and genotyped using molecular markers. An F_2 population of 122 plants was derived from a cross between the resistant isoline, IRBB5, and the susceptible recurrent parent, IR24. A second population consisted of 61 F_2 plants from the cross Chinsurah Boro II/IR64. Chinsurah Boro II is a land race from India that contains *xa5* (Ogawa and Khush 1989). IR64 is a widely planted variety throughout tropical Asia, which has the *Xa4* gene and several quantitative trait loci that provide BLB resistance (R. Nelson, IRRI, 1995, pers. commun.).

BLB inoculation

The populations and controls were inoculated with Philippine strains of *Xanthomonas oryzae* pv. *oryzae* to determine the resistant and susceptible phenotypes. Inoculations of the strains PXO 61 (race l), or PXO 86 (race 2), were used to study the segregation of *xa5* in the populations. All plants were inoculated at 55 d after planting. At 14 d after inoculation, the plants were scored as resistant or susceptible and lesion length was measured for six leaves. Seed was collected from the population individuals for F_3 family analysis. The controls used for these inoculations were the four parents of the populations, the F_1s of each cross, and the susceptible variety, IR8.

RFLP analysis

RFLP survey filters were made from DNAs of the recurrent parent, isoline, and donor parent digested with nine restriction enzymes (*Bam*HI, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Pst*I, *Sca*I, and *Xbn*I). Southern analysis was conducted with cDNA and genomic probes from the saturated Cornell map (Causse et al 1994) and the landmarker sets of the Japanese Rice Genome Program (NIAR 1993). DNA extraction and Southern analysis were conducted according to Causse et al (1994). Mapping filters were prepared with DNA from all the F_2 individuals for each population and from the two parents and the F_1 individual, as controls.

Microsatellite analysis

Fifty microsatellite primer pairs (Wu and Tanksley 1993; O. Panaud, X. Chen, and S.R. McCouch, 1995, unpubl. data; X. Chen, Cornell University, pers. commun.) were used to amplify genomic DNA from the recurrent parent, isoline, and donor parent. The PCR conditions and silver staining detection technique were described by O. Panaud, X. Chen, and S.R. McCouch (1995, unpubl. data). The PCR products from the three genotypes were run as adjacent triplets on 40-cm-long 4.5% denaturing polyacrylamide gels. Multiplexing, which is the loading of a single polyacrylamide gel electrophoresis (PAGE) gel multiple times (Fig. 1), was used for the simultaneous mapping of several different microsatellite loci. Two size standards (markers V and VII) from Boehringer-Mannheim were used in each PAGE gel at regular intervals. Linkage analysis for the markers and resistance gene was conducted with

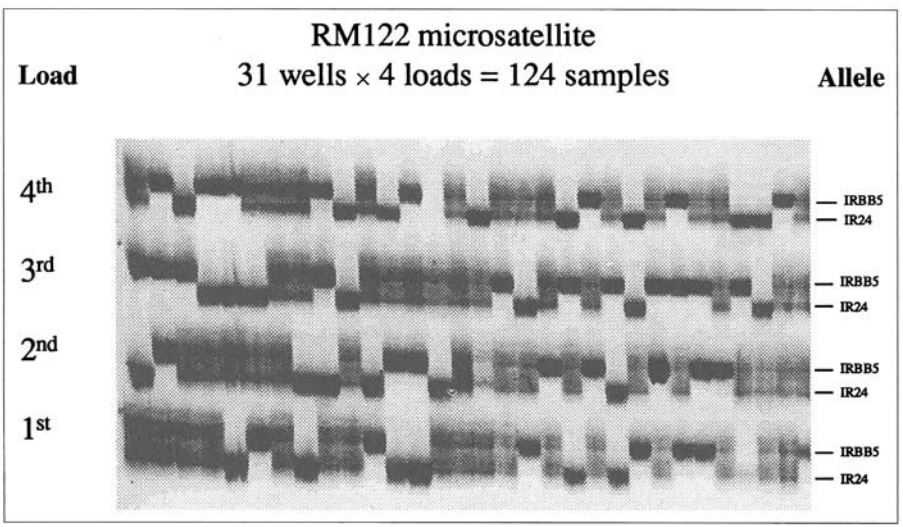

Fig. 1. Multiple loading of a single PAGE gel allowed the genotypic analysis of 121 F_2 individuals from the cross IR24/IRBB5 for the RM122 microsatellite locus. The two parental alleles are shown for each of the four loads.

MAPMAKER v.2.0. All map distances (cM) are reported in Kosambi units and the best marker order was determined (LOD score > 2.5) as in Causse et al (1994).

Results

Microsatellite survey of the NILs

Microsatellites were highly polymorphic and able to differentiate recurrent parent and donor parent alleles with greater frequency than RFLPs. Of the 50 microsatellite markers used in the current parental survey, 28 (56%) were polymorphic. RFLP probes used in previous surveys of the same parents detected much lower rates of polymorphism (McCouch et al 1992, Yoshimura et al 1995). When a marker was monomorphic, the presence or absence of an introgression could not be determined. When a marker was polymorphic, an introgression was determined to be absent when the isoline had a recurrent parent allele, and present when it had a donor parent allele (McCouch et al 1991). Of the 28 microsatellite markers showing polymorphism, 6 diagnosed the presence of an introgression, while 22 predicted the absence of an introgression. One introgression occurred at the top of chromosome 5, in the region containing the *xa5* gene, whose chromosomal position had been determined a decade ago by trisomic analysis and classical genetic studies (Yoshimura et al 1983). Three spurious introgressions were located on chromosomes 1, 6, and 11. The introgression on chromosome 1 had not been detected in the previous RFLP surveys (Yoshimura et al 1995) but was discovered by two linked microsatellites, RM5 and RM9. The introgressions on chromosome 11 could be diagnosed by the marker RM21.

Linkage analysis

Segregation analysis of the individuals in the IR24/IRBB5 population clarified the linkages of the microsatellites with *xa5* and with RFLP markers in the introgressions on four chromosomes. All the microsatellites were scored as codominant markers. The markers on chromosomes 1, 5, and 6 had segregation ratios that fit the expected segregation ratio. However, many of the markers in the chromosome 11 introgression had skewed segregation ratios, which deviated from the expected ratio in favor of the IRBB5-derived allele.

The linkage map for the chromosome 5 introgression (Fig. 2) shows the placement of two microsatellites in relation to *xa5* and to 10 RFLP markers. The positions of three JRGP markers, G292, C597, and G3396, were integrated with the seven markers from the Cornell map. Two RFLP markers, RZ390 and RG556, cosegregated completely with *xa5* in this population of 122 plants. Flanking markers were found on both sides of *xa5*. G292, C597, and RG207 were distal, at 4.1, 2.9, and 0.8 cM, respectively, from *xa5*, while the microsatellite, RM122, was 2.1 cM proximal to *xa5*. The second microsatellite identified in the region, RM13, was 18 cM away from *xa5*. In previous studies by Yoshimura et al (1995) and McCouch et al (1992), smaller numbers of F_2 individuals of the same cross were used and no recombinants were observed between RG207 and *xa5*. The end of this introgression was defined by the microsatellite, RM164, and the RFLP marker, RZ495. The three spurious introgressions

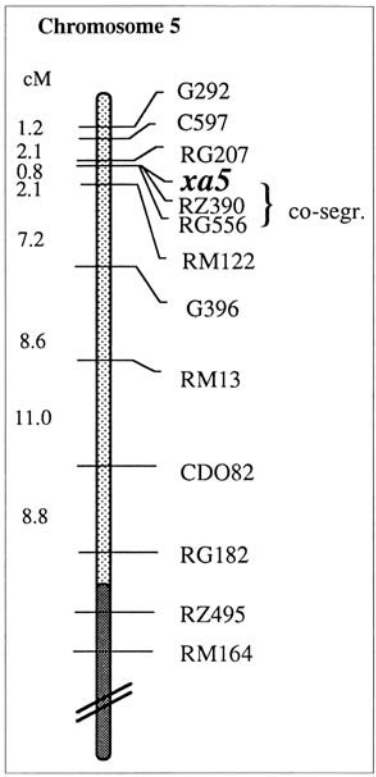

Fig. 2. Fine map of the *xa5*-containing region on chromosome 5 (lightly stippled), based on segregation data of the F_2 population (IR24/IRBB5), showing the placement of microsatellite markers (RM) within an RFLP framework map that includes Cornell markers (RG, RZ, or CDO) and Rice Genome Program markers (C or G). Marker alleles along the rest of the chromosome (darkly stippled) were derived from the recurrent parent (for RZ495 and RM164) or were monomorphic.

were on separate chromosomes and varied in size (Fig. 3). The two microsatellites in the chromosome 1 introgression, RM5 and RM9, were 1.7 cM apart. The end of the introgression was delimited on one side by two linked microsatellite markers, temporarily identified as RM(t1) and RM(t2).

Identification of an improved NIL with graphical genotyping

Graphical genotypes were prepared for the IRBB5 isoline and the 122 F_2 individuals of the IR24/IRBB5 cross using the microsatellite marker data and the computer program Hypergene (Young and Tanksley 1989). The objective was to select a recombinant individual containing only a small segment of donor parent DNA around *xa5* and without the spurious introgressions. RM13 was used to screen for recombination events that would reduce the size of the chromosome 5 introgression. RM122 and the BLB resistance reaction were used to identify lines that were homozygous in the immedi-

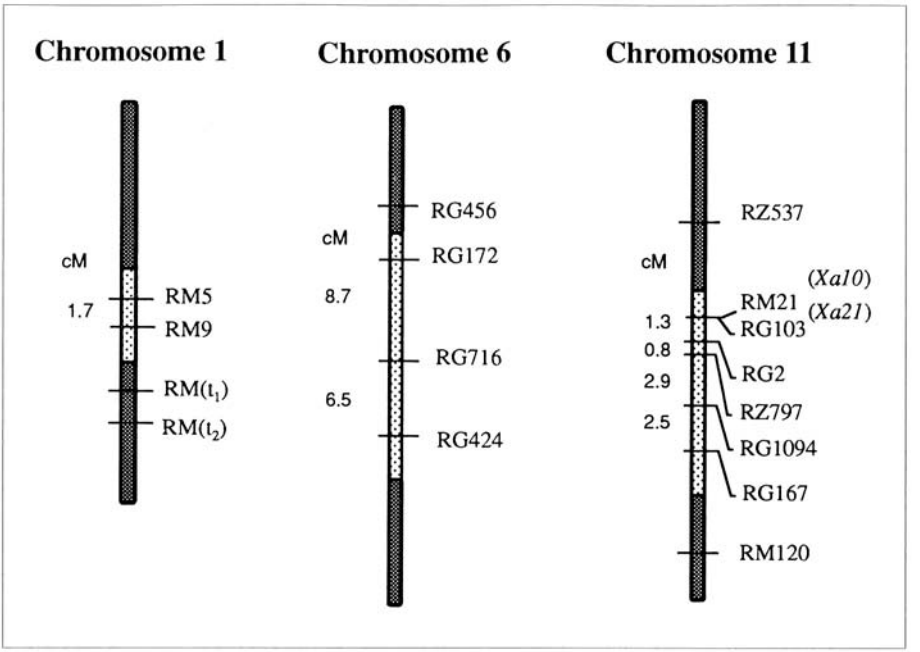

Fig. 3. Genetic linkage maps of three spurious introgressions on chromosomes 1, 6, and 11 in the near-isogenic line IRBB5. The extent of the introgression is shown by the lightly stippled segments of the chromosomes. Flanking markers delimit the surrounding nonintrogressed regions (darkly stippled). Genetic map distances (cM) were estimated from segregation data of the F_2 population (IR24/IRBB5).

ate region of *xa5*. Several resistant individuals with cross-overs between RM13 and RM122 were found, which were heterozygous for only one of the spurious introgressions. Seed was harvested from these plants to grow F_3 families and fix the desired genotype in a pure line. A single F_2 individual of this population had unexpected alleles for three microsatellite loci and was discarded as it was a probable seed mixture or outcross.

Discussion

Microsatellites were the ideal markers for tagging the targeted BLB resistance genes. In the parental survey, microsatellite markers detected a high level of polymorphism, despite the fact that both recurrent and donor parents were indica subspecies. This meant that microsatellites were more proficient than RFLPs at diagnosing the presence or absence of introgressions in the IRBBS isoline. Microsatellites were also useful in defining the ends of each introgression. Microsatellite markers were especially effective for whole genome analysis of NILs because many loci could be screened simultaneously on a single polyacrylamide gel at minimal cost and effort.

The utility of the microsatellites near BLB resistance loci for marker-assisted selection, fine mapping, and gene characterization was demonstrated. Graphical genotyping and marker-assisted selection were implemented to screen the IR24/IRBB5 population and to produce a more nearly isogenic line from the progeny. The improved isoline will be used for future molecular characterization of the *xa5* gene. The introgressions in the IRBB5 isoline were mapped and genotyped to determine if they may have had some role in resistance and to speculate whether they had been maintained by chance or by design during the backcrossing program of disease screening and selection. Of the three spurious introgressions carried along in IRBB5, the most interesting in this regard was the one on chromosome 11. In other varieties, this region is known to contain a cluster of blast and BLB resistance genes including *Pi7, Xa10,* and *Xa21* (Causse et al 1994). Although we could not find evidence for a major phenotypic effect of this introgression with our BLB infection conditions, the location of many resistance genes on chromosome 11 raises the possibility that this introgression may have contributed in some other way to fitness or BLB resistance and was inadvertently selected. Skewing in favor of the donor parent allele was observed for this introgression. By mapping the chromosome 11 introgression, the microsatellite marker RM21 was found to cosegregate with RG103, which is part of the recently cloned *Xa21* resistance gene (Song et al 1995).

We recommend that the RM21 microsatellite be used to select for *Xa21* and its cluster of linked BLB resistance genes. The present study mapped the microsatellite markers, RM122 and RM13, in relation to *xa5* and clarified the exact placement of *xa5* in relation to adjacent RFLP markers on chromosome 5. The mapping of two RFLP markers, G292 and C597, distal to *xa5* allowed us to estimate the genetic distance from the resistance gene to the telomere, which has been mapped with a telomere-specific repeat clone, TEL1 (Kurata et al 1994).

In summary, the three microsatellite markers discussed here could be used in the future for further characterizing BLB resistance genes and their interactions or for pyramiding effective combinations of these genes through marker-assisted selection.

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Genetic analysis and tagging of gene for brown planthopper resistance in indica rice

M. Mei, C. Zhuang, R. Wan, J. Wu, W. Hu, and G. Kochert

Brown planthopper (BPH) is one of the most serious insect pests in south China. About 3,000 cultivars and varieties from this area have been screened for BPH resistance, and more than 100 varieties have been identified to have high or moderate resistance. Genetic analysis of F_1 and F_2 populations derived from a cross between Sanguizhan, a resistant indica cultivar, and Taichung Native 1, a highly susceptible cultivar, indicated that resistance was governed by a single dominant gene. An allelism test revealed that this gene was nonallelic to and independent of *Bph1* and *Bph3.* For tagging this gene, F_3 lines were developed and evaluated for their resistance to BPH. Restriction fragment length polymorphism (RFLP) markers were used as probes to survey the bulked DNAs from homozygous resistant lines and homozygous susceptible lines, and cosegregation between RFLP patterns and resistance in F_3 lines was analyzed. The results obtained so far showed that RZ404, a rice cDNA clone located near the end of chromosome 9, correlated with resistance. No BPH resistance genes have been previously reported in this map position.

The brown planthopper (BPH) is one of the most serious pests of rice throughout Asia. To control this pest through varietal resistance, many investigators have studied the inheritance of resistance in rice cultivars. At least nine resistant loci have been reported, which include four dominant genes and five recessive genes (Khush and Brar 1991). By trisomic analysis, *Bph1* and *bph2* were mapped to chromosome 4 and *Bph3* and *bph4* to chromosome 10.

In south China, the main BPH population is biotype 1. This biotype causes heavy losses in rice production in this area every year. To evaluate local germplasm for resistance to this insect, Ceng and Wu (1989) screened about 3,000 accessions from south China based on their reactions to BPH biotype 1. More than 100 cultivars or varieties were identified to be highly or moderately resistant.

Studies of the inheritance of resistance indicated that different accessions appeared to have a single dominant gene, a recessive gene, or two genes (Liu et al 1992). The first molecular map of rice was published in 1988 (McCouch et al 1988). Recently, two maps with high-density molecular markers have been constructed simultaneously (Causse et al 1994, Kurata et al 1994). These maps were used to locate a number of important agronomic genes via linkage to restriction fragment length polymorphism (RFLP) markers.

For BPH resistance, an alien gene introgressed from wild rice *Oryza australiensis* into cultivated rice has been mapped to chromosome 12 through molecular tagging (Ishii et al 1994). The present study was undertaken to detect RFLP markers linked to a BPH resistance gene in Sanguizhan, a resistant indica cultivar. To verify results from RFLP analysis, allelism tests of the resistance gene from Sanguizhan with two known resistant loci were also conducted.

Materials and methods

Plant materials and evaluation of BPH resistance

Sanguizhan—a domestic cultivar (indica) from Guangdong Province, China, and identified to have BPH resistance governed by a single dominant gene (Liu et al 1992)—and Taichung Native 1 (TN1)—a cultivar highly susceptible to BPH—were used as parents. About 40 F_3 lines derived from the F_2 individuals were used for the mapping population. A bulk seeding test was performed to evaluate the population for BPH resistance (Wu et al 1984). The F_3 lines were classified as homozygous resistant, segregating, or homozygous susceptible.

RFLP analysis

Seedlings from the parents and each F_3 line were used for DNA extraction. To speed up screening, pools of DNA from six homozygous resistant F_3 lines (R pool) and six homozygous susceptible lines (S pool) were made. DNA from parents, pooled samples, and F_3 lines were digested with four restriction enzymes: *Eco*RI, *Eco*RV, *Hin*dIII, and *Xba*I. One hundred and thirty-seven RFLP markers (rice genomic clones, oat cDNA, and rice cDNA), provided by S.D. Tanksley of Cornell University, distributed across the 12 chromosomes were screened. After analysis of preliminary results, more probes were selected from chromosome 9 (including several clones located on the map constructed by Kurata et al [1994], and rice cDNA clones from Dr. Uchimiya and mapped by G. Kochert) to survey potentially linked markers.

MAPMAKER Macintosh Version 2.0 (Du Pont Company) was used for segregation data analysis and genetic distance calculation.

Allele tests

To determine the allelic relationship of resistance gene in Sanguizhan with *Bph1* and *Bph3*, crosses of Sanguizhan with Mudgo or Rathu Heenati were made. The reaction of F_2 progeny from these two crosses was evaluated by the bulk seedling test. Sanguizhan was the resistant check and TN1 was the susceptible check.

Results and discussion

Genetic analysis of BPH resistance

Table 1 summarizes the data for resistance of the F_1, F_2, or F_3 progeny to BPH biotype 1 in three crosses involving Sanguizhan. In the cross with TN1, all the F_1 plants were resistant, and the F_2 population segregated in the ratio of 3 resistant:1 susceptible in the primary study (Liu et al 1992), indicating that resistance was determined by a single dominant gene. The F_3 line from this cross in the present study segregated in the ratio of 1 resistant:2 segregating:1 susceptible, making the same inference, although the value of x^2 was higher than that from the F_2 segregating data. BPH resistance of F_2 populations from the cross Sanguizhan/Mudgo for *Bph1* or from Sanguizhan/Rathu Heenati for *Bph3* segregated in a ratio very close to 15 resistant:l susceptible, demonstrating that the single dominant gene in Sanguizhan was nonallelic to and independent of *Bph1* and *Bph3,* the dominant resistance genes in cultivated rice mapped so far.

The deviations from the 1:2:1 ratio in F_3 lines from the cross Sanguizhan/TN1, and from the 15:l ratio in the F_2 populations for the allelic tests may be due to misclassification of a few seedlings because of unfavorable growing conditions, such as low temperature and low light intensity in the test room. Under these conditions, some Sanguizhan seedlings also died.

Identification of RFLP markers linked to the resistance gene

Results of screening with 146 RFLP markers indicated that less than 20% of the markers detected different restriction fragment patterns between Sanguizhan and TN1 (Table 2). Most of the polymorphisms were detected by only one enzyme with a given probe. However, for a few clones from chromosomes 4 and 9, two to four enzymes detected RFLP patterns, suggesting greater variations in the genomic regions between the two parents. When bulked segregant analysis was performed, polymorphism between the resistant pool (R pool) and the susceptible pool (S pool) was found with one random amplified polymorphic DNA (RAPD) product which was then mapped close to one end of chromosome 9 by using a backcross mapping population. Flanking markers close to the RAPD marker from different maps produced identical restriction fragment patterns between the parents.

Cosegregation analysis of the BPH resistance phenotype and RFLP patterns of polymorphic clones RZ404, CD0412, and UCH170 in 40 F_3 lines revealed possible linkage between RZ404 and the resistance locus (x^2 = 9.65; $P<0.05$), but the recombination fraction was high (0.24) (Fig. 1). Markers tightly linked to this resistance locus thus could not be identified. The identification is difficult because of the small size of the mapping population (some F_2 individuals that produced the F_3 lines were infected by diseases) or the misclassification of resistance of some F_3 lines under unfavorable growing condition during the assays.

To verify whether the gene controlling resistance to BPH biotype 1 in Sanguizhan is a new dominant resistance gene and to determine its location in the genetic map,

Table 1. Reaction to brown planthopper biotype 1 of F_1, F_2, or F_3 populations from crosses of Sanguizhan with TN1, Mudgo, or Rathu Heenati.

Cross	F_1	F_2 seedlings (no.)		c^2		F_3 lines (no.)			c^2
		Resistant	Susceptible	3:1	15:1	Resistant	Segregating	Susceptible	1:2:1
Sanguizhan/TN1[a]	Resistant	409	138	0.015		12	36	15	1.491
Sanguizhan/Mudgo		335	33		4.648				
Sanguizhan/Rathu Heenati		349	41		12.09				

[a] Data on F_1 and F_2 reaction were from Liu et al (1992).

Table 2. Restriction fragment length polymorphism between Sanguizhan and TN1.

Chromosome no.	1	2	3	4	5	6	7	8	9	10	11	12
Markers screened	16	14	12	22	9	12	7	6	20	8	10	10
Polymorphic markers	6	3	4	5	1	0	1	0	5	2	1	0

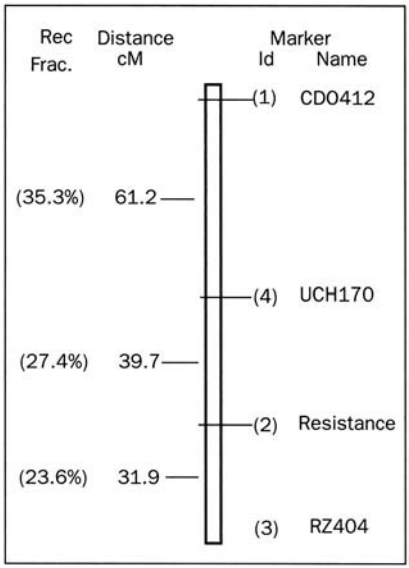

Fig. 1. Region of rice chromosome 9 in the vicinity of the gene for resistance to brown planthopper biotype 1 in Sanguizhan (indica cultivar).

further cosegregation analysis with a larger mapping population and allelic tests between this gene and the other reported dominant genes should be conducted.

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Tagging and mapping the thermosensitive genic male sterile gene of rice with molecular markers

B. Wang, W.W. Xu, J.Z. Wang, M.L. Weng, M.Y. Li, R.C. Yang, and H.T. Nguyen

Bulk segregant analysis of F_2 populations was used to identify random amplified polymorphic DNA (RAPD) markers linked to the thermosensitive genic male sterile (TGMS) gene of rice. Four hundred RAPD primers were screened for polymorphisms between the parents and between the two bulk populations representing fertile and sterile plants. Of these, four primers produced polymorphic products. Most of the polymorphic fragments contained repetitive sequences. Only one single-copy sequence fragment was found, a 1.2-kb fragment amplified by primer OPB-19, and subsequently named TGMS1.2. TGMS1.2 was mapped on chromosome 8 with a genetic distance of about 6.7 cM from the TGMS gene. TGMS1.2 was sequenced and changed to a sequence-tagged site marker. It is available for public use. A rice bacterial artificial chromosome library was screened with the labeled TGMS 1.2 as the probe. Nine positive clones were screened.

The rapidly increasing demand for rice and the continuous decrease in rice-growing areas have resulted in a search for ways to improve rice production. The use of hybrid rice has proven to be an effective and economical way to increase rice production output. It is easy to obtain about 10-20% higher yield just by growing hybrid rice instead of the common varieties. As the widely used three-line system for producing hybrid rice is extremely time-consuming and costly, a two-line system using thermosensitive genic male sterile (TGMS) rice has been developed. This system has become a simple and efficient breeding method for producing hybrid rice. However, little is known about the TGMS gene at the molecular level, which subsequently limits the wide application of TGMS rice.

The pollen fertility of TGMS rice is regulated by temperature. It is completely sterile at high temperature but fertile at lower temperature (Sun et al 1989). Therefore, TGMS plants can be used to produce hybrid seeds by interplanting them with normal

fertile lines under high temperature and allowing them to reproduce by selfing under low temperature. It can be used not only as a male sterile line, but also as a maintainer line, thereby providing the opportunity to produce hybrid rice by the two-line system. The two-line system developed with TGMS rice has many advantages. First, because TGMS rice can be used both as a sterile line and as a maintainer line, the expressly developed maintainer line is no longer needed. Second, TGMS rice has a broad spectrum of fertility restoration; almost all of indica rice and most japonica rice can be used as a pollen donor to produce fertile F_1 hybrids. A restorer line can easily be obtained from a wide range of germplasm available. Therefore, deliberate efforts to develop restorer lines in the traditional three-line method are also eliminated. Third, unlike in three-line hybrids, the performance of TGMS hybrid rice is not adversely affected by male sterile cytoplasm. These advantages make the two-line system simpler and more economical than the widely used three-line system. Its application in hybrid rice production will greatly reduce cost in labor, time, and resources.

Rice has a relatively small genome and well-developed linkage maps in addition to a well-developed transformation system. Previous research has indicated that the fertility of TGMS rice is controlled by one Mendelian locus (Yang et al 1992). Therefore, it should not be very difficult to develop new TGMS lines by transferring the TGMS gene to selected desirable cultivars. If the rice TGMS gene is cloned and transferred into other crops, it could greatly advance the practical application of hybrid production in the entire field of crop breeding. But before cloning the gene, we have to map it first. This research was aimed at tagging the rice TGMS gene with molecular markers and mapping it onto a specific rice chromosome.

Materials and methods

Plant materials

To identify random amplified polymorphic DNA (RAPD) markers linked to the TGMS gene, an F_2 population was developed from a single cross between 5460s, a TGMS mutant line, and Hong Wan 52, a normal fertile line. It was found that the fertility segregation in this F_2 population was controlled by one major locus, with fertility segregation fitting the Mendelian 3:1 ratio.

Bulk generation

Bulk generation was done using the method described by Michelmore et al (1991). Two bulk DNA samples, each containing equal amounts of genomic DNA from each of the 15 individuals, were used for RAPD analysis to identify polymorphisms. They were constructed on the basis of their fertility: the individuals in one bulk sample were all extremely fertile while those in the other bulk sample were all extremely sterile.

RAPD analysis

Genomic DNAs were used as templates for polymerase chain reaction (PCR) amplification as described by Williams et al (1990) and modified especially for rice in our laboratory (Zhang et al 1994).

Cloning of polymorphic fragments

Polymorphic DNA bands of interest resolved by agarose gel electrophoresis were purified with a GeneClean™ kit (Bio-101, La Jolla, CA) as recommended by the manufacturer. The purified products were then cloned with a Sureclone™ ligation kit from Pharmacia (Piscataway, NJ).

Linkage analysis

The RAPD marker TGMS1.2 was mapped in the F_7 recombinant inbred line population from the cross CO 39/Moroberekan using the program MAPMAKER (Lander et a1 1987). Previously mapped loci (Wang et a1 1994) were used as anchor loci to determine chromosomal location. The mapping result was reconfirmed with a doubled haploid (DH) line mapping population from the cross Azucena/IR64.

Results and discussion

Screening RAPD markers linked to the TGMS gene

Bulk segregant analysis was employed to identify RAPD markers linked to the rice TGMS gene. Four hundred primers were screened for their polymorphism between the two bulk and two parental DNA samples. Of these, four primers amplified polymorphic products. Unfortunately, most of the amplified polymorphic products contained repetitive sequences. Only the 1.2-kb fragment amplified by primer OPB-19 proved to be a single-copy sequence and was named TGMS1.2. It was further cloned into pUC18 and used as a probe in gene mapping.

Cosegregation between the TGMS1.2 marker and the TGMS gene

To confirm the putative linkage between the TGMS gene and TGMS1.2 and to convert TGMS1.2 into a restriction fragment length polymorphism (RFLP) marker, we further analyzed its parents by Southern blotting. More than 30 restriction enzymes were tested. An RFLP was found only when the parental DNAs were digested with *Sac*I. Cosegregation analysis was then performed. TGMS1.2 was found to cosegregate with and link to the TGMS gene. Because two recombinants were found, the genetic distance between the TGMS1.2 marker and the TGMS gene was estimated to be 6.7 cM in this F_2 population. This confirmed the result obtained from RAPD analysis of the same population.

Mapping TGMS1.2 onto a specific chromosome

To map the rice TGMS gene onto a specific chromosome, we used the anchor loci to map the RAPD markers onto a specific rice RFLP linkage group with the mapping population. The previously mapped RFLP loci in this population served as the anchor loci. TGMS1.2 was labeled with ^{32}P by the random primer labeling procedure and hybridized to the *Bam*HI- or *Eco*RV-digested DNA from F_7 RI lines. Data from TGMS1.2 were added to the data set and analyzed with MAPMAKER/EXP (IBM v.3.0) with the following linkage criteria: minimum LOD score = 3.0 and maximum recombination fraction = 40%. TGMS1.2 was grouped with RG333, RZ562, and

RG978. TGMS1.2 was located between RZ562 and RG978, linked to RZ562 by 12.6 cM, and linked to RG978 by 12.7 cM. The hybridization data of TGMS1.2 were further analyzed with MAPMAKER (Mac v.2.0) (minimum LOD score = 5.0 and maximum recombination fraction = 40%). TGMS1.2 was also grouped with RG333, RZ562, and RG978. Both analyses showed that the best order was RG333-RZ562-TGMS1.2-RG978. It has been reported that RG333, RZ562, and RG978 are in linkage group 8 (chromosome 8) (Wang et al 1994). The TGMS gene, therefore, must be located on chromosome 8. Later, TGMS1.2 was remapped at IRRI using a DH mapping population from the cross Azucena/IR64. It was confirmed that TGMS1.2 was located on the same region as chromosome 8.

Changing RAPD marker to STS marker

To make TGMS1.2 available for public use, it was sequenced and changed into a sequence-tagged site (STS) marker from an RAPD marker. Further, two 20-mer primers were designed and synthesized based on the sequences of the two ends of TGMS1.2.

BAC library screening

A bacterial artificial chromosome (BAC) library (from Texas A&M University, which is three times the size of the rice genome) was screened with the labeled TGMS1.2 as the probe. Nine positive clones have been screened. The screened molecular markers and positive BAC clones will be very useful in monitoring TGMS gene transfer in rice breeding and in TGMS gene isolation by means of map-based cloning.

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Inheritance and molecular mapping of the thermosensitive genic male sterility gene in rice

P.K. Subudhi, R.P. Borkakati, S.S. Virmani, and N. Huang

The inheritance of thermosensitive genic male sterility gene in IR32364TGMS developed at IRRI was studied in eight different cross combinations. The progenies in F_2, BC_1, and F_3 generations were grouped into sterile and fertile classes based on observations on pollen and spikelet sterility. Genetic analysis revealed that this trait is under the control of a single recessive gene. Bulk segregant analysis was employed in conjunction with random amplified polymorphic DNA (RAPD) technique in IR32364TGMS/IR68 cross combination and five RAPD markers (OPF18-2600, and OPAA7-550, OPAC1-300,OPC3-640, and OPB19-750) were identified to be closely linked to this gene on both sides. Only one of the RAPD markers, OPAC3-640, was fertile-specific and the rest were sterile-linked. All the RAPD fragments except OPF18-2600 were of multicopy sequences. The OPF18-2600 has been cloned and also showed polymorphism in Southern blot analysis. Sequencing of this clone is under way for converting it into a sequence-tagged site, which can help in marker-assisted selection while transferring this gene to a different background.

The wide-scale adoption of hybrid rice in tropics and subtropics has been limited mainly due to complexity in the seed production process associated with the commonly used cytoplasmic genetic male sterility (CMS). The discovery and application of thermosensitive genic male sterility (TGMS) are likely to have great impact in these areas because of its many advantages compared with the CMS system, such as easier multiplication of TGMS seeds, no need of maintainers or restorer parents, etc. Further, the cytoplasmic base can be diversified since the TGMS gene can be incorporated into any rice cultivar to be used as female parent for hybrid production. The incorporation of the TGMS gene through conventional breeding is quite cumbersome; it involves identification of TGMS plants in segregating generations and induction of

seed fertility by ratooning under an appropriate fertility-inducing temperature regime. Inheritance of this gene and subsequently mapping by molecular markers should help in exercising marker-aided selection for transferring the TGMS gene to different backgrounds.

Materials and methods

A TGMS mutant line, IR32364TGMS, developed at IRRI (Virmani and Voc 1991), was crossed with eight fertile testers: IR32364 Normal, IR68, Dular, Moroberekan, IR72, IR54752 B, N22, and BPI76. Observations on pollen fertility and spikelet fertility were taken in F_2, F_3 (only studied in IR32364TGMS/IR68, IR32364TGMS/Dular, IR32364TGMS/Moroberekan) and BC_1 generations for classification of plants into sterile and fertile groups. Single plants of these populations were evaluated for pollen fertility (determined by using 1% IKI solution) and spikelet fertility (determined by using 2 bagged panicles per plant). Individuals with less than 20% pollen fertility were grouped under sterile class. The c^2 test was performed for verifying the goodness of fit to 3:1, 1:1, 1:2 ratio for F_2, BC_1, and F_3 generations, respectively.

The cross, IR32364TGMS/IR68, was used for molecular mapping purposes. The DNA of two parents and 65 F_2 individuals was isolated following the procedure of Dellaporta et al (1983) and bulk segregant analysis (Michelmore et al 1991) was performed in conjunction with the random amplified polymorphic DNA (RAPD) technique. Equal amounts of DNA from 10 fully fertile (homozygous) and 10 fully sterile (homozygous) individuals were pooled to constitute the fertile and sterile bulk, respectively. The two bulked samples along with the parents were then subjected to RAPD analysis following conditions of Williams et al (1990) with minor modifications. Amplification reaction was performed in 25 µl containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.001% (w/v) gelatin, 1.9 mM $MgCl_2$, 100 mM each of dATP, dCTP, dGTP, and dTTP, 40 ng of primer, 50 ng of genomic DNA, and 1 unit of Taq polymerase in Techne thermocycler. Amplification profile was 94 °C for 2 min, followed by 45 cycles of 1 min at 94 °C, 1 min at 36 °C, 2 min at 72 °C with a final extension time of 7 min at 72 °C. Amplification products were resolved by electrophoresis in 1.5% ethidium bromide containing agarose gel in 1X TAE. A total of 389 random primers (10-mer) obtained from Operon Technologies, Inc. (Alameda, CA) were screened. The primers showing putatively linked RAPD fragments were again used for amplification of all homozygous sterile and fertile F_2 plants. The linkage relationship between loci were analyzed using the MAPMAKER program (Lander et al 1987).

Results and discussion

Mean pollen fertility of the F_1s ranged from 58.7 to 85.0% (Table 1) and mean spikelet fertility ranged from 55.4 to 80.6% (data not shown). Two distinct classes of fertile and sterile individuals, observed in the F_2, BC_1 populations of all eight cross combinations, clearly segregated in 3:1 and 1:1 ratios, respectively (Table 1), indicating

Table 1. Inheritance of TGMS in different crosses based on pollen fertility data.

Cross combination	Pollen fertility (%)			Observed segregation in F_2			Observed segregation in BC_1		
	P_1	P_2	F_1	Fertile	Sterile	c^2(3:1)	Fertile	Sterile	c^2(1:1)
IR32364TGMS/IR32364	2.8	79.4	77.3	165	39	3.46	52	56	0.08
IR32364TGMS/IR68	2.8	79.3	74.1	247	70	1.29	46	30	2.96
IR32364TGMS/Dular	2.8	83.4	85	154	42	1.15	38	42	0.11
IR32364TGMS/Moroberekan	2.8	83.1	60.7	138	59	2.32	39	45	0.3
IR32364TGMS/IR72	2.8	69.2	69.2	143	44	0.14	47	33	2.11
IR32364TGMS/IR54752 B	2.8	86.3	77.3	166	42	2.31	13	12	0
IR32364TGMS/N22	2.8	73.8	58.7	161	43	1.47	53	54	0
IR32364TGMS/BPI76	2.8	79.2	78.4	147	51	0.03	17	13	0.3

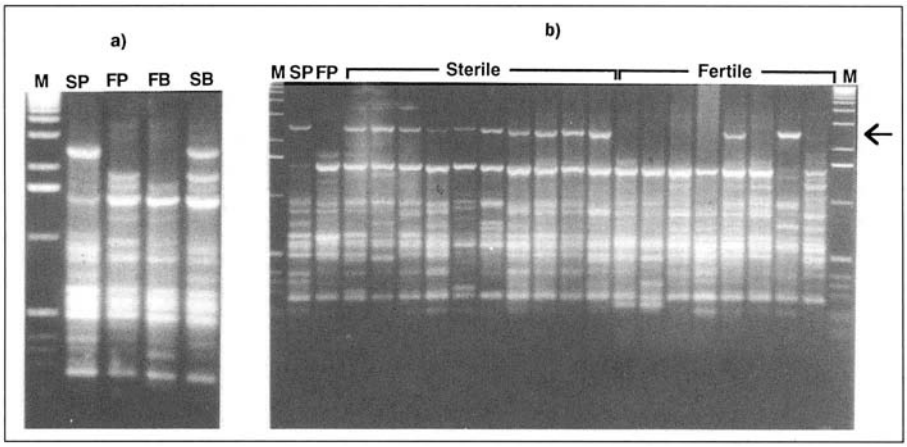

Fig 1. DNA amplification pattern obtained with random primer OPF18. a) Bulk segregant analysis: the polymorphic fragment of 2600 bp is indicated by an arrow. M = marker, SP = sterile parent IR32364TGMS, FP = fertile parent IR68, FB = fertile bulk, SB = sterile bulk. b) Amplification pattern of 10 homozygous sterile and 8 homozygous fertile individuals along with the parents IR32364TGMS and IR68. The RAPD fragment OPF18-2600 cosegregates with the TGMS trait.

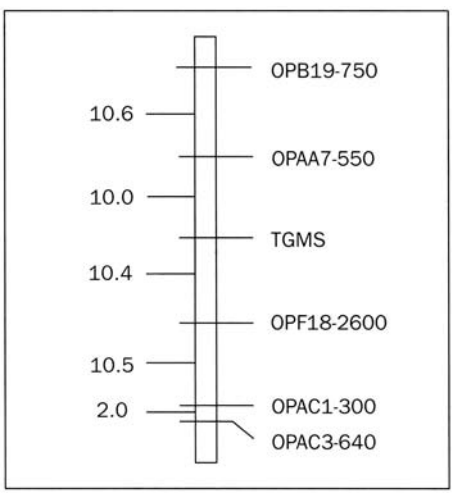

Fig 2. RAPD map of the region surrounding the gene for thermosensitive genic male sterility (TGMS). Distances (cM) are given on the left side of the vertical line and markers are on the right side.

the TGMS trait to be under the control of a single recessive gene. The pollen fertility data correlated well with the spikelet fertility data. F_3 segregation pattern in the crosses of IR32364TGMS with IR68, Dular, and Moroberekan also supported the monogenic recessive hypothesis. This report on inheritance concurs with the earlier observations of Maruyama et al (1991) in the TGMS mutant Norin PL12 and Yang et al (1990) in TGMS line R59TS.

After surveying 389 random primers, five RAPD markers generated by five primers (OPF18, OPB19, OPAC1, OPAC3, and OPAA7) were found to be putatively linked to the TGMS gene. Further analysis with all the homozygous sterile and homozygous fertile F_2 individuals showed cosegregation of these markers with the TGMS trait. A typical bulk segregant analyzer and the analysis of progenies with the RAPD primer OPFl8 are shown in Figure 1. Of five markers, four were sterility-specific (OPF18-2600, OPB19-750, OPAC1-300, and OPAA7-550) and one fertility-specific (OPAC3-640). Linkage analysis revealed that all these markers are flanking the TGMS gene from both sides (Fig. 2). OPF18-2600 and OPAA7-550 were located within 10 cM from the TGMS gene. All the polymorphic fragments were of multicopy sequences except OPF18-2600. This fragment is being cloned and a dominant polymorphism-like RAPD has been detected in genomic Southern blot analysis. Sequencing work is underway to convert the RAPD marker to sequence-tagged sites. Attempts to map this linked fragment in our doubled haploid mapping population of IR64/Azucena have failed because of a very skewed segregation pattern. Recently, the analysis of reciprocal crosses in IR32364TGMS/IR68 and other polymorphic crosses revealed that this marker is nuclear in origin in IR32364TGMS, but cytoplasmic in origin in other cultivars. It is speculated that this fragment is probably translocated from cytoplasm to the nucleus during irradiation of IR32364, which has this sequence. This will be clarified when the reciprocal cross of IR32364 Normal/IR68 is analyzed.

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Genetics of seedling-stage submergence tolerance in rice

D.J. Mackill and K. Xu

Submergence tolerance is a useful characteristic for rainfed lowland cultivars and has been suggested as a component of weed management strategies for water-seeded rice. Unimproved cultivars such as FR13A possess a high level of tolerance but are agronomically inferior and poor in combining ability. Tolerance was transferred from FR13A into improved indica lines through conventional breeding methods combined with screening in screenhouse tanks. Tolerance scores of segregating progeny suggested that the trait was controlled by one or two genes. Inheritance of submergence tolerance was investigated in a cross between indica line IR40931-26-3-3-5, which derived its tolerance from FR13A, and California japonica line P1543851. The nine most tolerant and most susceptible lines were used for bulk segregant analysis with random amplified polymorphic DNA markers. Of 640 primers used, six showed bands that were associated with submergence tolerance. Five of these bands mapped to a region of chromosome 9 close to the restriction fragment length polymorphism markers C1232 and RZ698. This chromosomal segment accounted for approximately 70% of the phenotypic variation for submergence tolerance. This locus, which we have designated *Sub-1*(t), thus accounts for a major portion of the submergence tolerance inherited from FR13A.

Submergence tolerance has long been regarded as an important breeding objective for rainfed lowland and deepwater rice areas (Mackill et al 1995). Despite this recognition, there has been limited success in developing improved submergence-tolerant cultivars. This has been attributed to the complexity of the trait as well as its association with undesirable characters. This paper reports the progress on breeding for improved submergence tolerance and identifies a major locus conferring this tolerance.

Transfer of submergence tolerance into improved lines

Major sources of submergence tolerance used in the rainfed lowland breeding program at IRRI included FR13A. FR43B, and Janki (previously designated Chenab 64-117). These cultivars were crossed with improved semidwarf or intermediate-height breeding lines, and the progeny were screened in concrete tanks in the screenhouse following the method of Vergara and Mazaredo (1975). The progeny were simultaneously selected for improved plant type in the field. Segregation in early-generation progeny indicated that tolerance was inherited by one or two genes.

Majority of the most tolerant lines developed in this program were derived from cultivar FRl3A (Fig. 1). These lines were evaluated in a yield trial with other improved cultivars and breeding lines (Mackill et al 1993). In general, lines with the best submergence scores were low-yielding. One line (IR49830-7-1-2-2), however, combined high yields with submergence tolerance. This seems to support the conclusion that low yields are a result of the linkage of undesirable genes to submergence tolerance in the parent FR13A, and these linkages can be broken by backcrossing.

Molecular mapping of a submergence tolerance locus

Previous genetic studies on submergence tolerance (Suprihatno and Coffman 1981, Mohanty and Khush 1985, Sinha and Saran 1988, Haque et al 1989) indicated that

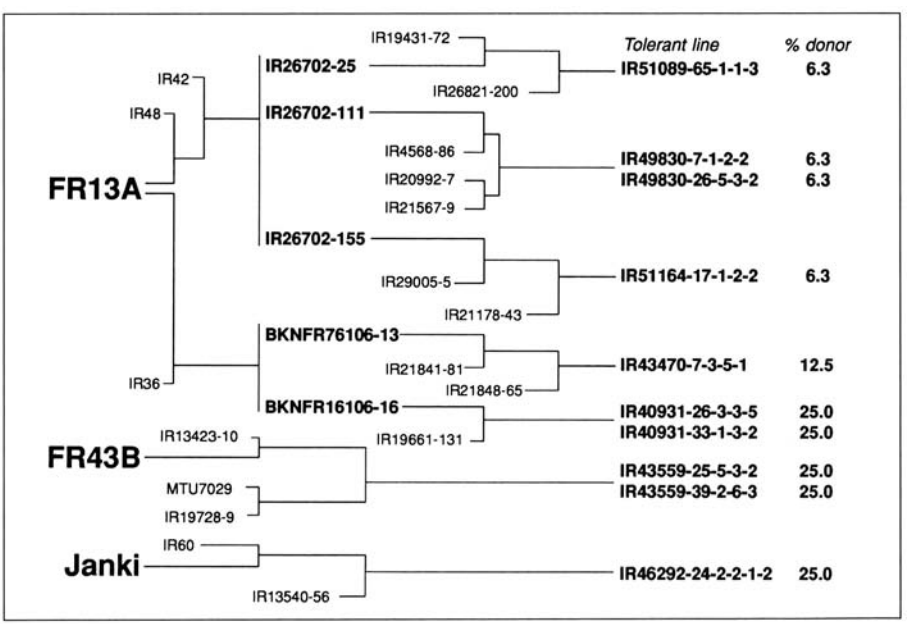

Fig. 1. Derivation of improved plant type lines with submergence tolerance. Submergence-tolerant lines are in boldface. The survival percentages are from screenhouse and field tests (Mackill et al 1993).

heritability was relatively high, but specific genes or loci involved in tolerance could not be identified. To identify loci involved in submergence tolerance, the most tolerant line identified in the above study, IR43931-26-3-3-5, was crossed with a susceptible japonica line, PI543851. IR40931-26 performed identically to FR13A in repeated screening experiments at IRRI. DNA was extracted from the F_2 plants of this population, and F_3 seeds were used for submergence screening. DNA extraction and molecular marker (restriction fragment length polymorphism [RFLP] and random amplified polymorphic DNA [RAPD]) analysis followed well-established protocols for rice (McCouch et al 1988, Mackill 1995). F_3 progeny were screened in four replications in greenhouse tanks at Davis, CA. Ten to fifteen seedlings per replication of 8-d-old plants were screened for 14-16 d under 55 cm water depth. One week after the treatment was terminated, individual plants were scored from 1 (tolerant) to 9 (susceptible) on a scale modified from Suprihatno and Coffman (1981).

F_3 mean survival scores ranged from 1.6 to 8.9, while the tolerant and susceptible parents scored 1.5 and 8.4, respectively (Fig. 2). The distribution was skewed toward the susceptible parent, with few lines showing equal tolerance for IR40931 (Fig. 3). While the distribution was more or less continuous, there was some indication of peaks near the tolerant and susceptible parents.

DNA from the F_2 plants resulting in the nine most tolerant and most susceptible F_3 lines was used for bulk segregant analysis (Michelmore et al 1991) with RAPD markers. These bulk populations were screened with 624 RAPD primers (Operon Technologies, Inc.), of which 63 showed polymorphic bands on the two bulk populations. Testing on individual plants within bulk populations identified six primers that produced bands strongly associated with either the tolerant ($OPH07_{950}$.$OPQ01_{600}$) or the susceptible ($OPN04_{1200}$, $OPS14_{700}$, $OPU15_{550}$, $OPAB16_{850}$) progeny. Testing

Fig. 2. Parents and F_3 plants subjected to submergence for 14 d. Tolerant plants on the left include tolerant parent IR40931-26 and the tolerant F_3 progeny. Susceptible plants on the right include susceptible parent P1543851, California japonica cultivar M-202, and the susceptible F_3 progeny.

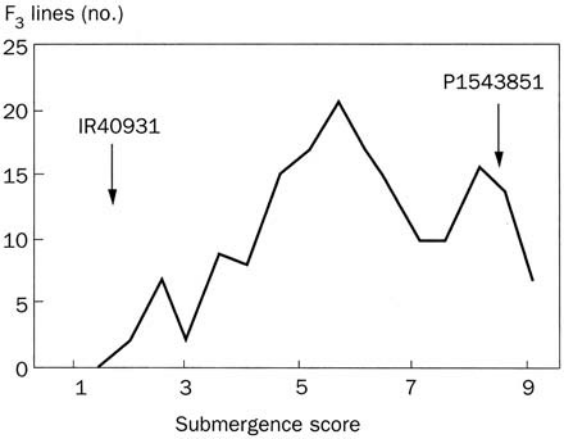

Fig. 3. Distribution of submergence tolerance scores for F_3 families of the cross IR40931-26/M-202.

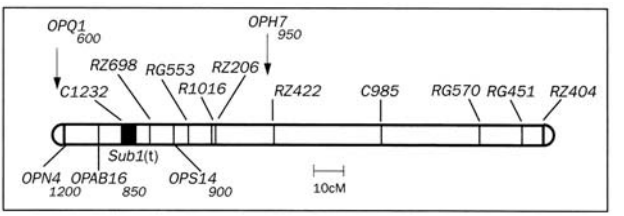

Fig. 4. RFLP map of rice chromosome 9 showing RAPD and RFLP markers and putative location of *Sub1*(t) (dark bar) based on MAPMAKER/QTL analysis.

of these primers on the entire F, population indicated that they were linked to each other ($OPU15_{550}$ was not mapped because of aberrant segregation).

RFLP probes obtained from Cornel1 University were assayed on the F_2 population. The RAPD markers associated with submergence tolerance were linked to RFLP markers on the end of chromosome 9 near RFLP markers RZ206 and RZ422. Additional probes from chromosome 9 were obtained from the Rice Genome Program, Japan. Analysis with MAPMAKER/EXP (Lander et a1 1987) indicated that the RAPD markers were located at the end of chromosome 9 near RFLP marker C1232 (Fig. 4). Analysis with MAPMAKER/quantitative trait locus (QTL) indicated that this locus had a peak log of odds (LOD) score of 36 and accounted for nearly 70% of the phenotypic variation for submergence tolerance in this cross. The results indicate the presence of at least one gene for submergence tolerance in this region although the possibility of several linked genes cannot be ruled out. This locus was designated *Sub1*(t). Additional analysis with MAPMAKER/QTL using 79 RFLP probes distributed throughout the other rice chromosomes did not detect any QTL affecting submergence tolerance.

Conclusions

Our results indicate that the *Sub1*(t) locus is responsible for a major portion of the tolerance of IR40931-26. The observation that IR40931-26 has a level of tolerance similar to that of FR13A suggests that *Sub1*(t) is probably the most important locus for submergence tolerance in this cultivar, which has been identified as perhaps the best source of this trait (HilleRisLambers and Vergara 1982). No other QTLs for tolerance were identified in this population, and *Subl*(t) accounted for nearly 70% of the phenotypic variation.

The identification of a major locus conferring submergence tolerance should stimulate intensified efforts to incorporate this trait into improved cultivars. Our efforts are focusing on fine-scale mapping of *Sub1*(t) using a cross between a tolerant F_2 plant from IR40931-26/PI543851 and a susceptible California cultivar, M-202. We plan to use the RAPD markers to incorporate this trait into a California japonica cultivar to determine if submergence tolerance will be useful as a component of our weed management strategy, where deep standing water (20 cm) at the time of seeding is used to suppress weed growth.

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Tagging an aromatic gene in lowland rice using bulk segregant analysis

S. Tragoonrung, J.Q. Sheng, and A. Vanavichit

Khao Dawk Mali 105, a traditional Thai rice (*Oryza sativa*) possessing unique jasmine aroma, was crossed with a nonaromatic rice cultivar CT9993-5-10-1-M for aromatic gene tagging. Eighty-one F_2 plants and their corresponding F_3 families were scored for aroma and classified into three groups by sensory test. The DNA from the segregating F_2 plants were bulked based on aromatic classification. Aromatic and nonaromatic groups were analyzed by bulk segregant method. Three hundred random primers were used to screen against the DNA of the two parental lines and the two bulk populations. Only one primer, 5'-GAACGGACTC-3', amplified the DNA fragment that was associated with the aromatic trait. The DNA fragment was modified and cloned into a vector for Southern blot analysis. The blot showed the same segregation result with more information on heterozygous genotypes.

The aromatic Thai rice cultivar Khao Dawk Mali 105 (KDML 105) has gained market shares in many areas of the world due to its superior grain quality. Conventional methods of aromatic identification depend on sensory evaluation—chewing seeds directly (Ghose and Butany 1952), eluting aroma from leaf tissue with diluted KOH (Sood and Siddiq 1978), or smearing partially scraped seeds with I-KI solution (Li 1991). Sensory methods, however, are prone to human error and difficult for aromatic comparison of different rice samples. Although volatile compounds in aromatic rice are influenced by several components including carbohydrate, aldehyde, acetaldehyde, and alcohol (Paule and Power 1989), 2-acetyl-1-pyrroline is widely accepted as a major aromatic compound (Buttery et al 1983). The method, however, requires expensive equipment and a large sample.

The availability of a saturated rice restriction fragment length polymorphism (RFLP) map offers a powerful tool to localize agronomically important genes. Recently, a fragrance locus was identified on rice chromosome 8 using a segregating population,

B8462T3-710/aromatic Lemont (Ahn et a1 1992). The fragrance (*fgr*) locus was 4.5 cM from the nearest RFLP marker, RG28. However, our preliminary results showed that the RG28 marker did not cosegregate with aromatic trait in an F_2 population, CT9993-5-10-l-M/KDML 105, suggesting that different loci may be involved. Therefore, the aromatic locus in a segregating population will be tagged via linkage with RFLP using derived F_2 population from CT9993- 10-5- 1 -M and KDML 105 using bulk segregant analysis (Michelmore et al 1991).

Materials and methods

A set of aromatic and nonaromatic rice cultivars was used in this experiment. KDML 105, RD15 (KDML mutant), and Basmati 370 are the most popular aromatic rice cultivars available in the market. Hawm Nai Pol, Leung Hawm, and Hawm Pama are traditional aromatic Thai rice varieties. Zhe 908 is an aromatic rice cultivated in Zhejiang Province, China. Azucena is an upland aromatic rice from the Philippines. IR28, IR50, RD10, RD23, Zhe852, Zhe9248, CT9993-5-10-1-M (CT9993), and CT6241 are nonaromatic rice cultivars. Eighty-one F_2 segregating plants derived from a single F_1 seed from KDML 105/CT9993 were used as a mapping population.

The aroma was evaluated using the procedure of Berner and Hoff (1986). Briefly, 20 F_3 seeds from a single panicle of each of the 81 F_2 plants were chewed one by one. The scented and nonscented phenotypes of F_2 plants were scored if all 20 F_3 seeds were aromatic or nonaromatic, respectively. F_2 plants containing a mixture of aromatic and nonaromatic seeds were considered as mild aromatic phenotype (heterozygous). To confirm the aromatic phenotyping of F_3 seeds, F_4 seeds were tested by the chewing-scoring method.

Total genomic DNA was extracted according to McCouch et al (1988) with some modifications. Random amplified polymorphic DNA (RAPD) analysis was performed in a 10 pl reaction containing 10 ng of DNA template, 1X PCR buffer (50 mM KC1, 10 mM Tris-HC1 pH 8.3, 1.5 mM $MgCL_2$, and 0.01% gelatin), 0.2 mM of each dNTP, and 0.2 pM of single random sequence decamer from Operon Technologies, Inc. The reaction was overlaid with a drop of mineral oil and subjected to one cycle of 95 °C for 2 min, followed by 45 cycles of 94 °C for 1 min, 35 °C for 1 min, and 72 °C for 2 min. The amplified products were run on 1.6% agarose gel containing ethidium bromide (0.5 pg ml^{-1}).

Total genomic DNA from each parental line was digested with eight restriction enzymes: *Bam*HI, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Xba*I, and *Hha*I according to the manufacturer's (Boeringer Mannheim) recommendations, using two units of enzymes per microgram of DNA. Digested DNA samples in agarose gels were transferred onto a positively charged nylon membrane (Boehringer Mannheim) and hybridized to digoxigenin-labeled probes. Rice genomic clones were from the Rice Genome Program (RGP), Japan, and Cornell University, USA. Hybridization was carried out overnight at 65 °C and detection of DNA bands was conducted according to the manufacturer's (Boehringer Mannheim) protocol. Selected DNA fragments

amplified from PCR were excised from an agarose gel and purified using GENE CLEAN II Kit (Bio 101 Inc.). The fragment was ligated into vector pUC 18 precut with *Sma*I.

Results and discussion

Phenotyping of aroma

The segregation of 81 F_2 plants indicated a single recessive gene controlling aroma in rice with c^2 = 4.447, 0.25>P>0.10, for 1:2:1 ratio of nonaromatic:mildly aromatic:aromatic traits model. This result of a single recessive gene controlling aroma in rice is in agreement with several reports (Ali et al 1993, Pinson 1994). To confirm the aromatic data, seeds derived from each F_3 family were evaluated for aroma.

Identification of markers associated with aroma

Three hundred random primers were screened against genomic DNA from aromatic parent (KDML 105), bulk aromatic F_2 segregants (mix A), bulk nonaromatic F_2 segregants (mix N), and nonaromatic parent (CT9993). Sixty-four percent of the primers gave polymorphisms between the two parents. Only one primer, however, Opc-06 (5' GAACGGACTC 3') amplified a corresponding 1.5-kb DNA band between the mix N sample and the nonaromatic parent (Fig. 1). No amplified product at this position was detected in both KDML 105 and mix A. The primer was then used to amplify DNA samples from individual F_2 plants and the linkage between the 1.5-kb fragment and aromatic trait was detected (Fig. 2). The majority of F_2 plants scored mildly aromatic and nonaromatic phenotypes, having the 1.5-kb fragment after amplification with the Opc-06 primer. This result suggested that the 1.5-kb fragment was in a coupling phase with the dominant nonaromatic allele. When the Opc-06 primer was used to amplify the DNA samples from several known aromatic and nonaromatic cultivars, the 1.5-kb fragment was observed only in nonaromatic cultivars (Fig. 3). Therefore, the primer may be used to discard nonaromatic rice from the aromatic germplasm.

Cloning of the RAPD fragment

The 1.5-kb fragment was labeled and used for hybridization against digested DNA samples from the two parental lines and some selected F_2 progenies. RFLP scoring was difficult due to the complexity of the banding pattern. The 1.5-kb fragment was digested into 1.0- and 0.5-kb fragments by using *NcoI*. The 0.5-kb fragment (jasmine 500) gave a very clear RFLP banding pattern whereas the larger one (1.0 kb) gave a more complex RFLP pattern (data not shown). When screening DNA samples from the 81 F_2 plants using the jasmine clone as probe, the heterozygous loci could be differentiated from the dominant homozygous loci (Fig. 4). Tightly linked markers are needed to locate the aroma loci precisely and for marker-aided selection. We are now generating a doubled haploid population from CT9993/KDML 105 for mapping the aromatic loci and jasmine marker.

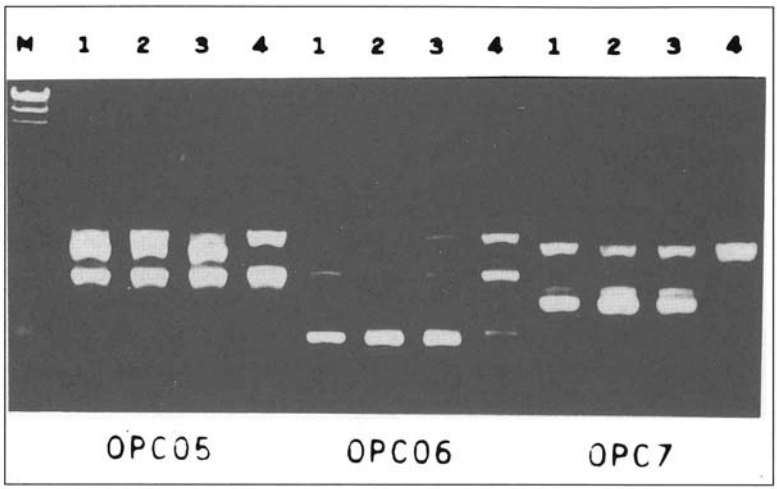

Fig. 1. Analysis of DNA patterns generated by random primers on an agarose gel against DNA samples from parents (lanes 1 and 4) and F_2 bulk populations (lanes 2 and 3). The 1.5-kb band, generated by Opc-06, was specific to nonaromatic genotypes in lanes 3 and 4. Lanes 1-4 are DNA samples from KDML 105, bulk aromatic F_2s, bulk nonaromatic F_2s, and CT9993. The marker (M) is lambda DNA cut with *Hin*dIII.

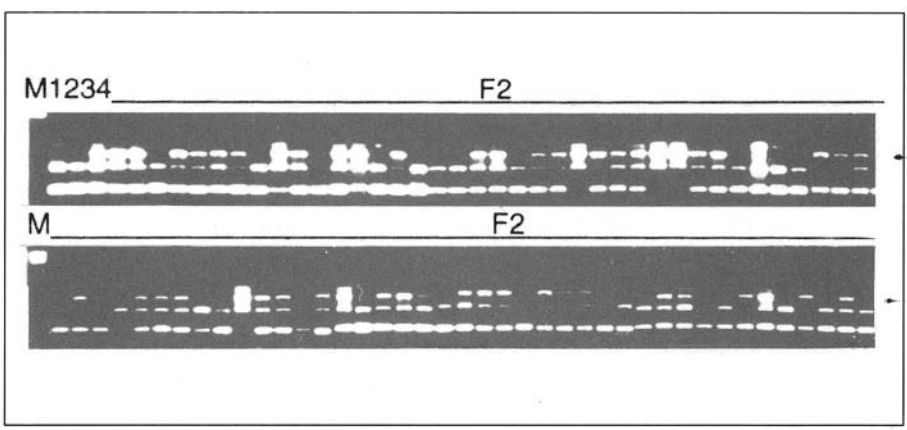

Fig. 2. Segregating patterns of amplified DNA using Opc-06 on an agarose gel from KDML 105 (lane 1), aromatic bulk (lane 2), nonaromatic bulk (lane 3), CT9993 (lane 4), and 81 F_2 individuals. The 1.5-kb fragment (shown by an arrow) was amplified from most of the DNA samples from nonaromatic F_2s. M=lambda *Hin*dIII.

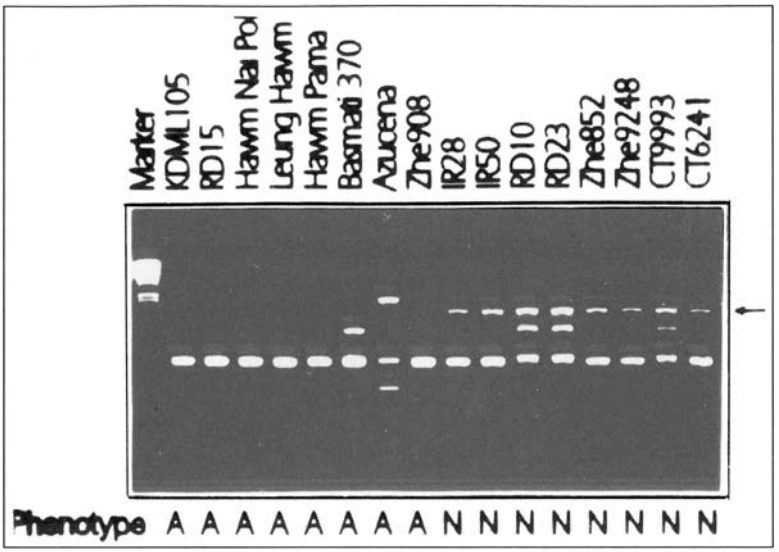

Fig. 3. RAPD patterns for the primer Opc-06 using DNA samples from a set of aromatic rice lines separated on an agarose gel. For the aromatic scorings, see Figure 2.

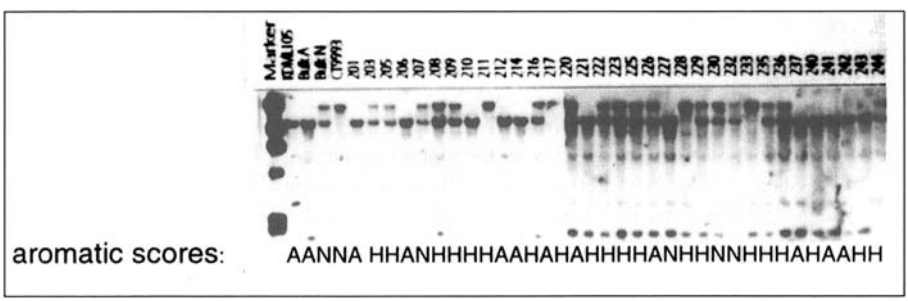

Fig. 4. Southern blot analysis of jasmine probe hybridized against *Bgl*II-restricted genomic DNA from F_2s derived from KDML 105/CT9993. The marker lane is lambda *Hind*III. Bulk populations A and N are DNA samples pooled from different F_2s with aromatic and nonaromatic scores, respectively. The three-digit numbers are individual F_2 samples.

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Quantitative trait locus analysis using recombinant inbred lines and restriction fragment length polymorphism markers in rice

H. Tsunematsu, A. Yoshimura, M. Yano, and N. Iwata

Five quantitative traits—heading date (HD), culm length (CL), panicle length (PL), grain length (GL), and grain width (GW)—were analyzed by using 71 rice recombinant inbred (RI) lines and 386 restriction fragment length polymorphism markers in different rice plots in 1993 and 1994. Chromosomal location of quantitative trait loci (QTLs) and their effects were estimated. For HD, three QTLs were detected on chromosomes 3, 6, and 8. It was suggested that the QTL identified on chromosome 6 was associated with photoperiod sensitivity. Also located were two QTLs for CL on chromosomes 1 and 4; four QTLs for PL on chromosomes 2, 3, 4, and 6; two QTLs for GL on chromosomes 3 and 7; and two QTLs for GW on chromosomes 5 and 9. The locations and effects of these QTLs must be confirmed by further experiments using near-isogenic lines or chromosomal segment substitution lines.

Since agriculturally important traits such as culm length and panicle length are, in most cases, quantitatively measured, it is important to analyze quantitative trait loci (QTLs) from a genetics and rice breeding viewpoint. Efforts have been made to analyze QTLs using a statistical approach. However, the number of the QTLs and their chromosomal locations is sketchy at best.

Recently, molecular marker technology has made it possible to obtain numerous genetic markers to aid in QTL analysis. Subsequently, studies of QTLs using molecular markers are now being conducted (Paterson et al 1988).

Recombination inbred (RI) lines have a number of advantages in QTL analysis. Because RI lines constitute a permanent population in which all members are homozygous, they can be evaluated under different environmental conditions and the dominance effect can be ignored. Since a genotype is represented by a line rather than by an individual, the error of measurement of the traits can be minimized.

RI lines were developed from the cross Asominori/IR24 and a restriction fragment length polymorphism (RFLP) linkage map was established. which contained 114 RFLP loci (Tsunematsu et al 1993) and 272 RFLP markers (Kurata et al 1994). The RI lines show phenotypic variations in many traits. In this study, QTL analyses of heading data (HD), culm length (CL), panicle length (PL), grain length (GL), and grain width (GW) were done using RI lines and segregation data of the RFLP markers.

Materials and methods

Plant material

Seventy-one RI lines derived from a cross between japonica variety Asominori and indica variety IR24 were used for QTL analysis. In 1993. F_7 RI lines were planted at Kyushu University (Fukuoka), Japan, in two plots sowed at different times—2 May and 27 May. The same lines were also planted at the Hokuriku National Agricultural Experimental Station (Joetsu), Japan, on 14 Apr. In 1994. F_8 RI lines were planted at Fukuoka following the 1993 planting pattern. To study photosensitivity, plots for short-day treatment (SDT) (9 h daylength) and long-day treatment (LDT) (16 h daylength) were established.

Assessment of traits

Twelve plants in each line were monitored for HD in 1993 and 1994. Five plants each were monitored in the SDT and LDT plots. Five individual plants in each line were measured for CL and PL in which the longest culm within a plant was used for measurement. Twenty F_7 seeds were measured for GL and GW. The mean of each line for each trait was used in the data analysis.

Estimation of QTL

To estimate the location of the QTLs on the RFLP linkage map, the RI lines were divided into two genotypic groups, Asominori homozygotes and IR24 homozygotes, at each RFLP locus. The *t* test was used to determine if the means of the two groups were significantly different ($P<0.01$). When a significant difference at the 1% level was detected in more than two plots, the QTL was located at the position of the RFLP marker. Allelic effect was estimated as the mean difference between the two groups. The allelic effect for each QTL was shown as the difference between the mean of the IR24 group and the mean of the Asominori group.

Results and discussion

Trait variation

The five traits (HD, CL, PL, GL, GW) evaluated at Kyushu University in 1993 showed continuous variation (Fig. 1). In the other plots, the variations for all traits were continuous as well.

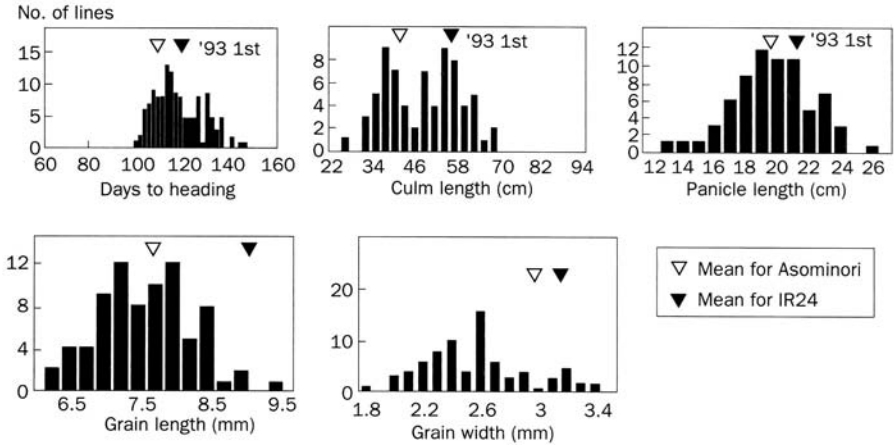

Fig. 1. Frequency distributions for five traits observed at Kyushu University.

Chromosomal locations and effects of the QTLs

Three QTLs for HD were located on chromosomes 3, 6, and 8 (Fig. 2). The QTLs on chromosomes 6 and 8 had alleles exhibiting effects opposite to those predicted by the parental phenotypes. The LDT and SDT analyses suggested that the QTL identified on chromosome 6 was associated with photosensitivity. The QTL on chromosome 8 was detected in all experimental plots. The stability of the gene expression under different environmental conditions should be noted. Li et al (1995) identified the QTLs for HD on chromosomes 3 and 8 through a QTL analysis using a different F_2 population. The region affecting HD on chromosomes 3 and 8 is not likely to be cross-combination specific. Mapping and near-isogenic line (NIL) development for the QTLs on these chromosomes are under way to analyze them more precisely.

Two QTLs for CL were identified on chromosomes 1 and 4 (Fig. 2). The QTL detected on chromosome 4 had alleles with effects that were opposite those predicted by the parents. The IR24 parent carries the semidwarf gene *sd1* and the RFLP marker XNpb93 is tightly linked to *sd1* (Ogi et al 1993). Therefore, the QTL detected on chromosome 1 was due to the effect of *sd1*.

Four QTLs for PL were identified on chromosomes 2, 3, 4, and 6 (Fig. 2); two QTLs for GL were located on chromosomes 3 and 7; and two QTLs for GW were identified on chromosomes 5 and 9.

The locations and effects of these QTLs will be confirmed through further experiments using NILs or chromosomal segment substitution lines.

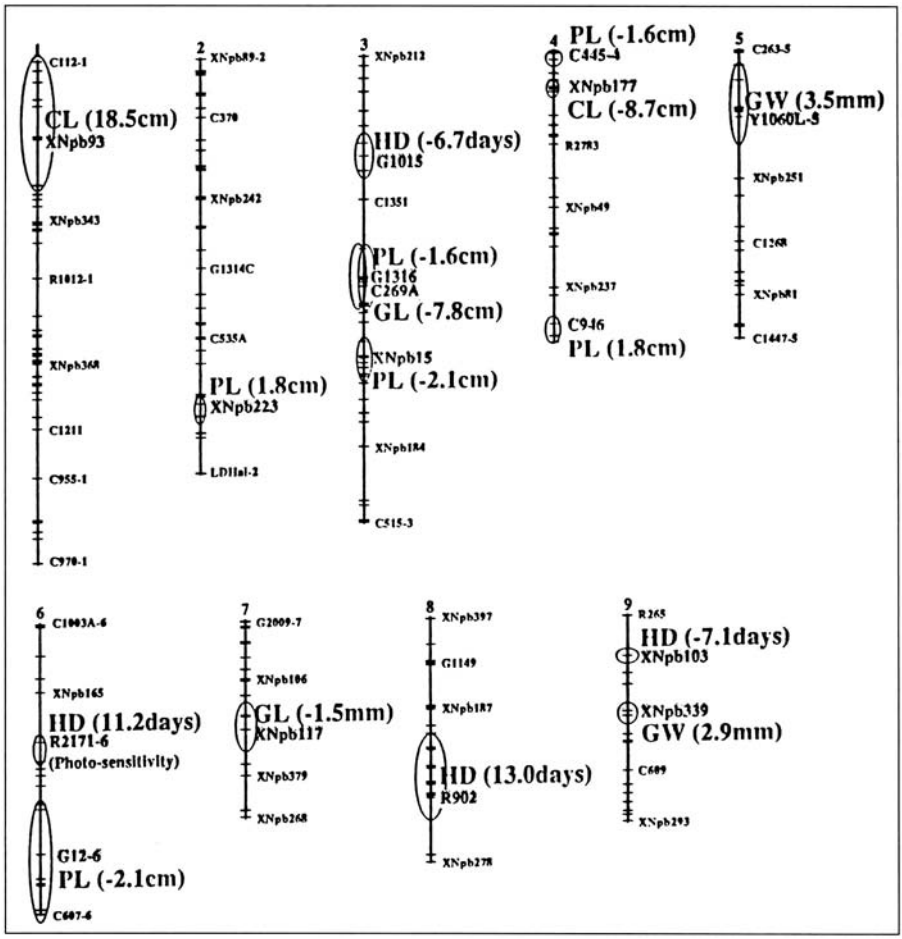

Fig. 2. The QTLs for heading date (HD), culm length (CL), panicle length (PL), grain length (GL), and grain width (GW) detected in RI lines. Circles indicate the location of QTLs estimated in more than two plots. Circle size indicates the relative magnitude of the gene effect. Shaded RFLP markers are located near each QTL. The allelic effect for each QTL is shown as the mean difference calculated by subtracting the mean of the Asominori group (japonica) from that of the IR24 group (indica).

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Notes

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Mapping of quantitative trait loci and quantitative-trait-modifying factors affecting three grain yield components in rice (*Oryza sativa* L.)

Z. Li, S.R.M. Pinson, W.D. Park, A.H. Paterson, and J.W. Stansel

The genetic basis for three grain yield components of rice *(Oryza sativa* L.)—1,000 kernel weight (KW), grain number per panicle (GN), and grain weight per panicle (GWP)—was investigated using 115 well-distributed restriction fragment length polymorphism markers and F_4 progeny testing from a cross between two commercial rice varieties Lemont (japonica from the southern United States) and Teqing (indica from China). Following identification of 16 quantitative trait loci (QTLs) affecting these traits, we sought to investigate the role of complementary epistatic loci affecting these phenotypes. Approximately 18-fold more statistically significant ($P < 0.001$) digenic interactions were found than would be expected from chance alone. Many quantitative trait-modifying factors (QTMFs) distributed throughout the genome appeared to be involved in digenic interaction. QTMFs acted primarily in a complementary manner. The low-heritability traits, GN and GWP, appeared to be more greatly influenced by epistasis than the highly heritable trait KW. Our results suggest that QTLs are those loci with relatively large phenotypic (quantitative) effects and/or those that act largely independent, and QTMFs are loci that contribute to quantitative variation through epistasis. The identification of QTMFs is a step toward resolution of discrepancies between QTL mapping and classical genetic dogma, contributes to better understanding of the maintenance of genetic variation for quantitative traits in populations, and impels reconsideration of optimal mapping methodology and marker-assisted breeding strategies for improvement of complex traits.

Epistasis, or interactions between nonallelic genes, is an important factor that affects phenotypic expression of genes and genetic variation in populations. Gene interactions are the core of Wright's theory of the genetic basis for evolution (Wright 1932, 1951) and play a key role in founder effect models of speciation (Templeton 1980). In

an effort to understand the role of epistasis in determining variation of complex quantitative traits, we investigated the influence of epistasis on three grain yield component traits of rice using replicated progeny testing and a virtually complete restriction fragment length polymorphism (RFLP) linkage map. Our primary objectives were to reassess the prevalence of digenic interactions, identify specific genomic regions involved in digenic interactions, and study patterns by which alleles at two loci interact with measured gene actions of quantitative trait loci (QTLs).

Materials and methods

Plant materials

Oryza sativa L. cultivars Lemont (japonica from the southern United States) and Teqing (indica from China) were used as parents. From each of 255 F_2 plants from the Lemont/Teqing cross, 7-11 (10 in most of the cases) F_3 plants were randomly selected and produced 2,418 F_2-derived F_4 lines for the phenotyping experiment, as described previously (Li et al 1995).

RFLP marker genotyping and the field experiment

Genotyping of the original 255 F_2 plants for 113 RFLP and two morphological markers (*C* and *gll*) was described previously (Li et al 1995). The resulting 115 loci were spaced at an average 19.1 cM across the 12 rice chromosomes. Phenotypic analysis of the 2,418 F_4 lines was conducted at Texas A&M University System Agricultural Research & Extension Center (Li et al 1995). Ten to thirteen panicles, each from a different plant in one of the F_4 lines were collected and dried at 50 °C for 72 h. Panicles from each of the F_4 lines were hand-threshed and assayed for the number of grains per panicle (GN), 1,000 kernel weight (KW, two samples of 200 grains were weighed and converted to 1,000 grain weight in grams), and grain weight per panicle (GWP). The "breeding values" of each of the 255 F_2 plants for these traits were obtained by averaging F_4 lines and used in the data analyses.

Data analyses

Mapping of QTLs used interval mapping (Lander and Botstein 1989). A log of odds (LOD) score ≥ 2.4 was used as a threshold for claiming the presence of QTLs and genomic regions with $2.4 > \text{LOD} \geq 2.0$ are noted as putative QTLs. All possible (4,465) two-way ANOVAs between 95 selected codominant markers were performed using SAS PROC GLM (SAS Institute Inc. 1987). The statistic used to detect significant digenic interactions was $pF_{\text{interaction}} \approx p\text{Ms}_{\text{interaction}} / \text{MS}_{\text{residual}}$ (Haley and Knott 1992). The digenic parameters t_{ij} involved in statistically significant interactions were estimated using the method of Graybill (1976). A *t* test was performed to determine if individual $t_{ij} = 0$ (due to chance) using $(s^2_e/n_{ij})^{1/2}$ as standard errors, where s^2_e, the error variance, was approximated by averaging the 4,465 observed s^2_{ij} and n_{ij} was the observed sample size of individual digenic genotypes.

When there were r independent QTLs segregating in the population with respective effects a_i ($i = 1,2,3,...r$), the expected genotypic value for traits arising from all

QTLs of an F_2 plant with a specific digenic genotype at any two markers I and J can be expressed as $G_{ijm} = Sa_i$. The expected genotypic value of the n_{ij} F_2 plants with the same digenic genotype is $G_{ij} = (1/n_{ij})\ SG_{ijm}$ with a standard error of $S_G = (S^2_{Gijm}/n_{ij})^{1/2}$ ($m = 1, 2, 3 \ldots n_{ij}$). Then, the *t* test, $t = (t_{ij} - G_{ij})/S_G$ ($df = n_{ij} - 1$), was used to examine if each statistically significant interaction was due to sampling variation in the segregating QTLs.

Results

Mapping of QTLs affecting KW, GN, and GWP

Sixteen QTLs contributing to KW, GN, and GWP were identified in this population and mapped to seven of the 12 rice chromosomes (Fig. 1). An additional seven genomic regions showed effects on these traits, which fell slightly below the stringent significance threshold ($2.4 > LOD \geq 2.0$).

Detection of digenic epistasis

Of 4,465 possible two-way ANOVAs between the 95 markers, the observed numbers of statistically digenic interactions for the three traits were much more than expected by chance (Table 1). After removing those due to linkage and nonrandom sampling of the identified QTLs, there were 14, 40, and 49 highly significant interactions between unlinked markers for KW, GN, and GWP, respectively. Decomposition of these interaction variances indicated that the additive component arising from double homozygous individuals (approximately 20% of the population) explained more than 65% of the total interaction variances for the three traits. Consistent results were obtained from *t* tests in which there was at least one significant additive epistatic *(aa)* effect in each of these interactions, but none of the nonadditive epistatic effects were significantly different from zero.

Magnitudes and characteristics of additive digenic epistatic *(aa)* effects

The mean R^2s of the 14, 40, and 49 interactions affecting KW, GN, and GWP were similar to those of the identified QTLs described previously. For KW, the mean R^2 explained by 14 interactions was 6.40%, ranging from 5.29 to 7.75%, which was slightly higher than the averaged R^2 (6.0%, obtained by one-way ANOVA) explained by the 8 KW QTLs. The average magnitudes of the 26 *aa* effects were 1.66 ± 0.53 g, equivalent to the doubled mean additive effects (1.62 ± 0.11 g) of the QTLs. For GN, the mean R^2 explained by 40 interactions was 6.11%, ranging from 3.34 to 12.46%, which was slightly smaller than the averaged R^2 (6.77%) explained by the 6 GN QTLs. The mean magnitude of the 80 *aa* effects was 15.5 ± 6.5, slightly smaller than the doubled mean additive effects of the QTLs (16.6 ± 4.1). For GWP, the mean R^2 explained by 49 interactions was 5.95%, ranging from 4.03 to 8.99%, which was slightly lower than the averaged R^2 (6.5%) explained by the two GWP QTLs. The mean magnitude of the 88 *aa* effects was 3.28 ± 1.3 g, slightly lower than the doubled mean additive effects of the two QTLs (3.50 ± 0.08 g).

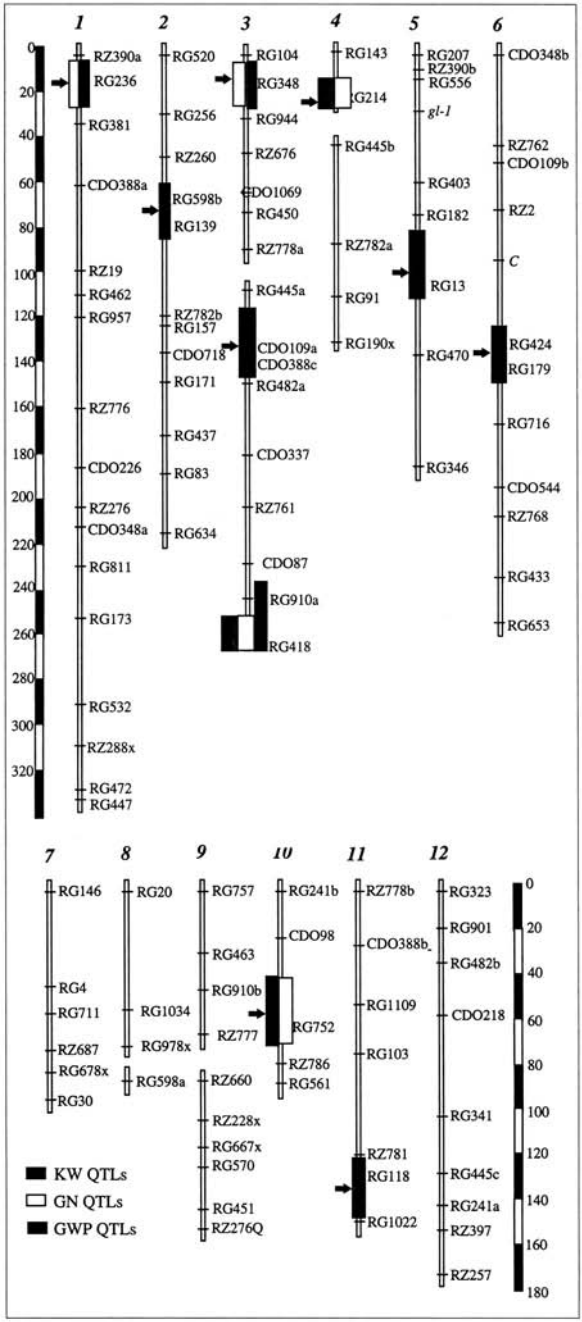

Fig. 1. The RRP map with 115 markers constructed from 225 F_2 plants of the Lemont/ Teqing rice cross. The boxes indicate the genomic locations (1 LOD confidence interval) for 16 QTLs identified for KW, GN, and GWP.

Table 1. The observed number and proportions of statistically significant interactions between 4,465 random RFLP marker pairs in the rice genome for KW, GN, and GWP in the Lemont/ Teqing cross.

Trait	P £ 0.05		P £ 0.01		P £ 0.001		P £ 0.0001	
	No.	%	No.	%	No.	%	No.	%
KW	561	12.57	253	5.67	87	1.95	20	0.45
GN	526	11.78	222	4.97	79	1.77	24	0.54
GWP	556	12.46	240	5.38	79	1.77	15	0.34
Average	547.7	12.27	238.3	5.34	81.7	1.83	19.7	0.44
Expected[a]	223.2	5.00	44.6	1.00	4.5	0.1	0.45	0.01
Obs./Exp.		2.5		5.3	1	8.2		43.8

[a] The number of significant interactions expected to be caused by chance alone.

The interactions between alleles from the same parents (the parental types) tended to result in increased fitness (GN and GWP) while interactions between alleles from different parents (the recombinants) tended to result in reduced fitness, which provided a reasonable explanation for the overall reduced fitness observed in the progeny of the Lemont/Teqing cross.

Discussion

Our results indicate that a substantial portion of the genetic variance inexplicable solely by QTLs may be due to epistasis. Moreover, "main effects" of individual QTLs may be somewhat modified, as a result of epistatic relationships beyond the resolution of many data (Paterson et al 1988, Tanksley and Hewitt 1988, de Vicente and Tanksley 1992, Edwards et al 1992, Stuber et al 1992). It should be pointed out that our results were based on the analyses of only digenic interactions on the three selected traits.

Distinction between QTLs and QTMFs

We proposed a new term—quantitative trait-modifying factors or QTMFs—to distinguish those loci that affect quantitative traits through epistasis, from traditional QTLs. To better demonstrate the differences between QTLs and QTMFs, a comparison was made between the summarized results from QTL mapping and the interaction analyses (Table 2). It appears that complex traits such as GN and GWP are indeed determined by greater number and more complicated epistatic loci. QTLs identified using one-way ANOVAs or interval mapping appeared to be those loci that have relatively large effects and/or act largely independently of other genes in the target genetic backgrounds and the environment. Fifty percent of the identified QTLs were involved in digenic interactions with QTMFs (background loci). Thus, the usual estimates of "main effect" of a QTL can be confounded by interactions.

The majority (90.4%) of the loci involved in the digenic interactions do not have significant main effects" on the three grain yield components. Thus, QTMFs may be

Table 2. Comparison of results from QTL mapping and analyses of digenic interactions for kernel weight (KW), grain number per panicle (GN), and grain weight per panicle (GWP) in the Lemont/Teqing cross.

Results	KW	GN	GWP
QTL mapping			
Estimates of heritability (h^2)	0.877	0.535	0.413
No. of identified QTLs	8	6	2
Mean R^2 (%) explained by individual QTLs	6.0	6.8	6.5
R^2 (%) collectively explained by all QTLs	82.9	36.5	12.5
Standardized mean doubled additive effect of QTLs	2.4	0.8	0.3
Analyses of digenic interactions			
No. of digenic interactions detected	14	40	49
Mean R^2(%) explained by individual interactions	6.4	6.1	6.0
No. of QTLs interacting with background loci	4	3	1
% of interactions between QTLs	0	0	0
% of interactions between QTLs and QTMFs	42.9	30.0	2.0
% of interactions between QTMFs (non-QTL)	57.1	70.0	98.0
No. of minimum putative QTMFs involved	25	45	55
Mean multiepistativity (ME) of putative QTMFs[a]	1.12	1.78	1.78
No. of putative QTMFs with ME > 2	0	9	11
Standardized mean additive epistatic effect	2.7	0.7	0.5

[a] The multiepistativity is defined as the number of other loci with which a QTMF interacts.

appropriately defined as loci that contribute to quantitative trait variation through epistasis and are detectable by two-way or multiway ANOVAs (two-dimensional or multidimensional interval mapping). Our results indicate that reduced fitness (yield) of the progeny from intersubspecific rice crosses may be due to the breakdown of the coadapted indica and japonica gene complexes, which are maintained by strong epistatic selection.

Implications for evolution and plant breeding

Many QTMFs affecting complex quantitative traits in a predominantly complementary manner have two important implications in evolution and plant breeding. First, prevalence of complementary loci affecting complex quantitative traits in rice implies that classification of alleles as “favorable” or “unfavorable” may be misleading since the effect of an allele may be positive, neutral, or negative depending on interactions with other loci, and on the environment. In practice, phenotypic similarity between parents may provide little information regarding the loci contributing to genetic variation of quantitative traits in their progenies. Thus, in future mapping and breeding efforts to improve complex traits such as yield, emphasis should be placed on identification and selection of “best” multilocus combination(s). Second, epistasis may have played an important role in maintaining genetic variation for quantitative traits in self-pollinated plant populations (Carson and Templeton 1984).

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Using doubled haploid populations of rice for quantitative trait locus mapping

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Doubled haploid (DH) populations derived from anther culture have many advantages for quantitative trait locus (QTL) mapping because of their perfect homozygosity and fixed genetic background. The phenotypic values for the quantitative traits can be precisely evaluated through repeated trials and the genotype-by-environment interaction can be easily studied by using DH populations. Two DH populations were established in this study. One was from indica rice variety Zhai-Ye-Qing 8 and japonica variety Jing-Xi 17. One hundred and thirty-three DH lines were evaluated for many important agronomic traits by growing in three locations. Using a restriction fragment length polymorphism map with 167 markers spaced 12.8 cM in average, 20 genomic regions were identified that affected six agronomic traits. Among these QTLs, eight were detected in all three environments, seven in two environments, and five in one environment. Another DH population was produced to identify chromosomal regions affecting fertility restoration for wild abortive (WA) cytoplasm. Eighty-one DH progeny from a cross between indica restorer line Gui 630 and japonica variety 02428 were evaluated for their restoring abilities. Four genomic regions on linkage groups 2, 3, 4, and 5 were found to be associated with fertility-restoring ability to WA cytoplasm.

Restriction fragment length polymorphisms (RFLPs) have been used in many plant species to dissect genetic factors underlying quantitative traits in segregating populations. Doubled haploid (DH) populations derived from anther culture offer a particular advantage for quantitative trait locus (QTL) mapping because all alleles in the population are fixed and homozygous, thus, the phenotypic value of each line is free of dominant effects and can be precisely evaluated through repeated trials in diverse environments and experimental conditions. In addition, the genotype-by-environment (G×E)interaction can be studied using DH populations. In this study, we have established two DH populations by anther culture and have identified a number

of QTLs for some important agronomic characters. The effects of different environments on QTL mapping were also studied through testing in three diverse environments.

Materials and methods

Two DH populations were used in this study. One population consisting of 133 DH lines was derived from the cross between indica variety Zhai-Ye-Qing 8 (ZYQ) and japonica variety Jing-Xi 17 (JX). The parents differed significantly in many agronomic characters including heading date, plant height, 1,000-grain weight, number of panicles plant^{-1}, number of spikelets panicle^{-1}, and number of grains panicle^{-1}. Phenotypes were evaluated for each of 10 plants from 133 DH lines by growing them in three diverse environments (1ocations)—Beijing (located 40° N), Hangzhou (located 32° N), and Hainan (located 18° N). Another DH population was used to identify QTLs for wild abortive (WA)-type cytoplasm fertility restoration. Eighty-one DH progeny were derived from a cross between indica restorer line Gui 630 and japonica variety 02428. The restoring abilities of the parents and their DH progeny were determined by test-crossing five individual plants of each line with Zhen Shan 97 A.

RFLP maps were constructed based on segregation data of the two populations. All procedures were the same as described by Causse et al (1994). The genome constitutions of the DH populations were estimated by using the computer program HyperGene (Young and Tanksley 1989). DNA markers were mainly provided by Dr. S.D. Tanksley from Cornell University and the Rice Genome Research Program of Japan. Other markers were prepared in our laboratory.

Mapping of the QTLs and estimation of their genetic effects were performed according to the interval mapping method (Lander and Bostein 1989) using MAPMAKER ver.1.1 software. Due to the large number of markers tested and the sparse linkage map, an extremely high log of odds (LOD) score threshold (3.0) was used to declare the presence of a significant QTL, but for comparative QTL mapping, we also considered LOD scores between 2.0 and 3.0 as "suggestive."

Results and discussion

Comparative QTL mapping in diverse environments

G×E interaction is an important aspect for the assessment of genetic effects as well as the prediction of performance in breeding programs because genotypic values must be inferred from phenotypic responses. Previous studies of QTL mapping were conducted mostly in fixed environments. Paterson et al (1991) and Stuber et al (1992) studied G×E interaction by growing F_3 or backcross families in different environments. Here, a permanent DH population was used to discern the degree of G×E interaction at individual QTLs by comparing QTL maps generated in three diverse environments.

An RFLP linkage map was constructed by 167 markers genotyped for 133 ZYQ/JX DH lines. This map covers all 12 rice chromosomes with an average spacing of 12 cM. The genome ratios of the ZYQ genome of each DH line varied between 0.17

and 0.83, averaging 0.496. Of the mapped markers, 47 (28.5%) showed significant deviation ($P < 0.05$) from the expected 1:1 segregation ratio. Most of these markers favored the JX alleles.

Interval mapping identified a total of 20 genomic regions affecting the following traits: heading date, plant height, 1,000-grain weight, spikelet number panicle^{-1}, number of grains panicle^{-1}, and seed set percentage. These QTLs accounted for 8.3-36.3% of the phenotypic variance in a trait and showed different gene effects (Table 1). Among these 20 QTLs, eight were detected in all three environments, seven in two environments, and five only in one environment. Of the eight QTLs detected in all the three environments, six were for spike characters: two for grain weight, two for spikelet number, and two for grain number. The QTLs only detected in one location were those affecting heading date (*hd1*), plant height (*ph3, ph10*), and seed set percentage (*ssp5, ssp7*). Interestingly, they were all detected in Hainan. This result is not surprising because these traits are more sensitive to environments. In contrast, environments have only small effects on other traits such as grain weight, grain number panicle^{-1}, and spikelet number panicle^{-1}. This suggested that marker-assisted selection for these traits will have equal effects across locations.

Identification of QTLs for fertility restoration

Fertility restoration for cytoplasmic genetic male sterility (CMS) underlies the successful hybrid rice technology in China. Genetic analysis of fertility restoration, therefore, will be of great value to rice improvement programs. The most widely used CMS lines were derived from wild indica rice with aborted pollen (WA type). Genetic studies of fertility restoration for this type of CMS have suggested that it was governed by two independent and dominant genes, *Rf1* and *Rf2,* and the continuous variation was attributed to the collective action of quantitative genes and environments (Virmani et al 1986). In this research, we have identified several QTLs associated with WA cytoplasmic fertility restoration.

The parents of the DH population differed significantly in WA fertility-restoring ability. When they were test-crossed with Zhen Shan 97 A, which represents the WA-type cytoplasm, the seed set percentage of the F_1 was 77.01% for Gui 630 and 0% for 02428. Five plants from each of the 81 lines were test-crossed with Zhen Shan 97 A; the seed set percentages showed continuous variation among the DH lines, ranging between 1.11 and 85.7%. These results predicted that major QTLs for restoring WA and BT cytosterilities could be identified.

An RFLP map with 233 markers was constructed based on this DH population. The average distance between adjacent markers was 9 cM. Skewed segregation ($P < 0.01$) was detected for 106 of the 233 markers, comprising 20 distinct regions distributed on 11 chromosomes other than chromosome 9. Nevertheless, the numbers of the markers that deviated toward the male and female parents were nearly equal, and the average proportion of "Gui630" genome was 49% in the population.

Four genomic regions were identified in linkage groups 2, 3, 4, and 5 with significant effects on fertility-restoring abilities to WA sterility (Table 2). Each of these regions accounted for 20.5-49.6% of the total phenotypic variation. The multiple

Table 1. Biometrical parameters of individual QTLs for some important traits in three-environment trial of ZYQ/JX DH population.

Locus[a]	Marker interval	Trial[b]	LOD score	Phenotypic variation (%)	Additive effect
hd1	C385-C949	HN	3.66	13.4	-5.72
hd8	RG885-BP127A	BJ	7.65	36.3	14.26
		HZ	6.02	34.7	7.95
hd10	G1084-G1125	HN	5.16	18.0	6.69
		HZ	3.97	18.4	5.72
ph3	G62-G144	HN	3.68	17.8	-8.47
ph4	RG214-C513	BJ	2.55	17.1	10.82
		HN	3.71	17.3	8.62
		HZ	3.16	19.4	11.34
ph8	RG885-BP127A	BJ	4.39	25.0	13.2
		HZ	2.59	11.3	8.63
ph10	G1082-G291	HN	3.96	17.9	8.48
gw1	C112-C225	BJ	3.45	13.8	2.12
		HN	3.20	15.9	0.46
		HZ	4.22	18.5	2.48
gw2	G1314A-G243A	BJ	4.27	19.0	2.49
		HN	3.27	17.0	0.48
		HZ	2.37	12.1	2.02
gw3	G164-RG756	HN	5.19	20.8	0.52
		HZ	2.24	9.2	1.75
gw6	C235-RG445B	BJ	2.37	8.3	1.69
		HN	3.00	11.5	0.40
gw8	RG885-BP127A	BJ	3.42	17.4	2.40
		HZ	4.14	15.6	2.35
spn4	RG620A-RG214	BJ	4.19	19.7	40.95
		HN	4.45	19.0	30.68
		HZ	5.12	24.5	38.70
spn6	G122-G1314B	BJ	4.14	13.4	-34.93
		HN	2.81	13.8	-26.86
		HZ	5.83	25.1	-39.92
gn4a	RG620A-RG214	BJ	2.66	12.9	35.97
		HN	4.65	22.0	34.70
		HZ	4.90	28.8	32.04
gn4b	C513-G271	HN	6.89	28.7	40.73
		HZ	4.89	22.7	29.12
gn6	G294-G122	BJ	3.61	13.0	-37.63
		HN	4.56	19.4	-33.06
		HZ	4.53	25.9	-30.37
ssp4	G271-C975	BJ	2.63	10.0	12.10
		HN	5.08	22.7	23.58
		HZ	3.38	17.9	13.55
ssp5	G366-C624	HN	3.84	13.9	19.35
ssp7	RG650-C285	HN	3.08	13.2	17.62

[a]QTLs are named after trait abbreviations and chromosome number. Trait abbreviations: heading date (hd), plant height (ph), 1,000-grain weight (gw), number of spikelets panicle^{-1} (spn), number of grains panicle^{-1} (gn), seed set percentage (ssp). [b]HN=Hainan, BJ=Beijing, HZ=Hangzhou.

Table 2. Biometrical parameters of putative QTLs affecting fertility restoration for WA-type cytosterility.

QTL[a]	RFLP markers flanking the QTL	Chromo-some	Maximum LOD score	Phenotypic variation (%)	Additive effects
Rfi2	RZ404C-RG241B	2	3.50	20.5	26.37
Rfi3	RG69A-RG413	3	4.44	49.6	33.34
Rfi4	C22-RG449D	4	4.85	35.4	29.55
Rfi5	RG435-RG172A	5	3.33	28.2	22.43
Multiple QTL model			7.43	70.2	

[a]QTLs are named after trait abbreviation and chromosome number.

QTL model indicated that these QTLs collectively accounted for 70.2% of the total variation for fertility-restoring ability to WA sterility. In each case, "Gui630" alleles increased seed set percentage. This result agreed with the fact that the restoring ability of the parents differed significantly: Gui 630 was a strong WA cytoplasm restorer while 02428 had no restoring ability.

The numbers of QTLs for restoring WA cytoplasm reported here must be considered a minimum estimate. The presence of further QTLs, most likely with smaller effects and lower LOD scores just below the threshold, has to be assumed. For example, the regions near G30 on chromosome 6 and G148 on chromosome 12 may be real QTLs for restoring WA cytoplasm. These regions merit further attention in larger populations.

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Molecular mapping of drought resistance traits in rice

R. Chandra Babu, H.G. Zheng, M.S. Pathan, M.L. Ali, A. Blum, and H.T. Nguyen

Drought stress is a major constraint to rice production and yield stability. In most breeding programs, the genetic improvement of adaptation to drought conditions is addressed through the conventional approach, which involves selection for yield and progeny testing for yield and its stability over locations and years. Because of the low heritability of yield under stress and the inherent spatial and temporal variations in the field stress environment, such selection programs are expensive and slow in attaining progress. Significant developments were recently achieved in understanding the physiology of drought resistance and in developing physiological screening techniques for drought resistance, which may reduce the extent of yield testing in selection programs. However, the assessment of drought resistance in selection work by measuring the phenotypic physiological response to environmental stress entails tedious procedures and is still prone to problems of repeatability due to environmental variability. Recent advances in plant genome mapping offer new opportunities for breeding for stress environments. Molecular markers should allow breeders to track genetic loci controlling drought resistance without having to measure the phenotype, thus reducing the need for extensive field testing over time and space. Progress has been made in identifying quantitative trait loci associated with osmotic adjustment and root characteristics as major drought resistance components in rice. It is anticipated that marker-assisted selection soon will be possible for these traits.

Rice is grown under a broad range of environmental conditions in terms of topography, soil type, water regime, and climate. About one quarter of the world's rice crop, extending over 41 million ha, is cultivated in areas classified as rainfed lowland (Khush 1984). In these areas, farmers generally do not have access to irrigation, and yields may be seriously reduced due to drought caused by unpredictable and insufficient

rainfall patterns. For instance, in the rainfed lowland ecosystem of eastern India, farmers identified drought stress as the foremost constraint to higher yields (Widawsky and O'Toole 1990).

Future increases in production area will come mostly from rainfed lowlands. These areas may experience frequent (intermittent) and severe (prolonged) water deficits at any time during the rice growing cycle. More than 8.5 million ha of rice are currently grown in highly drought-prone upland areas in South and Southeast Asia alone, with almost no surface water accumulation (Herdt 1991). Hardpans in the soil can block root growth and impede water extraction in rainfed lowland areas and even in irrigated fields. Drought, therefore, is the most important abiotic stress that limits rice yields in almost all major ecosystems (Toenniessen 1991).

There are several drought-adaptive mechanisms in the rice germplasm that may be exploited to enhance the crop's ability to establish, grow, develop, and yield during drought (O'Toole and Chang 1979, Fukai and Cooper 1995). However, the adoption of these putative traits in breeding programs has been slow. Among other reasons, the inability to rapidly screen breeding populations for drought resistance mechanisms limits progress toward incorporation of these traits as selection criteria.

Marker-assisted breeding

Recent advances in plant genome mapping and molecular biology techniques offer new opportunities for breeding for stress environments. Several recent reviews have provided excellent discussions on the theory and advantages of using molecular marker-based selection for crop improvement over selection based solely on the phenotype (Tanksley et al 1989, Paterson et al 1991). The majority of agronomically important traits are controlled by several genes known as quantitative trait loci (QTLs). However, superior alleles can be efficiently selected if molecular markers are closely linked to the allele or genomic regions of interest. Selection based on molecular markers alleviates difficulties associated with low heritability, recessiveness, and difficult screening assays. This indirect selection of desirable alleles was the impetus for the development of restriction fragment length polymorphism (RFLP) linkage maps in several crops. High-resolution molecular genetic maps are now available for rice *Oryza sativa* (Causse et al 1994, Kurata et al 1994). RFLP maps have been used to successfully characterize several QTLs in rice and other crop plants.

Current physiological knowledge suggests broadly that drought resistance in rice is most likely dependent on one or more of the following components: 1) moderated water use through reduced leaf area and shorter growth duration; 2) the ability of the roots to provide for evapotranspirational demand from deep soil moisture; 3) the capacity for osmotic adjustment (OA), which allows plants to retain turgor and protect meristems from extreme desiccation; and 4) the control over nonstomatal water loss. In this paper, we report recent progress toward characterization of genetic variation, population development, and molecular mapping of root traits and OA in rice.

Root system

Roots can moderate the effects of drought by increasing the water supply. Individual root characteristics such as thickness, depth of rooting, and the ability to penetrate compacted soils have been associated with drought avoidance (O'Toole and Chang 1979, Yoshida and Hasegawa 1982, Ekanayake et al 1985). O'Toole and De Datta (1986) suggested that increased root depth and root density increase the capacity to extract available soil water and may confer increased drought avoidance in some rice genotypes. Thangaraj et al (1990) reported that during drought, water use is primarily determined by root system density and depth. While exploitation of deeper soil horizons may be beneficial in avoiding drought, water in deeper horizons is often inaccessible due to the presence of compacted soil layers.

Genotypic variation in the ability of rice roots to penetrate compacted soil layers has been reported (O'Toole 1982). Root penetration through compacted soil layers or hardpans has been recognized as an important breeding objective for drought resistance improvement in rice (Hanson et al 1990). However, incorporation of root selection criteria such as root penetration ability into plant breeding programs is difficult due to the lack of reliable and efficient screening techniques and the time-consuming, laborious nature of measuring root characteristics. We have developed a reliable, consistent, and efficient method for screening root penetration ability using a wax layer system to simulate compacted soils (Yu et al 1995). Identifying and mapping molecular markers associated with the root traits will be of use in developing rice varieties better adapted to water-deficit environments.

Using the wax layer evaluation technique as a proxy to compacted soils, six QTLs, including nine RFLP markers (RG324, RG73, RG620, RG476C, RG329, RG653, CDO365, RG118, and RG360) located on five chromosomes (2, 4, 5, 6, and 11) tentatively linked to root penetration ability, have been identified in our laboratory (Ray et al 1995) in a population of CO 39/Moroberekan recombinant inbred (RI) lines. Individually, these QTLs accounted for a maximum of 13% of the variation in root penetration ability. Multiple regression showed that three markers (RG324 on chromosome 2, RG476C on chromosome 4, and RG360 on chromosome 5) accounted for 34% of the variation. However, the population was not an ideal one. The marker segregation is highly skewed toward the CO 39 parent (about 80:20) and this probably limited our ability to map other loci involved in root penetration.

We evaluated 109 doubled haploid (DH) lines of the cross IR64/Azucena for root penetration and related root traits. Using the RLFP data from Dr. Ning Huang of IRRI, seven RFLP markers (RG520, RZ318, RG104, CDO418, RZ617, RG472, and RZ262) located on chromosomes 1, 2, 3, 4, 7, and 8, which together explained 52% of the variation in root penetration ability, have been identified. The two markers (RG520 and RZ318) located on chromosome 2 alone explained 23% of the variation, indicating that chromosome 2 has a major QTL controlling root penetration ability in rice.

The exact mechanism by which some roots are better able to penetrate compacted soils is not well understood, but it may be associated with physiological and morphological changes such as reduced branching, a radial thickening of the root,

and changes in plant growth regulator levels in impeded roots. Identifying the genetic variation in root-related parameters and their genetic control may help to better understand penetration ability in rice roots.

Concurrently, Champoux et al (1995) have identified molecular markers linked to rice root morphology using the RI lines of CO 39/Moroberekan at IRRI. They studied variation in root thickness, root-shoot ratio, root dry weight tiller^{-1}, deep root dry weight tiller^{-1}, and maximum root length. For root thickness, the best three markers (RG214 on chromosome 4, RG197 on chromosome 1, and RZ398 on chromosome 6) explained 56% of the phenotypic variation. One major marker (RG214, on chromosome 4) influencing root thickness is closely linked to the major marker (RG476C) that affects root penetration in the same population, suggesting that there may be a linkage between root thickness and root penetration ability.

In collaboration with scientists at IRRI, we are now working further on another DH population from the cross CT9993/IR62266 to confirm the QTLs for root traits in rice. This population also is well suited for field testing under rainfed lowland conditions.

Osmotic adjustment

Osmotic adjustment as a process of active solute accumulation under drought stress is receiving greater attention as a probable component of drought resistance in crop plants. It has been shown that growth and yield under water-limited conditions can be improved by selecting lines with higher levels of OA in wheat (Morgan 1984), sorghum (Ludlow et al 1990), and barley (Blum 1989). Studies have shown that OA does occur in rice in response to water deficit (Hsiao et al 1984) and there is considerable variation in OA among rice lines (Turner et al 1986). Recent detailed experiment with 12 divergent rice lines conducted in our laboratory confirmed further a large variation in OA under drought stress. The cultivar IR62266 expressed higher OA (1.76 MPa) than the rest of the lines. In spite of the positive influence of OA as a drought tolerance mechanism, the adoption of this trait in breeding programs for crop improvement has been slow due to lack of screening technique for handling a large number of progeny. Screening for OA currently requires complex measurement procedures and is time-consuming. The large genetic variation in OA in rice offers a good possibility for identifying suitable molecular markers for this trait, and eventually for incorporating it as a selection criterion in rice breeding programs.

From the studies involving a sample of RI lines of CO 39/Moroberekan, Liley et al (pers. commun.) identified one major marker (RG1) on chromosome 8, explaining 33% of the variation in OA. They indicated that high OA was generally associated with CO 39 alleles and good root systems with Moroberekan alleles. We are working on the DH population from CT9993/IR62266, whose parents are different in both root characteristics and OA to further identify molecular markers and study the genetic linkage between these traits.

Conclusion

On a global basis, the cumulative drought-induced yield losses in rice rank alongside major pests and diseases. Although the effect of water deficits on rice growth and yield is complex, lack of water is recognized as perhaps the most widespread constraint to increasing rice yields. The need to increase rice productivity is even more pressing in the coming decades and the increase has to come primarily from less favorable production environments such as rainfed lowlands. Efforts to incorporate higher levels of drought resistance into this major food cereal need no justification. Molecular markers should allow breeders to directly track genetic loci controlling drought resistance without having to measure phenotype, thus reducing the need for extensive field testing over time and space. In the long term, these important genes can be isolated using map-based cloning techniques for direct genetic engineering to improve drought resistance in rice.

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RFLP mapping of genes controlling root morphology in an indica/japonica doubled haploid population

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Drought is a major problem for upland rice. Deep and thick roots have been shown to contribute to drought tolerance. Difficult to measure, these traits have not been used in breeding programs. Marker-aided selection could help overcome the phenotyping problem. We evaluated 105 doubled haploid lines derived from an indica/japonica cross in a greenhouse experiment, in a completely randomized design with four replications. We measured root thickness, maximum root depth, root dry weight per tiller, root dry weight below 30 cm per tiller, and root-shoot ratio. We used a restriction fragment length polymorphism map of this population with 135 markers to identify markers linked with the different traits. Markers associated with root morphology were located mostly on chromosomes 1 (same location as the *sd1* gene, which controls the semidwarfism of IR64), 2, 7, and 9. The comparison of the detected quantitative trait loci (QTLs) with another team's results shows that, for each trait, between 1 and 3 QTLs were common between populations. The limited number of common QTLs can be due to allelic differences between populations, lack of polymorphism at the QTL locations, and/or thresholds chosen for the statistical tests.

Drought is a major problem for rice grown under upland conditions. Numerous studies demonstrate that a deep and thick root system, a high deep root-shoot ratio, and a high root length density are useful in increasing the volume of soil exploited by the roots and contribute to drought tolerance in upland rice. For traits as difficult to evaluate as these, marker-aided selection is likely to enhance the efficiency of selection. Progress has been made in identifying restriction fragment length polymorphism (RFLP) markers associated with the genes that control root morphology (Champoux et al 1995). However, it is not known whether a quantitative trait locus (QTL) would be found at the same position in a different genetic background or would show the same magnitude. Not very much is known on the QTL × environment interaction in

rice. The goal of this study is to identify the QTLs controlling root morphology in a doubled haploid (DH) line population derived from an indica/japonica cross and to compare with those found by Champoux et al (1995) in a different indica/japonica cross.

Material and methods

Material

A population of 105 DH lines derived from a cross between the irrigated indica variety IR64 and the upland tropical japonica Azucena (Guiderdoni et al 1992) was used in the experiment. An RFLP map of this population was established by Huang et al (1994) from an initial population of 135 DH lines with 135 RFLP markers well staggered on the 12 chromosomes.

Methods

The DH lines were evaluated in IRRI greenhouse together with the parents in a completely randomized design with four replications of one plant, distributed in two runs of two replications each staggered in time. The plants were grown in well-drained plastic bags set in polyvinyl chloride cylinders 1 m long and 0.2 m diameter filled with fairly uniform sandy loam soil and watered three times a week with 500 ml of Yoshida's culture solution. Thirty-five days after sowing, shoots and roots were separated. The number of tillers and the shoot dry weight were evaluated. The soil column was cut in three pieces (0-30, 31-60, 61-90 cm). The maximum root depth reached by the nodal roots (MRL) was determined. The roots from each section were then carefully washed. The average thickness of five nodal roots 2 cm under the tillering plateau (THK), the root length density, and root dry weight in different sections were measured. The total root weight tiller^{-1} (TRW T^{-1}), the dry weight below 30 cm tiller^{-1} (B30 T^{-1}) and root-shoot ratio (R/S) were derived from these parameters.

Statistical analysis. Analysis of variance (ANOVA) was performed to check the existence of genetic variability for the different traits. Genotypic correlations between traits as well as heritabilities were calculated using standard procedures.

QTL analysis. The QTL controlling the five traits was determined using single marker analysis, well adapted to replicated trials, with a threshold of 0.01. Only incidentally did we use MAPMAKER QTL to refine the QTL location. The comparison between the results obtained with this population and data from Champoux et al (1995) was done using the linkage map established by Causse et al (1994) as a bridge to determine the relative order of the two sets of markers. The probability threshold was set at 0.005 for IR64/Azucena against 0.0001 for CO 39/Moroberekan (due to the extreme bias of this population).

Results and discussion

Statistical parameters

The ANOVA revealed highly significant genotypic effect for all traits. Transgressions in both directions were observed for all traits. Broad-sense heritabilities ranged from 0.58 (R/S) to 0.84 (THK). The genotypic correlations between root dry weight and root length densities in the different horizons were extremely high (0.95-0.98), showing that root dry weight, while easier to measure, is an excellent predictor of root length density.

Identification of QTLs associated with root morphology

Figure 1 shows the locations of the QTLs detected using single-marker analysis. A more precise localization of these QTLs, found by combining single-marker analysis and interval mapping, is given in Table 1. It appears that genes controlling different aspects of root morphology such as MRL, RW T^{-1}, and B30 T^{-1} are concentrated in the same genomic areas (particularly on chromosomes 1, 2, 7, and 9). These results are consistent with the values observed for the genotypic correlations. The proportion of phenotypic variance in the individual markers explains the range from 5 to 28%. Surprisingly, Azucena, while the best parent in terms of root system, nevertheless does not concentrate all favorable alleles (negative effect of Azucena alleles on at least one QTL controlling THK, TRW T^{-1}, B30 T^{-1}, and R/S). The RZ801-RZ19 segment of chromosome 1, known to bear the *sd1* gene that controls plant height, tillering ability, and, therefore, biomass development, is exerting an important effect on root development. Correlations between aboveground and underground growth, hormonal as well as nutritional, are well established (Klepper 1991). Other QTLs that control plant height detected by Courtois et al (1995) are also mapping at the same place as the QTLs involved in controlling root weight per tiller (RZ318, RG908, and RZ12 on chromosomes 2, 4, and 9, respectively). The comparison of the root systems of known mutants for plant height with their wild counterparts could be an interesting field to explore.

Comparison with previous results

The comparison of the results of the two populations shows that common QTLs exist for all traits (Table 2). The magnitude of these common QTLs varies. Most of the detected QTLs are different because there are allelic differences at the QTL locations between populations and because both parents carry the same allele at the QTL location in the population with no detected QTL. The choice of the statistical thresholds (power of the tests) and the genetic background itself (nonallelic interaction not tested here) can also contribute to the variability. Lowering the threshold would, in some cases (R/S notably), increase the number of common QTLs. More populations should be studied to get a comprehensive idea of the pattern.

Table 1. QTLs controlling root morphology in IR64/Azucena DH population (single marker analysis and interval mapping combined).

		Root thickness		Maximum root length		Total root dry weight tiller^{-1}		Deep root dry weight tiller^{-1}		Root-shoot ratio	
	Chr[a]	Prob	Wgt	Prob	Wgt	Prob	Wgt	Prob	Wgt	Prob	Wgt
RZ730-RZ19[b]	1	0.00001	+	0.00046	+	0.00000	+	0.00001	+	0.00002	+
RG276-RG146	1			0.00179	+						
RZ318-RG157	2	0.00382	+	0.00000	+	0.00147	+	0.00233	+	0.00190	+
RG163-RG675	4					0.00227	+				
RG908-RG190	4					0.00012	-	0.00135	-		
RZ556-RG313	5	0.00108	+	0.00002	+						
RZ67-RZ70	5	0.00033	-			0.00008	-				
RZ144-RZ667	6					0.00290	+				
RZ667-RG648	6	0.00047	-								
CD0544-RG653	6									0.00000	-
RZ978-CD0497[b]	7			0.00000	+	0.00013	+	0.00000	+	0.00354	+
RZ337-CD059	7									0.00064	+
RZ66-CD099	8	0.00005	+								
CD0590-RZ206	9					0.00022	+				
RZ228-RZ12	9			0.00001	+	0.00136	+	0.00059	+		
CD093-RZ625	10	0.00176	-								

[a]Chr = chromosome; Prob = probability of the F value for the main marker; Wgt = contribution of Azucena alleles, increasing (+) or decreasing (-) the mean. [b]Segment with three markers.

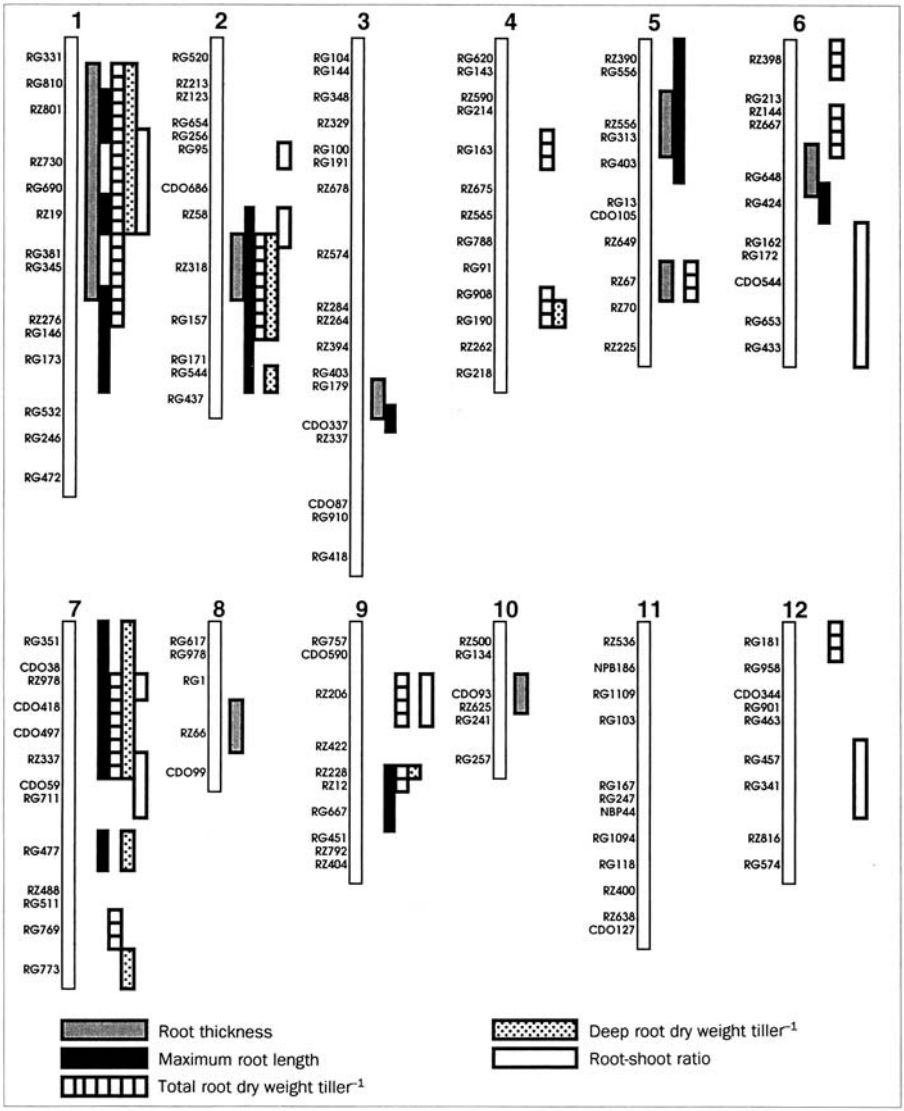

Fig. 1. QTLs controlling root morphology in IR64/Azucena DH population (single marker analysis; *P*<.01).

Table 2. Comparison between QTLs identified in the CO 39/Moroberekan and IR64/Azucena populations.

	Number of QTLs		Common QTL	Localizatlon of common QTLs				
	IR/AZ[a]	CO/MORO[b]	(no.)	Chr	IR/AZ	% var	CO/MORO	% var
Root thickness	7	18	2?	1	RG690-RZ19	8	RG197	25
				8	RZ66-CDO99	6	RZ66	22
				8	RZ66-CDO99	6	RG136	21
Maximum root length	6	2	1	9	RZ12-RG667	10	RZ12	?
Total root dry weight per tiller	6	14	3	2	RZ318-RG157	7	RG139	12
				7	CDO418-CDO497	18	RG351	11
				9	RZ206-RZ422	6	RG553	12
Deep root dry weight per tiller	5	8	3	2	RZ318-RG157	7	RG139	12
				7	CDO418-CDO497	18	RG351	6
				9	RZ228-RZ12	6	RZ12	8
Root-shoot ratio	5	16	2	9	CDO590-RZ206	6	RG553	22
				12	RG547-RG341	6	RZ397	15

[a]Probability threshold < 0.005. [b]Probability threshold < 0.0001.

Future steps

A parallel evaluation of the genotype of elite parents (using sequence-tagged sites) and of their root phenotype is under way to assess the allelic diversity of the markers and to identify alleles with major effect. These alleles will be eventually introgressed into interesting backgrounds to create substitution lines whose effect on the root phenotype will be assessed.

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QTL analysis as an aid to tagging genes that control heading time in rice

M. Yano, H. Yoshiaki, Y. Kuboki, S.Y. Lin, Y. Nagamura, N. Kurata, T. Sasaki, and Y. Minobe

Quantitative trait locus (QTL) analysis has been carried out to identify genes that control heading time in rice. One hundred and eighty-six F_2 plants derived from a cross between japonica variety Nipponbare and indica variety Kasalath were used as a primary population for QTL mapping of heading time and more than 850 markers were employed to identify QTLs. QTL scanning analysis revealed the existence of two QTLs with large effects, *Hd1* in the middle of chromosome 6 and *Hd2* in one end of chromosome 7. In both loci, the Kasalath allele reduced days to heading and the mode of inheritance of both loci was most likely additive. The two QTLs explained about 65 and 15% of total phenotypic variation of the F_2 population, respectively. In a secondary scanning analysis, which was carried out by fixing the position and phenotypic effects of the two detected major QTLs, three more QTLs *(Hd3, Hd4,* and *Hd5)* with minor effects were found to be located on chromosomes 6, 7, and 8, respectively. At these loci, the Nipponbare allele has the effect of reducing days to heading. The mode of inheritance of these three QTLs at chromosomes 6, 7, and 8 was most likely recessive, dominant, and additive, respectively.

In rice, heading time is one of the most important traits for adaptation to different cultivation areas and crop seasons. Controlling heading time of rice plant is a major objective in breeding programs. Large variation in heading time has been found among cultivated varieties (Vergara and Chang 1985). So far, many genes have been reported to control heading time (Chang et a1 1969, Yoko et a1 1980, Yamagata et a1 1986, Sato et a1 1988, Poonyarit et a1 1989, Sano 1992, Ohshima et a1 1993). However, except for *Sel,* the allelic relationships among these genes and their chromosomal locations remain uncertain. It was very difficult to analyze genetically the naturally occurring variation in heading time due to the complexity of the genes controlling this trait.

The recent development of molecular markers, for example, restriction fragment length polymorphism (RFLP), makes it possible to identify individual loci that control quantitative traits (Tanksley 1993). In this study, we have used densely mapped DNA markers to genetically dissect phenotypic differences in heading time in an F_2 population as an aid to molecular tagging of the involved genes.

Materials and methods

Plant materials

An F_2 population of 186 individuals, derived from a cross between japonica variety Nipponbare and indica variety Kasalath, was used. All plants were cultivated under natural daylength conditions. Heading times of each F_2 plant and parental plant were monitored individually for appearance of the first panicle. QTL analyses were carried out using days to heading (the number of days required from seeding to heading).

RFLP data analysis

The F_2 population used in this analysis is the same population used for the construction of a high-density linkage map with 1,383 DNA markers (Kurata et al 1994). All data for genotype of markers were available directly for this QTL analysis.

QTL analysis

To carry out QTL analyses, we have selected 857 independent loci, covering the whole rice genome from 1,383 DNA markers (Kurata et al 1994). The map location of putative QTLs were determined by interval mapping using computer software MAPMAKER/QTL ver. 1.1 (Lander and Botstein 1989). LOD score 3.0 was employed to detect putative QTLs in the analysis of single or multiple QTL model. To identify the mode of inheritance, reexamination of putative QTL regions was carried out by three constrained genetics, such as dominant, recessive, and additive, using MAPMAKER/QTL software. The phenotypic effects, such as the additive ones, the dominance effects, and the percent of total phenotypic variation explained by each putative QTL, are also estimated by the aforementioned software. Analysis of variance for each genotype class was employed to identify the existence of interaction, such as epistasis and suppression, between possible pairs of QTLs detected.

Results

Phenotypic variation

The mean values of days to heading of Nipponbare is 122 d (range 121-125 d), while that of Kasalath is 117 d (115-118 d). The difference between days to heading of both parental lines is very small. However, variation in days to heading of the F_2 plants was very large (ranged from 104-164 d) and continuous (Fig. 1). The frequency distribution of days to heading was likely to be bimodal and showed transgressive segregation.

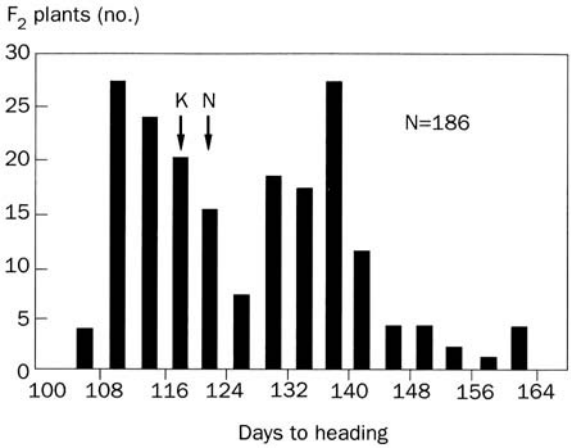

Fig. 1. Frequency distribution of days to heading in the F_2 population. K and N (arrows) indicate the mean values of parental varieties, Nipponbare and Kasalath, respectively.

Table 1. Putative QTLs that control heading time detected in the F_2 population derived from varieties Nipponbare and Kasalath.[a]

QTL	Chromosome	NML	LOD		Effects on the phenotype				MOI
					AE	DE	PVE	DGA	
Hd1	6	R1679	44.2	(0)	-14.5	2.3	66.7	N	A
Hd2	7	C728	6.2	(0)	-7.0	2.8	14.2	N	A
Hd3	6	C226A	64.4	(0)	2.1	-1.9	79.9	K	R
Hd4	7	L538T3	65.3	(0)	2.9	-1.9	80.4	K	D
Hd5	8	R902	65.7	(1.4)	3.4	0.9	80.6	K	A

[a]*Hd1 and* Hd2 were detected in the scanning analysis using a single QTL model. *Hd3, Hd4, and Hd5* were detected in the scanning analysis using a multiple QTL model with fixed *Hd1* and *Hd2.* NML: the nearest marker locus linked to putative QTL. Figures in parentheses indicate the genetic distance (cM) between the peak position of LOD curve and NML; LOD: log likelihood value calculated by MAPMAKER/QTL ver. 1.1 software in the condition of unconstrained genetics; AE: additive effect of Kasalath allele on days to heading; DE: dominant effect of Kasalath allele: PVE: percent of total phenotypic variation explained by the QTL (PVE of *Hd3, Hd4,* and *Hd5* are cumulative values in the multiple QTL model); DGA: direction of gene action. N and K indicate that Nipponbare and Kasalath alleles, respectively, increase the number of days to heading; MOI: mode of inheritances. A, D, and R are additive, dominant, and recessive, respectively.

QTLs that control heading time

In the analysis using the single QTL model, two distinct loci with relatively large effects were detected. One QTL, *Hd1* (LOD = 45.3), linked to marker R1679 of chromosome 6. The other QTL, *Hd2* (LOD = 7.5), linked to C728, the distal end marker of chromosome 7 (Fig. 2, Table 1). In both loci, Kasalath allele reduced days to heading and mode of inheritance of both loci was most likely additive (Table 1). In the single QTL model, the estimated additive effects of Kasalath allele were -14.5 d

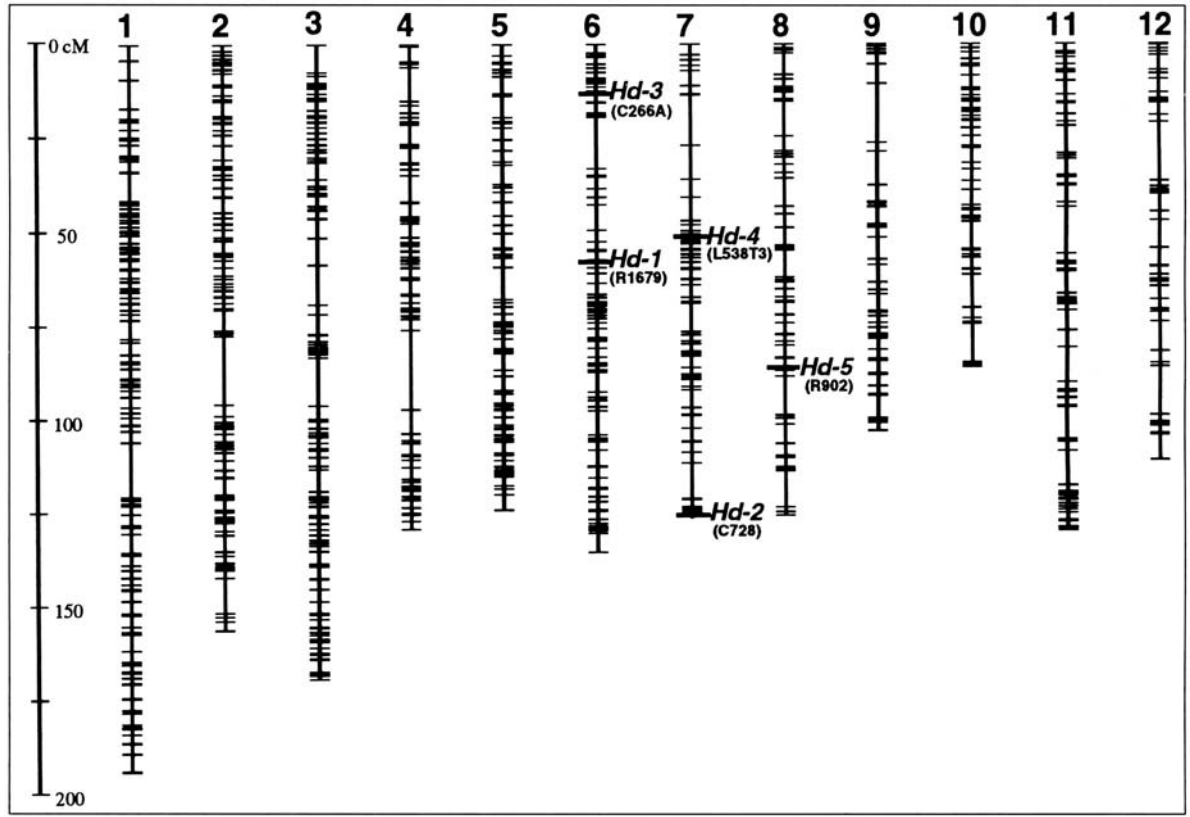

Fig. 2. RFLP linkage map of 12 rice chromosomes (Kurata et al 1994) showing locations of the putative QTLs detected in the analysis of the F_2 population derived from Nipponbare and Kasalath. Markers in parentheses are the nearest marker loci linked to the putative QTLs.

on *Hd1* and -7.5 d on *Hd2.* The two QTLs explained about 65.0 and 15.0% of total phenotypic variation of the F_2 population.

In the analysis by the multiple QTL model, while fixing simultaneously the positions and effects of the two putative QTLs detected in the first analysis, three other putative QTLs, *Hd3, Hd4,* and *Hd5,* with relatively small effects, were found on chromosomes 6, 7, and 8, respectively. *Hd3* linked to C266A (chromosome 6), *Hd4* to L538T3 (chromosome 7), and *Hd5* to R902 (chromosome 8) (Table 1). In all these loci, the Nipponbare allele reduced days to heading. The estimated additive effects of the Kasalath allele on *Hd3, Hd4,* and *Hd5* were 2.1, 2.9, and 3.4 d, respectively (Table 1). The mode of inheritance of these three putative QTLs *(Hd3, Hd4,* and *Hd5)* was most likely recessive, dominant, and additive, respectively. To detect the existence of gene interaction, genotype data of the nearest markers of each QTL were used for the analysis of variance for the classified genotype classes. All possible combinations of detected QTLs fitted the linear model well (i.e., additive model). However, the combination of *Hd1* and *Hd3* on chromosome 6 may involve gene interaction such as suppression or enhancement.

Discussion

Beavis et a1 (1991) hypothesized that some of the qualitative trait loci (e.g., dwarfing gene) should be the same as the quantitative trait loci. This QTL analysis has also supported this hypothesis for heading time in rice. The major photoperiod sensitivity gene, *Se1,* has been found to be located on chromosome 6 and to be closely linked to the isozyme marker *Pgil* (Kinoshita 1993) and to the blast resistance gene *Piz* (Yokoo et a1 1980). In this study, *Hdl,* with a large effect on heading date, was found to be located in the middle of chromosome 6. One of the cDNA clones that encode *Pgi* was also mapped in the vicinity of R1679, linked closely to this putative QTL (unpubl. data). Based on the location of both loci on chromosome 6, *Hdl* might be at the same locus as *Sel.* In this study, *Hd3* (affecting gene expression of Hdl) was also detected on chromosome 6. Sano (1992) has shown that the strong photoperiod sensitivity in one accession of *Oryza rufipogon* (W593) is controlled by *Sel* and its dominant enhancer, *En-Sel.* The gene *En-Sel* was found to be located on chromosome 6 and to be linked to the *wx* locus. In this study, *Hd5* was also found to be located in the vicinity of marker C266A and was linked to the *wx* locus on chromosome 6. Moreover, one of the photoperiod sensitivity genes, *El,* was found to be located on chromosome 7, as our *Hd4,* and was linked to the morphological marker *Rc* (Okumoto et a1 1992). In our study, the marker L538T3 was linked to *Hd4,* also located in the vicinity of *Rc* on chromosome 7 (Lin et a1 1994). Further analysis will be necessary to determine the allelic relationship between these known loci and *Hd5* and *Hd4.*

This QTL analysis is also the first case of high-resolution QTL mapping. More than 850 independent markers, mostly composed of codominant markers, have been used to detect putative QTLs and the mean genetic distance of marker intervals was less than 1.8 cM. As a result, in the vicinity of the LOD score peaks, average genetic distances of intervals between markers were less than 1.0 cM (data not shown) and so

most of the LOD score peaks were at the marker positions. Densely mapped markers also enabled us to detect putative QTLs with small effects (*Hd4*) and with interactive effects (*Hd3*). These results indicated that QTL analysis using densely mapped markers will provide us an effective and a precise gene mapping system.

Genes controlling heading time are involved in the transition of the plant from the vegetative to the reproductive growth phase. Isolation of genes involving heading time is needed to understand this phenomenon. Recently, a photoperiod sensitivity gene, *co,* has been isolated in *Arabidopsis* by map-based cloning (Putterill et al 1995). Map-based cloning will be one strategy for the isolation of genes corresponding to the QTLs detected in this study. Several near-isogenic lines (NILs) that carry the Kasalath alleles for each putative QTL in the genetic background of Nipponbare are currently being developed by marker-aided selection (Yano et al 1994). Once these NILs are available, it would be possible to detect putative QTLs as a single Mendelian factor and to map the QTLs more precisely. In this way, these QTLs might be used for map-based cloning.

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Using quantitative trait locus analysis for studying genetic regulation of shattering

Y. Fukuta, Y. Harushima, and M. Yano

This study aimed to clarify the mechanism of genetic regulation of shattering in rice. Quantitative trait locus (QTL) analysis was performed on an F_2 population, using molecular markers and histological analysis of the abscission region in the spikelets of three shattering-resistant mutant lines induced from indica variety Nanjing 11. The F_2 population was derived from a cross between Nipponbare (japonica) and Kasalath (indica). QTL analysis identified five putative QTLs on chromosomes 1, 2, 5, 11, and 12. The locus on chromosome 1 expressed very strongly, but the other four loci expressed more weakly. In three mutant lines whose resistance to shattering was controlled by single recessive genes, histological analysis showed that the degrees of resistance to shattering were related to the development of the abscission layer and the structure of the spikelets. It was hypothesized that shattering is controlled by at least three genes and perhaps five or more, which control the development of the abscission layer or the structure of the spikelet, at least, and that one, which expresses very strongly, is located on chromosome 1.

Wide genetic variation in the degree of shattering has been found in cultivated rice (Uchiyamada et al 1985). Shattering habit itself may be controlled by major gene(s), however, the genetic mechanism is difficult to analyze. Three genes that control shattering—*shl* (Nagao and Takahashi 1963), *sh2* (Oba et al 1990, Ogi et al 1993), and *Sh3* (Eiguchi and Sano 1990)—have been located on rice chromosomes 11, 1, and 4, respectively.

The development of restriction fragment length polymorphism (RFLP) markers and RFLP linkage maps have enabled the detailed analysis of quantitative trait loci (QTLs) (Lander and Botstein 1989). RFLP linkage maps and RFLP markers have also been developed for rice (McCouch et al 1988, Kurata et al 1994). In this study,

QTL analysis using molecular markers (RFLP; RAPD, random amplified polymorphic DNA) was applied to study the genetic mechanism that controls shattering.

Mutant and isogenic lines are useful materials for conducting genetic analyses of quantitative traits. Therefore, three shattering-resistant mutant lines—SR- 1, SR-5, and SR-2—were used to study the development of the abscission layer in the spikelet. These lines showed very high, high, and moderate resistance to shattering, respectively (Fukuta et al 1994a). Shattering resistance in these lines was controlled independently by a single recessive gene (Fukuta et al 1994b). The recessive gene in mutant SR-1 was located on chromosome 1 (Fukuta 1995).

In this paper, a hypothesis for the genetic mechanism of shattering in cultivated rice is proposed, based on QTL analysis and histological observations of the mutant lines.

Materials and methods

QTL analysis

One hundred and eighty-six F_2 plants, which were derived from a cross between japonica variety Nipponbare and indica variety Kasalath, were used for QTL analysis. The degree of shattering in the F_2 plants was determined using a shattering habit tester (TR-II; Ichikawa et al 1990). The shattering degree of each plant was measured by its breaking tensile strength (gf). Southern and RAPD analyses were done on 609 molecular markers.

The QTL analysis was done to detect loci of the shaterring gene on an RFLP linkage map using computer programs MAPMAKER/EXP ver 3.0 (Lincoln et al 1992a) and MAPMAKER/QTL ver 1.1 (Lincoln et al 1992b). The RFLP linkage map used in this analysis was presented in Kosambi cM units. A likelihood map based on the log of odds (LOD) scores for shattering degree was developed by interval mapping (Lander and Botstein 1989). The LOD score indicated the strength of the data supporting the hypothesis about the existence of the loci of the shattering gene. When the LOD was higher than 2.4, it was postulated that the locus of the RFLP marker was linked with the locus of the shattering gene, and that the maximum likelihood position of the two loci corresponded to the highest point of the LOD score's curves. The average value of F_2 plant phenotypes for Nipponbare/Nipponbare homozygotes, the additive effects and the dominance effects of the Kasalath allele, and the variance explained by the QTL were calculated at the putative loci.

Histological analysis using shattering-resistant mutant lines

Three kinds of shattering-resistant mutants lines that were induced from indica variety Nan-jing 11 were used for histological analysis of the development of the abscission layer and the structure of the spikelet. Longitudinal sections in the spikelets, which were collected periodically from each of the mutant lines and the original variety Nan-jing 11 (from beginning of flowering to 30 d after flowering), were observed microscopically.

Results and discussion

QTL analysis

Five putative QTLs were identified on chromosomes 1, 2, 5, 11, and 12, respectively. The locus on chromosome 1 expressed very strongly, but the other four loci expressed more weakly. The value of the LOD score and the variance explained by QTL at the putative locus on chromosome 1 were 45.5 and 68.6%, respectively. The values at the other putative loci were low, and the LOD scores and explained variances ranged from 2.9 to 3.3% and from 6.9 to 8.6%, respectively. The Kasalath alleles at the putative QTL on chromosomes 1, 2, and 5 appeared to express for shattering, but those on chromosomes 11 and 12 exhibited resistance to shattering (Fig. 1).

Histological analysis

Microscopic observations of the longitudinal sections demonstrated that the development of the abscission layer and the structure of the spikelets in the mutant lines and original variety were different. Nan-jing 11 developed a clear abscission layer at the base of sterile glumes in the spikelet, whereas SR-1 lacked an abscission layer. SR-5 also lacked an abscission layer, and the cells at the base of the sterile glume underwent partial degeneration. In SR-2, cell degeneration was less pronounced than that in SR-5 and the region between sterile glumes and rudimentary glumes was longer or more slender than in the other lines (Fig. 2).

These results indicate that the shattering habit in cultivated rice is controlled by several major genes and that the genes were related to the development of the abscission layer and the structure of the spikelet. QTL analysis showed that the putative locus on chromosome 1—located in the neighborhood of *sh2*, which was identified by Ogi et al (1993)—expresses very strongly. It has also been found that the shattering-resistant mutant gene in SR-1 with special characters of very high resistance to shattering and nonformation of an abscission layer was located in the same chromosome region where Ogi et a1 (1993) mapped *sh2* (Fukuta 1995).

We hypothesize that shattering is controlled by at least three genes and perhaps five or more, which control the development of the abscission layer or the structure of the spikelet, at least, and that one, which expresses very strongly, is located on chromosome 1.

Important characters in cultivated rice are mostly quantitative and are expressed continuously in various phenotypes. QTL analysis with RFLP and RAPD markers and analysis of mutant lines are useful tools in breeding for these characters.

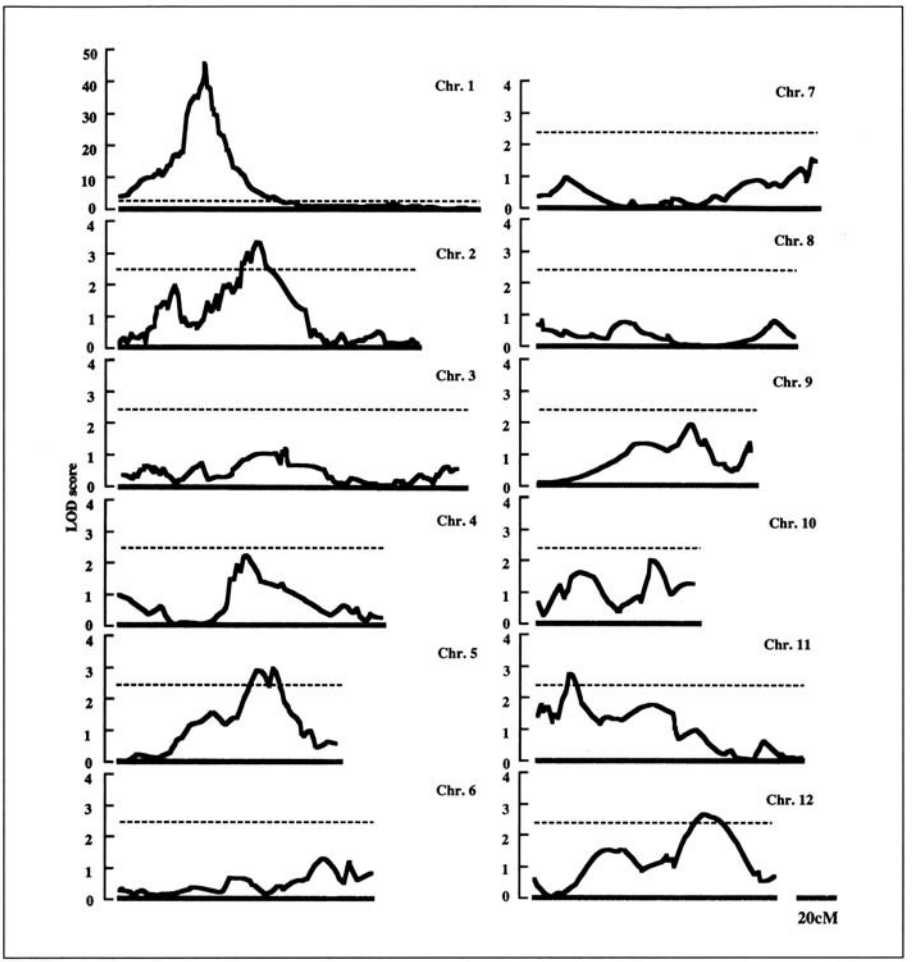

Fig. 1. A likelihood map indicated by LOD scores for shattering degrees in the F_2 population derived from the Nipponbare/Kasalath cross. The RFLP linkage map used in the analysis is presented along the abscissa in Kosambi cM. The maximum likelihood position of shattering degrees is the highest point in the LOD score curves. The dotted line, at point 2.4 of the LOD score axis, indicates the required significance level. The length and number of markers: chromosome 1: 198.2 cM, 100 markers; chromosome 2: 165 cM, 90 markers; chromosome 3: 195.7 cM, 86 markers; chromosome 4: 146.1 cM, 37 markers; chromosome 5: 123.1 cM, 41 markers; chromosome 6: 137.9 cM, 49 markers; chromosome 7: 151.1 cM, 36 markers; chromosome 8: 141.1 cM, 35 markers; chromosome 9: 117.9 cM, 29 markers; chromosome 10: 87.5 cM, 31 markers; chromosome 11: 146 cM, 46 markers; chromosome 12: 129 cM, 29 markers.

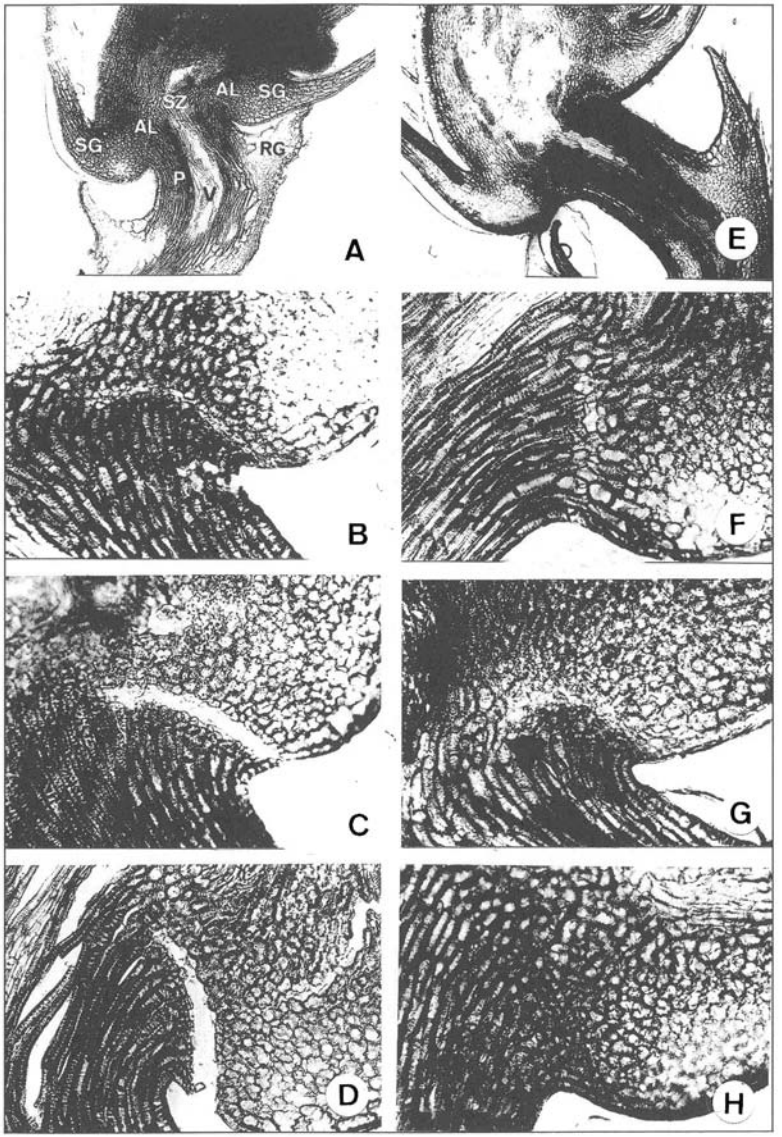

Fig. 2. Longitudinal section of the abscissa region between the pedicel and rachilla of three kinds of shattering-resistant mutant lines and original variety Nan-jing 11. A) Nan-jing 11 (AL=abscission layer, P=pedicel, RG=rudimentary glume, SG=sterile glume, SZ=supporting zone, V=vascular tissue); B) Nan-jing 11 (2 d after flowering); C) Nan-jing 11 (15 d after flowering); D) Nan-jing 11 (30 d after flowering); E) SR-2; F) SR-2 (15 d after flowering); G) SR-5 (15 d after flowering); H) SR-1 (15 d after flowering).

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RiceGenes, an international genome data base for rice

E. Paul, M. Goto, and S.R. McCouch

RiceGenes is an internationally accessible computer data base containing a wide variety of information on rice genetics. It was organized around the concept of a genetic map, with markers on the map providing "hot links" to related pieces of information such as marker sequences, vectors, homology to known genes, and map locations in other species. The links allow the researcher to move seamlessly among related items of interest. The data base currently consists of information on more than 3,000 DNA markers, five genetic maps of rice, a genetic map of maize, relevant bibliographic citations, digitized images of autoradiograms, and more than 1,300 DNA sequences. Restriction fragment length polymorphism profiles of rice germplasm, sequence information related to markers or known genes of rice, and detailed comparisons between maize and rice maps have also been included. RiceGenes is accessible via the Internet in four formats—Gopher, a C. *elegans* data base (ACEDB), World Wide Web (WWW), and electronic mail.

RiceGenes is a public repository of genetic information pertaining to rice. The effort to create, maintain, and extend this data base is funded through the United States Department of Agriculture's (USDA) Plant Genome Research Program (Paul 1995), and relies on the willingness of rice scientists worldwide to share their research results in a public forum.

A key goal of RiceGenes is to maximize the value of the information through the intelligent structuring and linking of data items, and the consistent presentation of information from multiple sources. A parallel goal is to maximize global access to the information by making it available in a variety of formats and by encouraging researchers to use the data base as a platform for publicizing their studies. With user support, the data base could help minimize redundancy in research efforts and enhance international cooperation and data exchange.

The data base is not intended to be a static archive of rice information, but rather an interactive tool that grows and changes to meet current research interests. The first release of the data base focused on genetic maps and molecular markers. To date, four molecular genetic maps of rice (McCouch et al 1988, Causse et al 1995, Kurata et al 1995, Zhu, pers. commun.), one of maize (Ahn and Tanksley 1993), and the classical linkage map of rice (Kinoshita 1993) have been added. For each DNA marker on the molecular maps, details concerning the origin and construction of the probe are provided, allowing users to amplify the probe in their own facility using polymerase chain reaction, or to intelligently deploy available probes publicly. DNA sequence information is also available for many of the markers. Autoradiographic images of commonly distributed markers have been loaded, making it possible for researchers to compare and crosscheck results generated in their respective laboratories. A key point is that all the information is structured so that the user can easily and intuitively move through it even without an understanding of the underlying structure of the data base (Fig. 1).

The second release of RiceGenes was marked by the incorporation of detailed molecular profiles for a large, genetically diverse sample of cultivated rice varieties

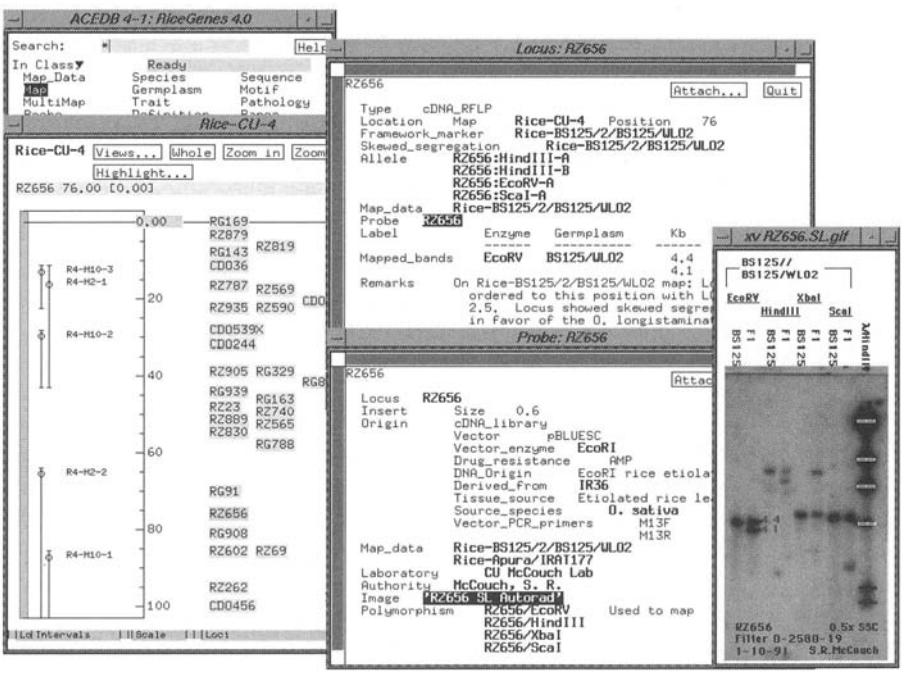

Fig. 1. Illustration of how a user can progress from a genetic map, to a specific locus, to the DNA probe for that locus, and to the image of the probe on a parental survey filter. Links to related information are highlighted by bold text. Each window is invoked by clicking on a boldface item. The genetic map display is an interactive graphic tool and almost every part of this display can be further expanded by clicking on a specific item.

(G. Second, pers. commun.) This data set allows a user to detect the unique banding patterns for a given probe/enzyme combination across a large germplasm sample, and to identify the accessions showing each pattern. As we learn more about markers that are linked to traits of interest, it can be a very powerful associative tool. In addition, specific accessions can be compared in such a way as to identify probe/enzyme pairs which will give polymorphic (or monomorphic) results (Fig. 2). We continue to be

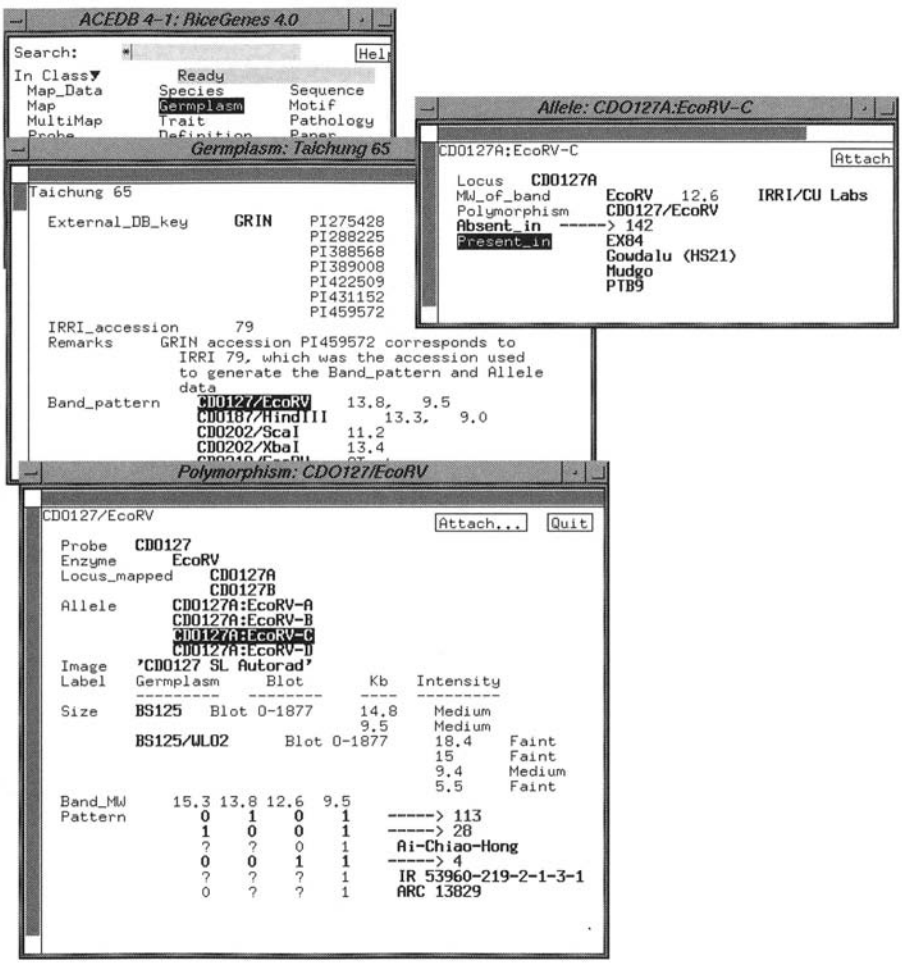

Fig. 2. A typical germplasm record and a related polymorphism and allele record. In the example of a polymorphism record, the most common banding pattern included a 13.8 and a 9.5-kb band (the arrow and number indicate a collapsed list of items, in this case, 113 accession names). Looking down the columns, it is evident that the small band (9.5 kb) is uninformative, appearing in all accessions. The 12.6-kb band, corresponding to the *C* allele, however, was successfully scored in only four accessions.

interested in this class of information, and are eager to extend its scope through international collaborations.

In the most recent release of RiceGenes, the use of rice as a tool for comparative mapping was explored through the addition of a maize-rice comparative map. By using a common set of rice, oat, and barley cDNA probes, it was possible to identify segments of chromosomes displaying conserved gene order between the two genomes. In several instances these homeologous segments contained genes of known function in their predicted position. As more comparative maps are added, we can determine if or how these same segments are recombined in other grass genomes. The value of comparative maps is increasingly obvious as quantitative trait loci are added to the maps. Being able to relate map positions across genomes will allow scientists to take advantage of the progress being made in multiple crops and to direct research efforts more precisely to particular places in a genome of interest (Fig. 3).

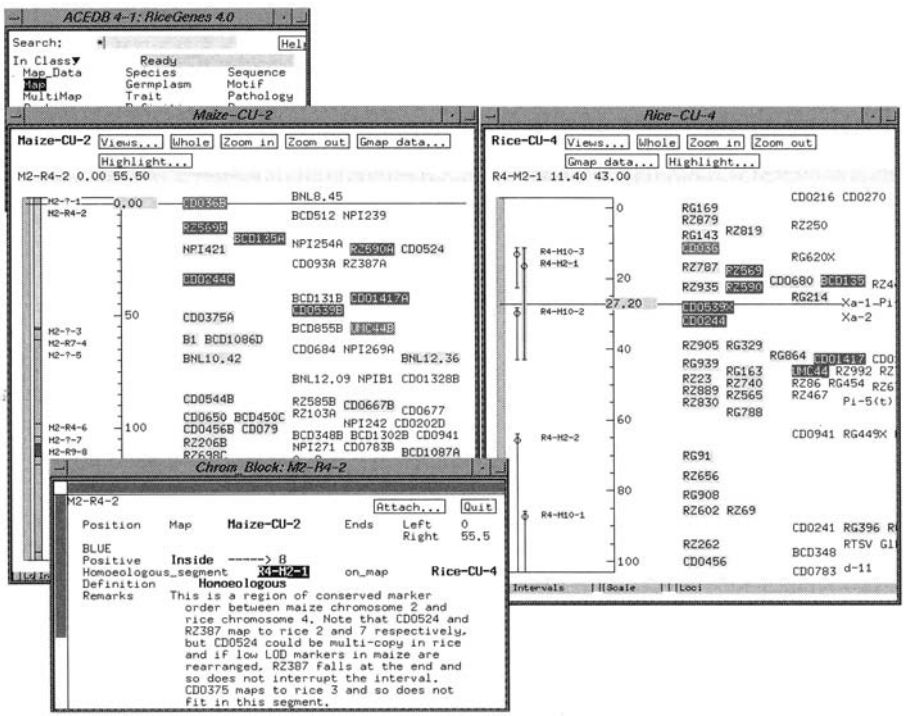

Fig. 3. Maize chromosome 2 divided into segments (e.g., M2-R4-2, M2-R7-4), sharing their gene order with a chromosomal segment from rice. Clicking on the segment highlights the markers known to reside within the segment, and provides further information about the nature of the segment and where the rice counterpart is located (in this case, chromosome 4). Segments are portrayed as vertical lines on the rice map to better facilitate additional comparisons between rice and other grasses. Again, clicking on the line highlights the markers within the segment; the marker order is preserved between maize and rice. This segment from rice 4 exists as one large segment on maize 2, and as two smaller segments on maize 10.

Accessing the data base

The contents of the RiceGenes data base are accessible through a number of different computer software interfaces. The figures shown thus far were taken using "a *C. elegans* data base" (ACEDB) software (Durbin and Thierry-Mieg 1991). The software is graphically based and includes many features and displays that meet the unique requirements of genetic information. It supports the display of image files as well as textual data, and allows the data base curator to create links between data items, making navigation easier for the end user. The current RiceGenes ACEDB distribution requires a UNIX host machine to run, and can also be accessed from personal computers if they have a direct TCP/IP network connection to the UNIX host and X11 capability. We hope an ACEDB version of RiceGenes for the Macintosh will soon be available through collaboration with the Korean Rice Genome Project. The UNIX ACEDB distribution can be downloaded over the Internet via an anonymous ftp from the site probe.nalusda.gov, directory pub/ricegenes.

A second method of accessing the information is the Gopher, a menu-driven, easy-to-use interface that does not require any special graphics or windowing capability, and which allows the user to effortlessly move among public information sources. The Gopher can be accessed most simply by a modem connection to an Internet host computer. In addition, it is an excellent tool for providing additional text or image files that do not easily fit into the ACEDB structure, but which might be of interest to rice researchers. With this in mind, we have made available on the RiceGenes Gopher the entire text of the first nine volumes of the Rice Genetics Newsletter. Because the Gopher supports full-text indexing, a user can now search an entire volume for a specific word or phrase. The RiceGenes Gopher is maintained at nightshade.cit.cornell.edu, port 70, and also at probe.nalusda.gov, port 7007.

Another interface to the information is the World Wide Web (WWW). This also requires a full Internet connection, but again allows the user to move seamlessly among data sources located around the world. The WWW also allows activation of "hot links" between data bases, allowing a user in RiceGenes to click on a link and immediately see the full record from another data base. This feature has been used to establish links from RiceGenes into sequence data bases (DDBJ, GenBank, EMBL, etc.), germplasm data bases (Germplasm Resources Information Network [GRIN]) and the USDA-sponsored maize data base (MaizeDB). The USDA maintains the WWW server that acts as an umbrella to all USDA-supported plant genome data bases. The server is located at the following electronic address: http://probe.nalusda.gov:8000.

The USDA also maintains an electronic mail server, allowing users to submit searches of the plant genome data bases via electronic mail. For instructions on formatting these searches, send a message to waismail@probe.nalusda.gov, which contains only the word "help" as the body of the message. You will receive a message back with instructions for using this service.

Finally, to ensure this information is available to all interested users, the USDA has published a CDROM containing all the plant genome data bases, including

RiceGenes. This CDROM is compatible with UNIX, PC and Macintosh computers, and is available free of charge upon request from the following electronic address: pgenome @ nalusda.gov or the following postal address: Plant Genome Database; USDA, NAL, PGD; Room 013, NAL Building; 10301 Baltimore Boulevard; Beltsville, MD 20705-2351; USA.

Future developments

Current projects under development include the modeling of rice quantitative trait loci data and the incorporation of microsatellite marker data. We believe both of these efforts will significantly enhance the usefulness of the data base as a tool for rice research. We continue to solicit suggestions for improvements and data submissions from the international user group, with the hope that this data base can foster collaborations, increase access to valuable information, and ultimately speed up the genetic improvement of rice.

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Cloning a blast resistance gene by chromosome walking

K.S. Wu, C. Martinez, Z. Lentini, J. Tohme,
F.G. Chumley, P.A. Scolnik, and B. Valent

We are progressing toward cloning a blast resistance gene using a map-based cloning strategy. Bulk segregant analysis was used to identify random amplified polymorphic DNA (RAPD) markers tightly linked to the *Pi62*(t) gene in a doubled haploid population. Screening 1,440 RAPD primers on pooled DNAs from resistant and susceptible plants identified 22 polymorphic bands. Eighteen markers turned out to be linked to the *Pi62*(t) gene, and three showed no recombination with *Pi62*(t) in 120 progeny. To facilitate fine-structure genetic and physical mapping, the RAPD markers have been converted into restriction fragment length polymorphisms (RFLPs) and sequence-tagged sites. Five of the *Pi62*(t)-linked markers have been mapped to the Cornell rice RFLP genetic map, and all are linked to RG869, an RFLP marker that is linked to the blast resistance gene *Pi4*(t) on chromosome 12. An F_2 population consisting of 700 individuals is being used for high-resolution genetic mapping. A bacterial artificial chromosome (BAC) library, consisting of 20,160 independent clones with an average insert size of 110 kb, has been constructed. The total content of the library is equivalent to five haploid genomes of rice. BAC clones have been identified using a single-copy probe, SP7C3, a marker that cosegregates with the *Pi62*(t) gene. A physical map encompassing *Pi62*(t) is being constructed.

Understanding the molecular signaling involved in recognition of the rice blast pathogen, ***Magnaporthe grisea,*** and the subsequent resistance mechanisms that block infection will lead to novel strategies for controlling this serious disease of rice. Recognition in classical "gene-for-gene" interactions is mediated by a dominant plant resistance gene and a corresponding dominant pathogen avirulence gene. Several ***M. grisea*** avirulence genes have been mapped and cloned (Valent and Chumley 1994).

Recent breakthroughs in cloning disease resistance genes in plants (Staskawicz et al 1995) raise the possibility of manipulating blast resistance by genetic engineering.

Even though more than a dozen blast resistance genes have been identified through conventional genetic analysis, no blast resistance gene has yet been cloned from rice. Our study focuses on two gene-for-gene interactions involving the Japanese differential rice cultivars Yashiro-mochi, with resistance gene *Pi-ta,* and Tsuyuake, with resistance gene *Pi-k*^{m} (Yamada et al 1976). A cloned *M. grisea* avirulence gene *AVR2-YAMO* encodes a protein with 223 amino acids that specifically prevents infection of Yashiro-mochi. The product of the *AVR1-TSUY* avirulence gene, which has been mapped but not yet cloned, prevents infection of Tsuyuake. Herein, we report the genetic identification of rice blast resistance genes with functional correspondence to *AVR2-YAMO* and to *AVR1-TSUY* and the progress of work toward cloning these genes.

Identification of molecular markers linked to blast resistance genes

We predicted that polymorphism at the resistance gene loci of interest in cultivars Yashiro-mochi and Tsuyuake could be detected by bulked segregant analysis (Michelmore et al 1991), even though these closely related japonica varieties would not be suitable for building a map. A doubled-haploid (DH) population of 429 independent lines was generated at the Centro Internacional de Agricultura Tropical from reciprocal crosses between Yashiro-mochi and Tsuyuake. Inoculation of the DH population with two pathogen strains—4360-R-62, containing *AVR2-YAMO,* and 4360-R-67, containing *AVR1-TSUY*—identified two independently segregating dominant resistance genes designated as *Pi62*(t) and *Pi67*(t), respectively. DNA from 10 lines that are resistant or susceptible to both isolates were then combined to form resistant or susceptible pools and used as templates for screening using random amplified polymorphic DNA (RAPD) primers (Williams et al 1990).

A total of 1,440 RAPD primers were screened using the pooled DNAs as templates. Twenty-two primers appeared to define markers linked to the resistance genes. Confirmation of linkage was first carried out on individuals forming the pools and then extended to other members of the mapping population. In this way, 19 positives were finally confirmed. Figure 1 shows the banding patterns of a few positive RAPD primers. Eight markers (SP1B8, SP2C12, SP3G6, SP4A5, SP4B9, SP6G10, SP8B8, and SP15B12) segregated in coupling and the remainder segregated in repulsion to the resistance genes.

All the positive RAPD markers have been converted to RFLP markers and some of them have been converted to sequenced-tagged sites (STS). Polymorphic RAPD fragments were eluted from gels, purified using Geneclean II (Bio101, CA), and cloned in the TA-cloning^{TM} vector (Invitrogene, CA). Polymerase chain reaction (PCR) products amplified from plasmids using SP6 and T7 primers served as probes for Southern blot analysis. Nine of sixteen RAPD markers tested were single-copy or low-copy probes in RFLP analysis. STS markers were developed by sequencing and extending the 10-mer RAPD primers to 24-mer specific primers. Even though all 10 STS markers developed so far gave discrete PCR products, only four were polymorphic between the mapping parents.

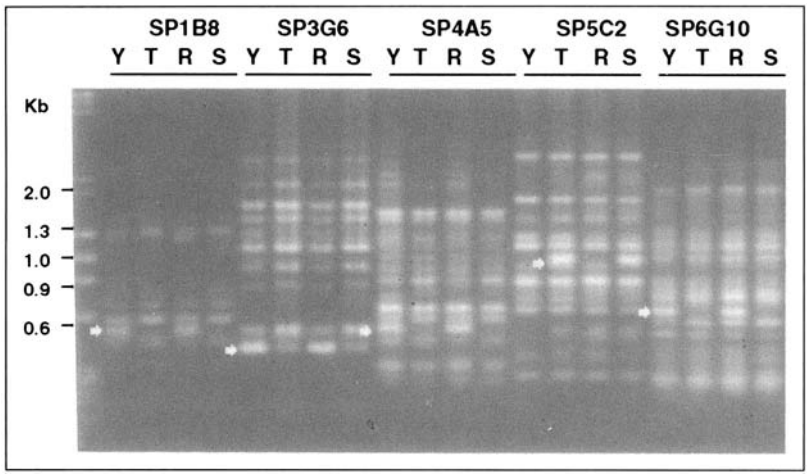

Fig. 1. Banding patterns of some positive RAPD markers. Y, Yashiro-mochi; T, Tsuyuake; R, resistant pool (10 plants); S, susceptible pool (10 plants). The arrows indicate the polymorphic bands.

Genetic mapping

Primary genetic mapping

Primary mapping was conducted using a population of 120 DH lines. Reactions of the DH lines to two fungal isolates were scored. Allelic data of blast genotypes were then tested against allelic data of each RAPD marker scored. This was achieved with computer program MAPMAKER version 2.0. Two-point analysis classified all the markers into two distinct groups: those that are linked to *Pi62*(t) and those that are linked to *Pi67*(t). Eighteen markers were linked to *Pi62*(t) and one marker was linked to *Pi67*(t). Since only one marker was identified for *Pi67*(t), we focused our work on the *Pi62*(t) gene. A linkage map of the chromosomal region containing the *Pi62*(t) was established based on multiple-point analysis (Fig. 2a). An LOD score higher than three was used as the criterion for linkage. Three markers showed no recombination with *Pi62*(t) in 120 DH lines, and eight more mapped within 1 cM of *Pi62*(t) (Fig. 2b).

Assignment of *Pi62(t)* to a rice chromosome

The *Pi62*(t) gene was mapped to rice chromosomes indirectly using flanking RFLP markers that have been derived from RAPD markers as described above. A recombinant inbred mapping population developed by Xiao et a1 (1995) was used for this purpose. The population was derived from a cross between LH422 and 9024. Survey of the parents with a combination of eight probes and five enzymes identified five polymorphic markers (SP3G6, SP4A5, SPlB8, SP8C6, and SP7C3). Two-point analysis established that all the markers were linked to a number of RFLP markers on chromosome 12. Their relative positions corresponding to the RFLP markers on the chromosome were determined by multiple-point analysis. All the markers mapped

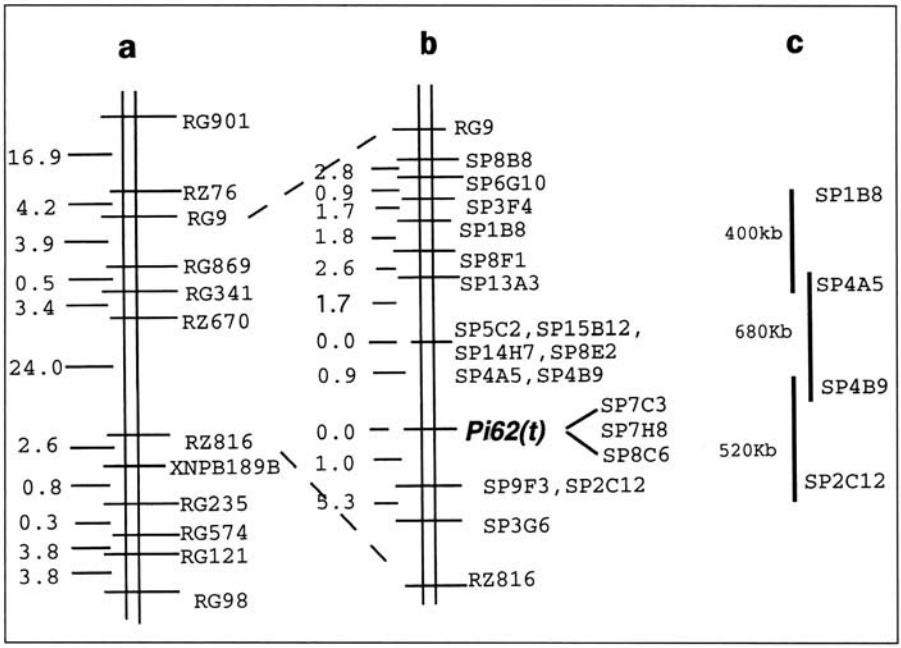

Fig. 2. a) A portion of the genetic map of chromosome 12 containing the *Pi-ta* gene (courtesy of J. Xiao). b) A regional genetic map constructed in this study with RAPD markers. Genetic distances in cM are given on the left. c) Physical distances among RAPD markers as revealed by pulsed-field gel electrophoresis.

to the genetic interval between RG9 and RZ816 (Fig. 2), demonstrating that the *Pi62*(t) gene mapped to the same genomic region as *Pi4*(t) (Yu et al 1991) and *Pi-ta* (Inukai et al 1994).

High-density mapping

For high-resolution mapping, a large F_2 population was developed from a cross between two DH lines (YT10/YT4) in which the *Pi62*(t) gene was segregating. Inoculation of 700 F_2 individuals with blast strain 4360-R-62 revealed that 526 were resistant and 174 were susceptible, a ratio nearly perfectly matching 3:l. One hundred and fifty susceptible plants were rescued after symptom development, and DNA was extracted from these plants using a miniprep procedure (Wu, unpubl.). DNA was also isolated from 150 more DH lines. Altogether, samples representing 600 F_2 individuals and 270 DH lines were used for high-resolution mapping. To reduce the work load, pools of DNA were made from the susceptible F_2 individuals and DH lines after blast genotypes were evaluated. Once a recombinant was detected in a pool, individuals forming the pool were further evaluated. To speed up the mapping process, we also employed an automatic gel loading and imaging recording system. As the population size increased, recombinants were detected for markers that previously cosegregated with the *Pi62*(t) gene. An accurate high-resolution map is in progress but has not been completed at this point.

Physical mapping by PFGE

Efforts were made to construct a physical map encompassing the blast resistance gene by using pulsed-field gel electrophoresis (PFGE). High-molecular-weight DNA was isolated from leaf protoplasts as previously described (Wu and Tanksley 1993) and digested with a number of restriction enzymes recognizing eight-basepair sequences, including *Asc*I, *Rsr*II, *Sfi*I, and *Not*I. DNA blots were then hybridized with markers from the *Pi62*(t) region. Most of the single-copy probes hybridized to relatively small fragments with all the enzymes except *Asc*I, which gave rise to relatively large fragments. This has made it difficult to construct an accurate physical map using the "top down" strategy. Nevertheless, we were able to find some common fragments shared by closely linked markers as shown in Figure 2c.

Construction of a BAC library

To facilitate the isolation of the *Pi62*(t) gene, a bacterial artificial chromosome (BAC) library was constructed using genomic DNA from leaf protoplasts of a DH line carrying both *Pi62*(t) and *Pi67*(t). Protoplasts were isolated from young leaves and sheaths as previously described (Wu and Tanksley 1993). The DNA was partially digested with *Hin*dIII in agarose plugs and fractionated on contour-clamped homogeneous electric field (CHEF) gels. DNA fragments ranging from 100 to 150 kb were eluted and subjected to a second size selection to remove small molecules. Size-selected DNA was then released from agarose by treating with GELase (Epicentre Tech) and ligated to the *Hin*dIII site of pBeloBACII vector (Shizuya et al 1992). Transformation of *Escherichia coli* DH10B cells was carried out by electroporation using cell porator electroporation system I (BRL). BAC colonies were selected on LB plates with chloramphenicol at 12.5 μg mL^{-1}. A total of 20,160 independent colonies were picked and stored in 96-well microtiter plates containing 100-μL LB with chloramphenicol and 10% glycerol.

The average insert size of clones in the library is 110 kb based on analysis of 28 randomly selected colonies. The library contains approximately five haploid genome equivalents of rice DNA. We have identified positive clones with single-copy probes using a two-way pooling scheme. Thus far, two positive BAC clones have been identified using probe SP7C3, a marker that cosegregates with *Pi62*(t) in 120 progeny. Ten positive clones were identified with SP2C12 and 12 with SP4B9. A regional clone physical map is being constructed with BAC clones. To speed up the screening process, the library, contained in 210 microtiter plates, is being spotted onto high-density filters using Biomek.

Summary and future plans

We have identified a resistance gene in rice cultivar Yashiro-mochi, named *Pi62*(t), that functionally corresponds to the cloned fungal avirulence gene *AVR2-YAMO*. Mapping results suggest that *Pi62*(t) may correspond to the previously identified resistance gene *Pi-ta* (Yamada et al 1976). We are continuing high-resolution genetic

and physical clone mapping to identify candidate cDNAs for complementation studies in susceptible plants. Cloning this resistance gene will facilitate molecular studies on specific resistance mechanisms in rice blast disease.

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Positional cloning of rice blast resistance genes

S. Kawasaki, K. Rybka, M. Miyamoto, Y. Tanaka, M. Kataoka, A. Saito, and I. Ando

Rice provides a good experimental plant that represents the monocotyledons well. We are trying to positionally clone rice blast resistance genes as a model. The resistance genes introgressed from an indica to a japonica background provide an excellent system for the precise mapping of genes using nucleic acid markers. We have mapped *Pi-b* and *Pi-ta*^{2} utilizing the numerous near-isogenic lines by graphical genotype analyses and obtained several cosegregating and very close flanking markers upon analysis of the F_2s. To have a contig that spans the flanking markers, we constructed a bacterial artificial chromosome library with a large insert size (175 kb), which makes the preparation of contigs and their DNA very convenient. An efficient test system of complementation with genome fragments by *Agrobacterium* transformation was also developed.

To understand the mechanism of gene-for-gene interaction between plant and pathogen, cloning of the resistance gene is indispensable. We have targeted rice blast resistance genes for positional cloning because

- the genome size of rice is the smallest among the major crops, ca 400 Mb;
- the physical length of the genetic distance per unit is small, ca 200 kb cM^{-1};
- rice has the most established transformation system among the monocotyledons;
- there are abundant restriction fragment length polymorphism (RFLP) markers and genetic information about rice blast resistance.

Three indica-derived resistance genes introgressed to japonica, ***Pi-ta*^{2}, *Pi-ta, and Pi-b,*** were selected as the targets for positional cloning because they provide excellent conditions for mapping with polymorphic markers. Their positions were precisely determined and we were able to establish the bacterial artificial chromosome (BAC) library and an efficient transformation system for rice.

Materials and methods

Graphical genotype analysis of the *Pi-b* position

The pedigrees of the introgression of *Pi-ta* and *Pi-ta²* into japonica cultivars to develop their near-isogenic lines (NILs) are presented in Figure l. RFLP probes were provided by A. Saito (Saito et al 1991) and Cornell University (Causse et al 1994). Plant genome DNAs were prepared using K-xanthogenate to solubilize the cellulose.

RAPD marker screening

Randomly amplified polymorphic DNA (RAPD) sequence markers common to the NILs were searched from 800 random primers. Analysis was done as described by Williams et al (1990).

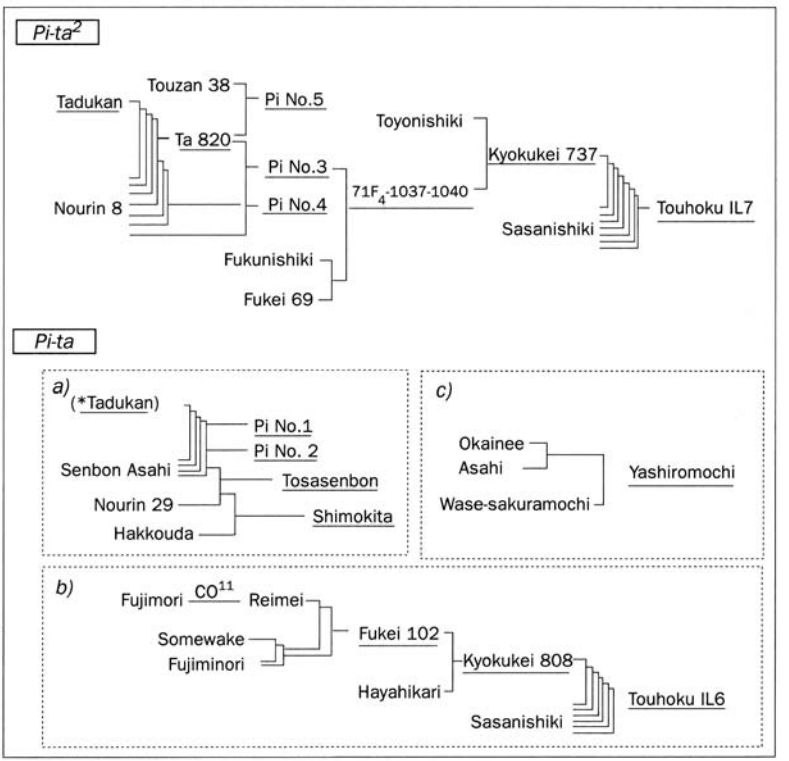

Fig. 1. Pedigrees of the introgression of *Pi-ta* and *Pi-ta²* into japonica cultivars to develop their NILs. Cultivars underlined are resistant. Asterisks indicate indica donor parents. Dashed lines of *Pi-ta* are doubtful connections.

F_2 analysis for precise mapping of *Pi-b*

Map distances between the *Pi-b*, *Pi-ta*^{2}, and the nearby RFLP/RAPD markers were determined by analyses of 122/170 susceptible F_2 individuals, respectively.

Long fragment RFLP analysis

Protoplasts of green rice leaves were prepared as described by Nomura and Kawasaki (1992). High-molecular-weight DNA was isolated in the agar block by protein digestion of the embedded protoplasts, and then digested with rare-cutter restriction enzymes. DNA fragments were contour-clamped homogeneous electric field (CHEF)-electrophoresed and subjected to Southern blot analysis with the nearby markers.

Construction of cosmid and BAC genome libraries

Rice genome libraries were constructed with a cosmid vector pWE15 and a BAC vector. High-molecular-weight DNAs were prepared by mild liquid digestion of the frozen leaf powders for the cosmid, or by in-agar digestion of green leaf protoplasts. These were CHEF-electrophoresed to 45-55 kb or 200-500 kb, respectively.

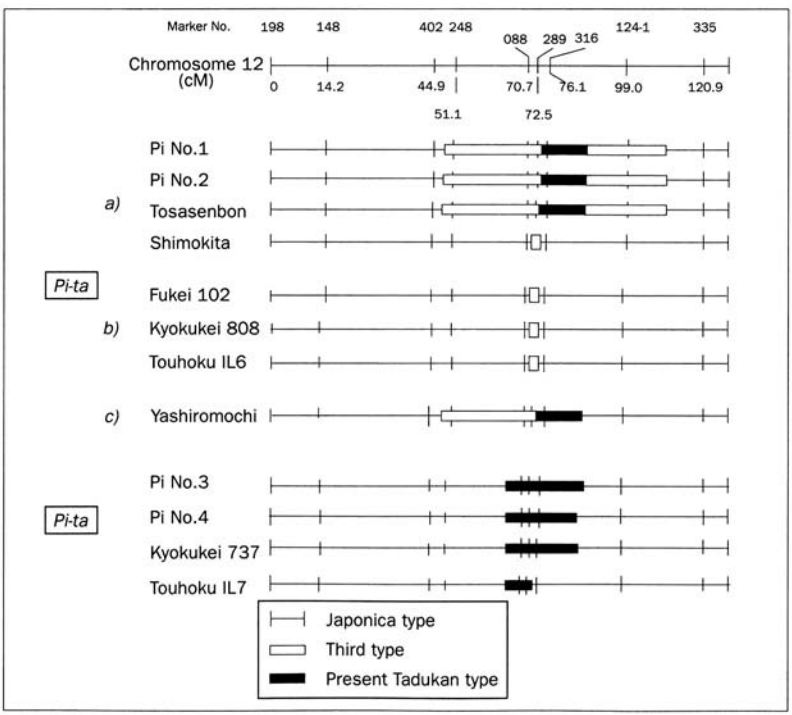

Fig. 2. Graphic genotype representation of the indica-derived chromosome regions in the NILs of *Pi-ta* and *Pi-ta*^{2}.

Results

Pi-ta^2 was mapped on chromosome 12 using graphical genotype analysis (Fig. 2). Through F_2 analysis of 170 recessive homozygous individuals, *Pi-ta*^{2} was found to cosegregate with four RFLP markers. and to be flanked with 0.3 and 0.7 cM distant RAPD and RFLP markers on the left and right side of the gene, respectively (Fig. 3). The left side markers were estimated to be within 40 kb from a restriction fragment analysis with CHEF-EP and rare-cutter enzymes. Theretore, we have constructed a cosmid library at first and a contig is being constructed. Simultaneously, the *Pi-ta* gene, which was also shown to be allelic with *Pi-ta*^{2} within 0.7 and 1.4 cM by the graphical genotype analysis (Fig. 2a), will be pursued in the same manner.

Pi-b was mapped on the OcM end of chromosome 2, and was also found to cosegregate with an RAPD and an RFLP marker, and 0.5 and 1.9 cM of the flanking markers were also found on the right and left sides of the map, respectively. This gene will be also a good candidate for positional cloning.

To facilitate the physical mapping of the genes, a BAC library of the rice genome was constructed. Its DNA can be prepared with an automatic plasmid extractor (Kurabo P1-100 sigma) after one night of culture, and antibiotic selection enables simple manipulation of the bacterial clones. BAC is also known for the low percentage of chi-

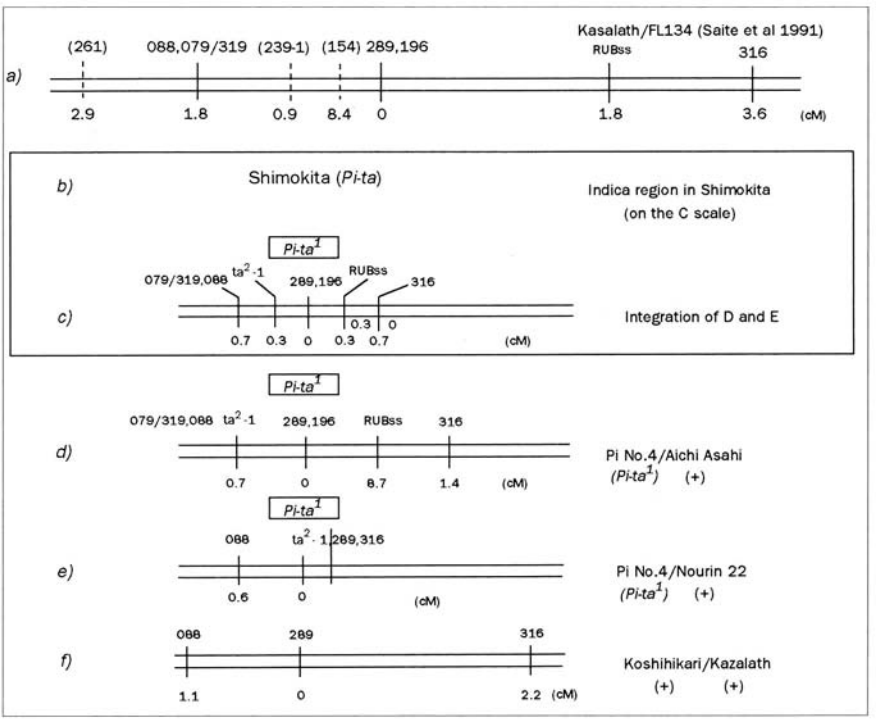

Fig. 3. Fine-mapping of *Pi-ta* and *Pi-ta*^{2} by F_2 analyses.

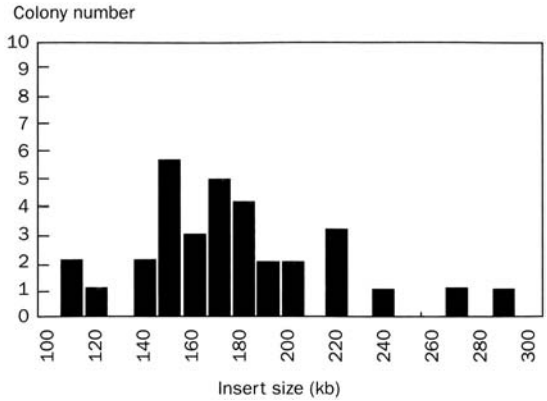

Fig. 4. Insert size distribution of the rice BAC library. Average insert size is 175 kb.

mera clones (Shizuya et a1 1992). These characteristics are in good contrast with the yeast artificial chromosome (YAC) library.

We have constructed a rice-genome BAC library with the average insert size of 175 kb (Fig. 4), much larger than the reported ones of 100-120 kb, with the efficiency of 10^5 clones μg^{-1} DNA. The clones were kept in the 96 hole plates, and assorted on 7 high-density membranes as the 6 × 6 array on the 8 × 12 hole positions of the microplate. Thus we assorted 21,504 clones corresponding to about 8.8 genome of the rice (430 Mb). Average 8-9 clones were positively selected by the hybridization of the above reported nearby markers of *Pi* genes. The walking of the clones was easily done by the thermal asymmetric interlaced-polymerase chain reaction method (Liu et a1 1994).

For the complementation test of the candidate genome fragments, efficient rice transformation system with *Agrobacterium* was established following the method of Hiei et a1 (1994). To facilitate the subcloning of the BAC clone and transformation via *Agrobacterium,* shuttle vectors mediating between *Escherichia coli* and *Agrobacterium* were also developed.

By using these BAC and transformation system, an efficient gene cloning system will be established in rice, and it will make the rice as the real experimental model plant of monocotyledons.

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Molecular cloning of a rice blast resistance gene and its genetic transformation

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A new gene that codes for the rice proteinase inhibitor has been amplified and cloned from *Oryza sativa* var. *japonica* (cv Zhonghua 8) using the polymerase chain reaction technique. The primers were designed based on the terminal amino acid sequences of rice proteinase inhibitors and the preferred codons of rice genes. The gene contains 408 bp and encodes 133 amino acid residues. The deduced amino acid sequence with a duplicated Bowman-Birk type proteinase inhibitor structure and active sites specific to trypsin has relatively high homology with that of proteinase inhibitors from wheat and beans. Its deduced amino acid sequence shares 74.8% homology with a rice bran trypsin inhibitor sequence reported previously. Chimeric plasmids containing the ubi promoter from maize and the DNA coding for transit peptide synthesized according to a pathogen-related protein from tobacco were constructed. The microprojectile transformation of rice embryogenic calli and suspension culture cells has been conducted. An antisense construction of this gene has also been transferred to rice.

Plant proteinase inhibitors play an important role in a plant's defense system to ward off diseases. However, there have been very few reports on whether these inhibitors can aid in the resistance to fungal infection (Kolaczkowsk 1980). When it was found that a proteinase inhibitor, isolated from a rice grain, could inhibit the growth of certain pathogenic fungi, the gene that encodes this proteinase warranted closer study.

Materials and methods

Germplasm

Oryza sativa var. *japonica* cv Zhonghua 8 and Taibei 309 were provided by the Chinese Academy of Agricultural Sciences and The Scripps Research Institute, respectively.

PCR amplification

With a few modifications, total rice DNA was extracted as described by Doyle and Doyle (1990). The primers were designed based on rice preferential codons (Murray et al 1989) and the amino acid sequences of the rice proteinase inhibitor (Liu et al 1994). They were synthesized on an ABIDNA synthesizer. Polymerase chain reaction (PCR) amplification was performed in a 100-µl reaction solution with 100 ng of rice total DNA as the template; 5' and 3' primers, 50 pmol, respectively; dNTP, 2.5 mmol each; 10x buffer, 10 µl; 2 unit Taq polymerase (Promega PCR kit); sealed with 50 µl mineral oil (Sigma Co.). Thirty-five cycles were carried out in a DNA thermal cycler (PR90 Institute of Genetics, Beijing). The amplified product was recovered from agarose gel.

Cloning, DNA sequencing, and analysis

The amplified fragments digested by *Eco*RI and *Cla*I were cloned into the *Eco*RI and *Cla*I sites of plasmid pBluescript SK (Stratagen Co.). *Escherichia coli* DH5α (Sino-American Co.) was used as the host bacterium and the recombinant plasmids with a 400 to 500-bp insertion were selected. The inserted fragments were sequenced on an ABI 373A DNA automatic sequencer by the dideoxy chain termination method using the Taq Dye Primer Cycle Sequencing Core Kit (PE-ABD). One fragment that we cloned, pBrbbi, was analyzed intensively. Sequence analysis was completed with DNASIS (Hitachi Software Engineering Co. Ltd). The sequence comparison was performed using a CD-ROM disc of the EMBL Data Library (EMBL Nucleotide Sequence Database Rel. 35; SWISS-PROT protein sequence database Rel. 25).

Construction of prokaryotic expression vectors

The *Eco*RI and *Xho*I fragment containing the target gene was cloned into the pTrcHisB (Invitrogen), which was digested by *Eco*RI and *Xho*I, forming the recombinant pTrbbi. The orientation of pTrbbi was confirmed by the digestion of the same enzymes.

Construction of eukaryotic expression vectors

The *Xho*I and *Bam*HI fragment containing the target gene was ligated with the large fragment of the *Sal*I- and *Bam*HI-partially digested pHAC17 (The Scripps Research Institute). The recombinant was named prbbiU.

The *Hin*cIII and *Bam*HI fragment containing the *rbbi* gene was cloned into the *Sma*I- and *Bam*HI-digested pPSK7 (The Scripps Research Institute) with the sequence coding for a transit peptide of a pathogen-related (PR) protein from tobacco. The recombinant was named pPRrbbi. The orientation and open reading frame (ORF) were confirmed by sequencing. The *Xho*I and *Bam*HI fragment containing the transit peptide and *rbbi* gene was ligated with the large fragment of *Sal*I- and *Bam*HI-partially digested pHAC17. The construction was named pPrbbiU.

The *Hin*cII and *Bam*HI fragment containing the *rbbi* gene was cloned into the *Sma*1I- and *Bam*HI-digested pRTL202 (The Scripps Research Institute) containing an IgG heavy chain transit peptide gene. The orientation and ORF of the recombinant prbbi202 were confirmed by sequencing. The *Nco*I and *Bam*HI fragment containing

the IgG heavy chain transit peptide gene and *rbbi* gene was cloned into the *Small* site of pBluescript SK, and then digested with *Eco*RI and inserted into *Eco*RI-digested pMON316. The orientation of the recombinant, pMR2, was confirmed by digestion with *Eco*RI and *Cla*I, respectively.

Construction of expression vector with antisense *rbbi* gene

The *Bam*HI fragment containing *rbbi* gene was cloned into the *Bam*HI-digested pAHC 17. The antisense orientation was confirmed by *Nco*I digestion.

Expression of *rbbi* in *E. coli*

The cultures of *E. coli* DH5a with pTrbbi was grown in LB medium at 37 °C and treated with IPTG for inducing the expression of the transformed gene. The expressed products were examined on sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) stained with Coomasie brilliant blue R250.

Plant transformation

The recombinants, prbbiU, pPRrbbi, prbbi202, and pMR2, were mixed, respectively, with 0.88 µg hygromycin and selective marker pHX4 (The Scripps Research Institute), and coated with gold particle (1.0 µm and 1.8-2.3 µm, 60% each). The mixture was shot into rice calli with the BIOLISTIC PDS-1000/He particle delivery (BIO-RAD) system at 1100 PIS. The transgenic calli were selected and regenerated under the conditions as described by Li et al (1993). The gene integrated into regenerated rice plants was detected by PCR amplification under the same conditions stated above except that the template was the DNA extracted from the regenerated transformed rice plants.

Results and discussion

Sequence and structure analysis of *rbbi*

Because the primers were based on amino acid sequences, the PCR-amplified products were a mixture of specific and nonspecific DNA fragments. The DNA fragments, each about 400 bp, were recovered from agarose gel. The clone pBrbbi has a complete ORF. The gene has 408 bp encoding 133 amino acid residues (Fig. 1). The deduced amino acid sequence was compared with those in the EMBL Swiss-port data base and it was found to be a new member of the Bowman-Birk type proteinase inhibitor (BBI) family. It shared a 74.8% sequence homology with a trypsin inhibitor isolated from rice by Tashiro et al (1987). It also had sequence homology with BBI in other plants. Further sequence comparison showed that it had the typical conserved sequence of BBI (Fig. 2). BBI was first used to describe a group of double-headed proteinase inhibitors isolated from legume plants (Laskowski and Ikunoshin 1980). Odani et al (1986) included trypsin inhibitors isolated from wheat germ in the BBI family. When the sequence described in this paper was compared with those of BBIs from soybean, wheat, and rice bran, it was found that it had four typical domains, i.e., positions 1-34 (I), 35-69 (II), 70-98 (III), and 99-133 (IV), of which I and III are homologous to the

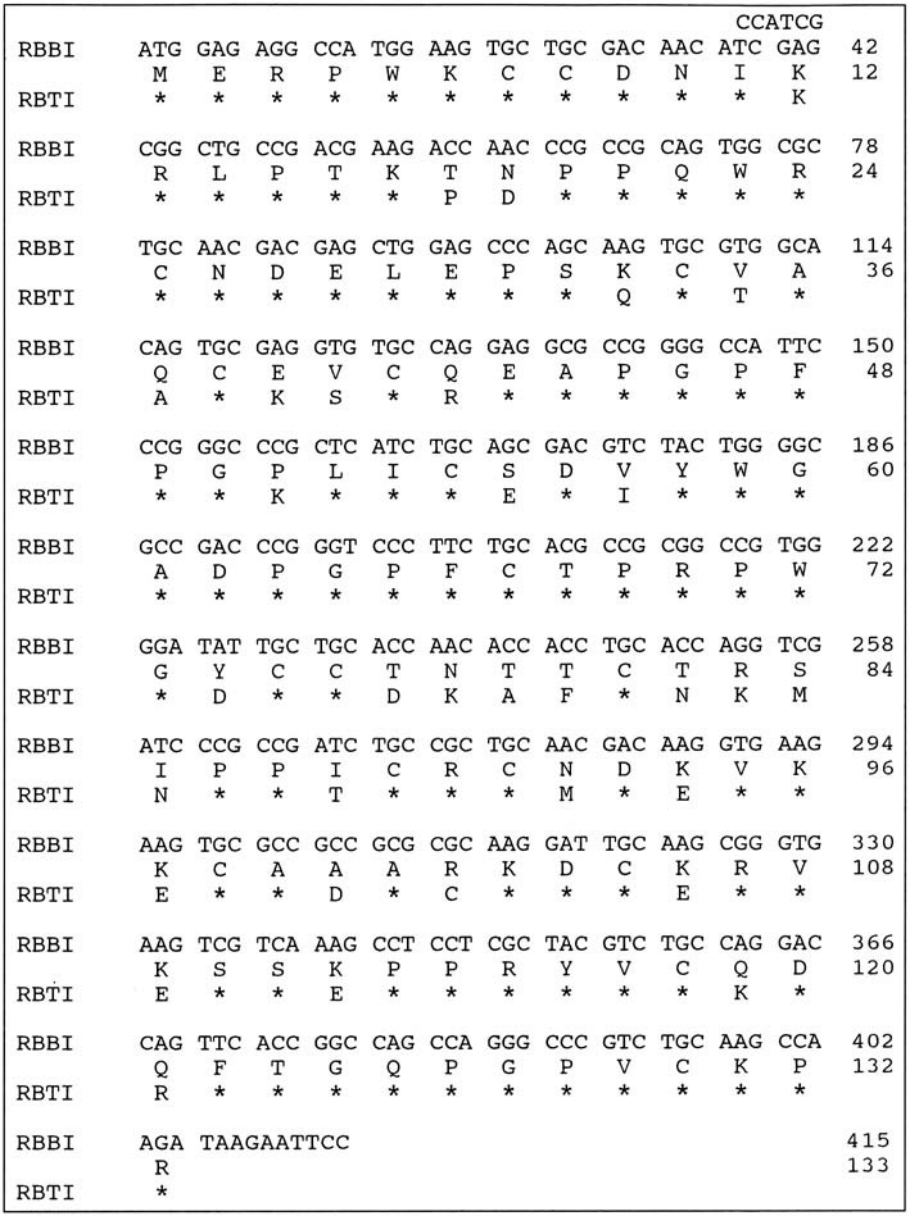

Fig. 1. The DNA sequence of rice RBBI and the comparison of its deduced amino acid sequence with that of bran trypsin inhibitor (RBTI). An asterisk indicates the same amino acid as the one above it.

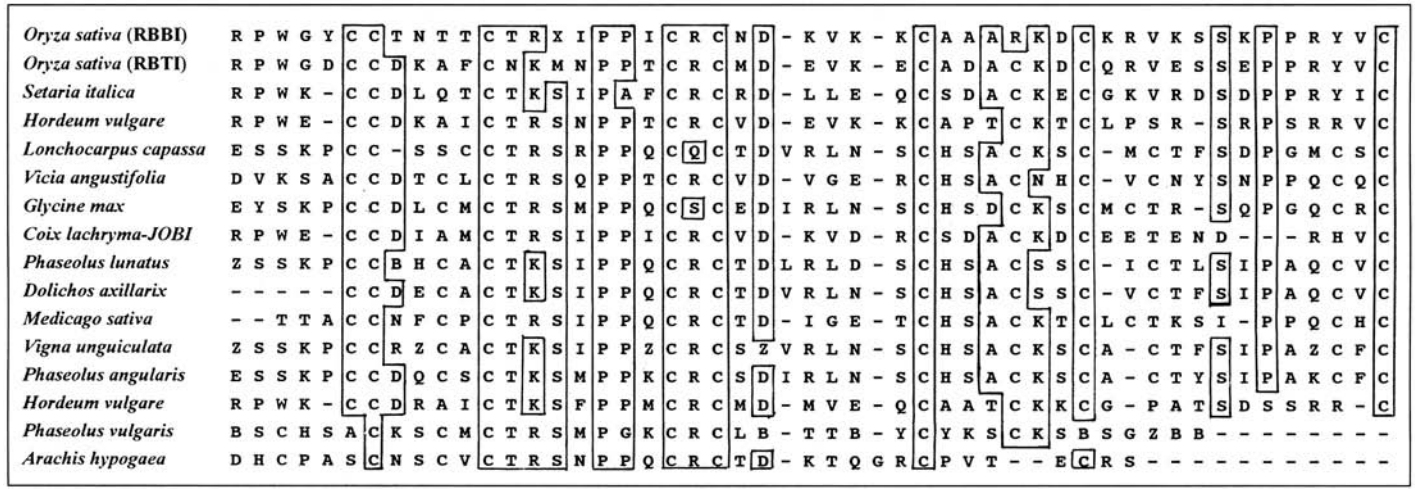

Fig. 2. Comparison of homologous regions among members of BBI family. Identical residues are enclosed in blocks.

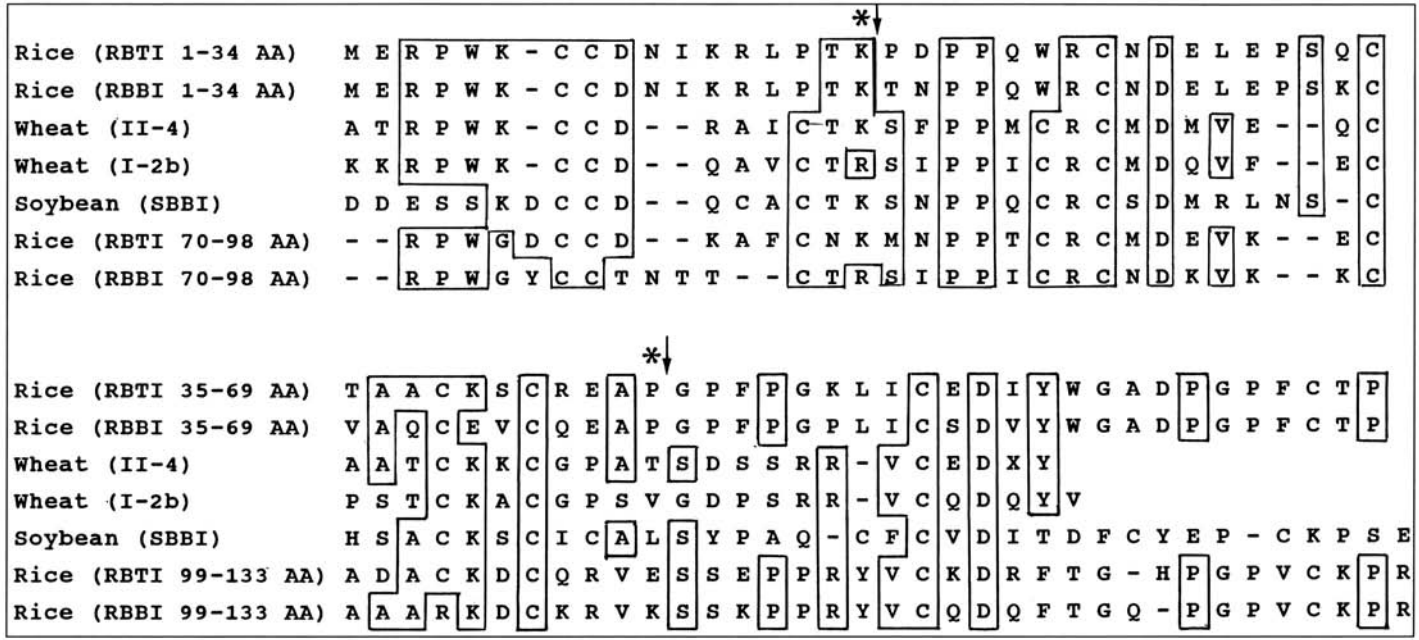

Fig. 3. Comparison of active domains among RBBI, wheat germ trypsin inhibitors (I-2b and II-4), and soybean BBI. ¯: the putative reactive sites; *: P1 positions; identical residues are enclosed in blocks.

first domain of soybean's BBI, and II and IV are homologous to the second domain of soybean's BBI. It has been reported that the inhibition specificity of proteinase inhibitors was usually determined by the amino acid composition at the active site. If the active site is composed of lysine (K) or arginine (R), its activity will be related to the inhibition of trypsin or similar substrates (Jering and Tschesche 1976, Tan and Kaise 1977). RBBI has three putative active sites at positions 17 (K), 83 (R), and 109 (K), respectively (Fig. 3). It has been postulated that the double-headed BBIs have evolved from a single-headed BBI through gene duplication (Odani et a1 1986). This study supports the conclusion that the genes of the BBI family have evolved mainly via gene duplication.

Expression of *rbbi* gene in *E. coli*

The expression of pTrbbi was induced by adding IPTG to culture medium. The expressed products were examined on SDS-PAGE. A band of about 15 kd specifically appeared in the transformed *E. coli* total protein.

Transformation of rice

The *rbbi* gene was transferred into rice and 57 regenerated rice plants were obtained. The positive transformants were confirmed by PCR amplification of the *rbbi* gene. Twenty-two rice plants had the specific amplified product. Because the proteinase inhibitor has been used as an agent against insects, it is postulated that the product of the *rbbi* gene may have the same function. The gene is being transferred into dicot plants. These transformed plants will be exposed to insect pests to see if any resistance has been transferred along with the gene.

The hyphae of most pathogenic fungi grow between the cell walls of their hosts. To prevent fungal infection effectively, the DNA encoding transit peptide of PR protein from plants and IgG heavy chain from animals have been linked with *rbbi* gene, respectively. The seeds from these rice plants have been harvested and plants generated from them will be exposed to pathogenic fungi in the greenhouse.

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Notes

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VIII. MOLECULAR GENETICS
OF CYTOPLASMIC MALE STERILITY

Molecular genetic studies on the rice mitochondrial genome

K.K. Narayanan, P. Senthilkumar, S. Venmadhi, G. Thomas, and J. Thomas

Our investigations show that the rice mitochondrial (mt) genome is organized as several circular, subgenomic molecules or mt chromosomes. More than 12 genetic loci have been located on the different mt chromosomes and the physical maps of two such entities are complete. A scrutiny of specific genetic regions, their organization, and expression indicated that the pseudogene, *cob2,* is absent in the male sterile cytoplasm (WA). Interestingly, this region is differentially organized in the fertile lines examined. Most of the mt genetic regions are transcribed separately while the *cox3* and *orf25* loci are transcribed as a single dicistronic transcript. Comparison of the genetic organization in fertile and sterile rice line mitochondria showed that the *cox1* locus was polymorphic. Studies are in progress to understand the role of this rearrangement in the elaboration of male sterility.

The plant mitochondrial (mt) genomes have a complex multipartite organization. They are variable in size between species, ranging from about 200 kb in *Brassica* spp. to more than 2000 kb in some cucurbits. This is many times bigger than animal or fungal mt genomes; however, the genetic information carried by mt genomes across taxa is conserved to a great degree. The plant mt genomes are known to code for more than 20 proteins, about 15 tRNAs and three kinds of ribosomal RNAs. In addition, there are several open reading frames (ORFs) conserved between species.

The rice mt genome has a genetic complexity of about 300 kb (Narayanan et al 1993) and this is smaller than that of several other cereals like wheat (more than 400 kb; Quetier et al 1985) or maize (more than 500 kb; Lonsdale et al 1984).

The rice mt genome is organized as mt chromosomes

The plant mt genomes were long believed to be organized as master circle molecules from which subgenomic molecules arise through homologous recombination (Lonsdale 1989). Based on this model, the physical maps of master-circle molecules have been constructed using overlapping cosmid clones of mt DNA fragments. However, there is no direct evidence, as yet, to prove that the master circle exists in the in vivo plant mitochondria.

We have used pulsed-field gel electrophoresis to analyze the physical organization of the rice mt genome. Our experimental strategy involves partial digestion of the rice mt DNA using rare-cutting restriction enzymes that have sites between linked genetic regions and Southern analysis using probes specific to those genetic loci (Fig. 1). The size of the largest linear fragment to which these probes hybridize define the size of the molecular entity on which the genetic loci are located. The data on the

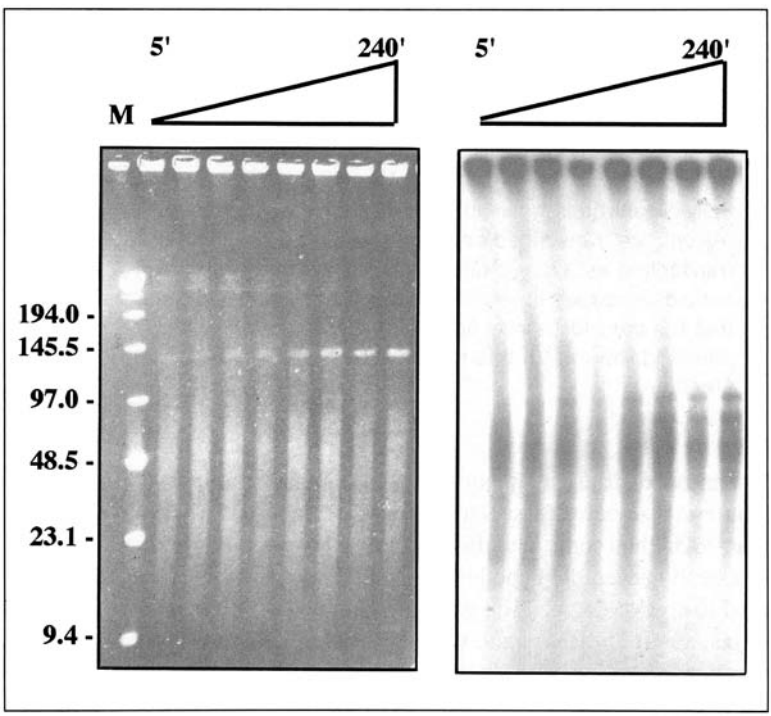

Fig. 1. PFGE mapping of the rice mt genome. IR36 mt DNA was digested for 5-240 min with *Asc*I and the partial fragments were resolved on a CHEF gel. Left panel, EtBr stained gel. M, size marker. The fragment sizes in kb are indicated on the left. Right panel, autoradiogram showing partial fragments hybridizing to *cox3* specific probe. The largest linear fragment hybridizing to the probe denotes the size of the molecular entity on which the gene locus is located.

sizes of the partial fragments, which hybridize to the gene probes, some of which are common to both the probes, and others, unique to either of them, are then used to construct a physical map of the molecular entity.

Using this approach, we did not find any master circle-sized molecule in the native mt DNA preparation. On the other hand, the genome was organized into at least five subgenomic circles of sizes 130, 117, 95, 70, and 56 kb. These molecules, we believe, are independently replicating and carry a set of gene loci forming a linkage group. Hence, we call them mt chromosomes. However, there is no evidence to suggest that these entities have a complex physical structure like the nuclear chromosomes.

The 117-kb mt chromosome has six genetic loci—*cox1, atp1, rrn26, cob2,* and a copy each of *rrn18* and *atp6.* Its physical map has been determined (Narayanan et a1 1993). The map of the 95-kb mt chromosome carrying the *cox3* and *orf25* loci is also ready. The major loci on the other mt chromosomes are *cob1* and possibly *nad2* on the 130-kb molecule, *cox2* and a copy of *atp6* on the 70-kb molecule, and *atp9* on the 56-kb molecule. The search for additional subgenomic molecular entities and locations of more gene loci on these mt chromosomes is in progress.

The *Cob2* pseudogene is differentially organized in different rice lines

Plant mt genomes commonly contain rearranged genetic regions; in many instances, sequences of expressed genes are involved in such rearrangements (Bailey-Serres et al 1986, Schuster and Brennicke 1986, Morikami and Nakamma 1987, Fragaso et al 1989). In IR36 rice, the apocytochrome b gene (*cob*) is present in two copies, an intact copy, *cob1,* and a chimeric copy or pseudogene, *cob2.* The fragment carrying the *cob2* copy does not hybridize to the *cob1-3'* specific probe (Fig. 2) suggesting that the sequence divergence of *cob2* from *cob1* is at the 3' end. The rice *cob2* was first cloned and characterized by Kaleikau et a1 (1 992).

We have examined the organization of the *cob1* and *cob2* regions in several lines of rice. The *cob1* organization was highly conserved whereas the *cob2* varied between rice lines (Narayanan et al 1995). The pseudogene copy could not be detected in the CMS cytoplasm, WA, using Southern hybridization (Fig. 3). However, the region that could have recombined with *cob1* to produce *cob2,* was present. Interestingly, the *cob2* organization was different in the different fertile lines examined. All the *cob2* copies diverged from the *cob1* sequence at a conserved "break-point." However, the 3' sequence of *cob2* from different lines was variable. In all *cob2* regions examined, there was a conserved 192-bp sequence immediately downstream of the breakpoint, which was not part of the *cob1* sequence. It is proposed that the *cob2* regions could have been produced by recombination or insertion events involving *cobl* and the 192-bp fragment which may be present at different locations in the mt genomes of various rice lines.

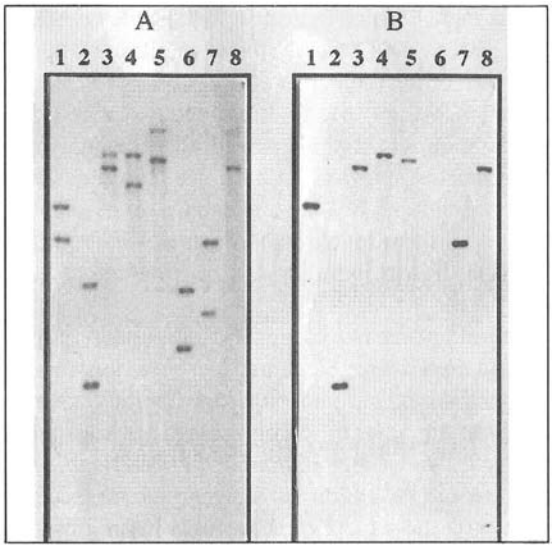

Fig. 2. *Cob2* diverges from the *cob1* sequence at the 3' end. IR36 mt DNA was digested with the restriction enzymes, *Stu*I, lane 1; *Xho*I, lane 2; *Xba*I, lane 3; *Bam*HI, lane 4; *Bgl*I, lane 5; *Eco*RI, lane 6; *Sma*I, lane 7, and *Sal*I, lane 8. a) Autoradiogram with probe specific to *cob1,* 5' region. b) Autoradiogram with probe specific to *cob1,* 3' region. The additional band in A is the pseudogene *cob2.*

The *Cox3* and *Orf25* loci are cotranscribed

Polycistronic mRNA transcripts are not uncommon in plant mitochondria. Dicistronic transcripts for pairs of protein-coding genes have been found in the mitochondrion of several species including *Zea mays* (Dewey et al 1986). *Oenotheru* (Wissinger 1988), and *Triticum* (Gualberto et al 1988). Such messages are used for coordinate regulation of genes in prokaryotes; for example, the genes constituting the lactose operon in *Escherichia coli* are transcribed as a single mRNA.

In rice, the loci encoding the third subunit of cytochrome oxidase, *cox3,* and an ORF conserved in many plant species, *orf25,* are cotranscribed (Liu et al 1992). The cotranscript contains an intergenic region 379 bp long and a long 5' untranslated region as well as a postulated 3', double stem-loop structure. The gene products of *cox3* and *orf25,* the gene of unknown function, do not seem to be related in function and there is no evidence to suggest that they are required in identical stoichiometry as in other related proteins in biochemical pathways. Therefore, the biochemical significance of the *cox3-orf2.5* cotranscript is not yet clear.

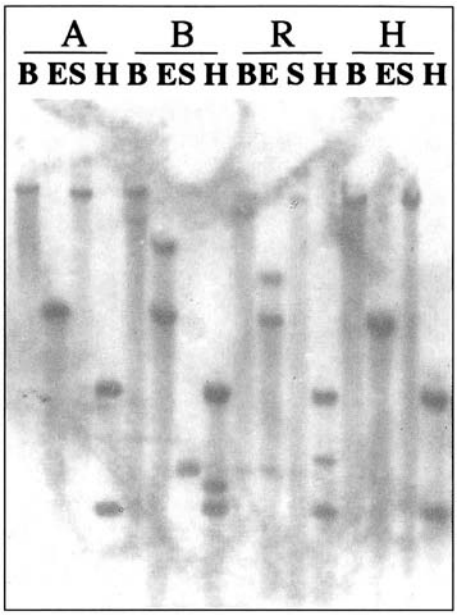

Fig. 3. *Cob2* is absent in the male-sterile line. Mitochondrial DNA from the male sterile line, A; its maintainer, B; the restorer line, R; and the fertility restored line, H, was digested with *Bam*HI (B), *Eco*RI (E), *Sal*I (S) and *Hin*dIII (H). The fragments were resolved in an agarose gel, blotted on to a nylon membrane (FlashTM, Stratagene) and hybridized to a *cob1/cob2* probe. Hybridizing fragments were detected using a chemiluminescent method (Flash Prime kit, Stratagene). The additional band in the lanes with maintainer and restorer mt DNA is the *cob2* copy.

Gene rearrangements and cytoplasmic male sterility

There are many reports of mt genetic rearrangements being associated with cytoplasmic male sterility (CMS) in plants (Hanson 1991). We have examined the organization of several mt genetic loci in the WA-type CMS line of rice, its maintainer, the restorer line, and the fertility restored line. While most of the loci showed a conserved genetic organization, a major rearrangement was observed for the gene encoding subunit 1 of cytochrome oxidase, *cox1*. A few other loci such as *cox2,* which are present in more than one copy in the rice mt genome, showed a conserved organization for one of the copies but a rearrangement in the other copy (Fig. 4).

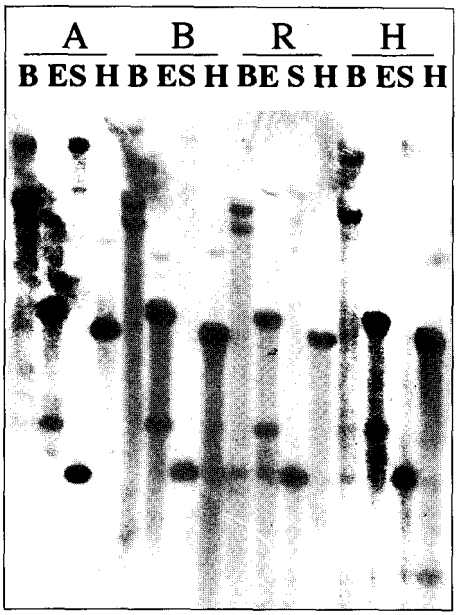

Fig. 4. One copy of *cox2* is rearranged between the fertile and sterile lines. The same blot as in Figure 3 was probed with a rice *cox2* specific probe and the hybridizing fragments detected using the same chemiluminescent method. While one copy of this gene is conserved in the different lines, the other is different in the fertile (B and R) and sterile (A and H) cytoplasms.

We have examined the transcription of the *cox1* locus and there appears to be a difference in the size of the transcript between the fertile and sterile lines. Experiments to establish whether the *cox1* rearrangement and the consequent changes in its expression is responsible for CMS in rice, or whether it is merely incidental, are in progress.

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Agrobacterium-mediated production of transgenic plants from mature embryos of commercial rice varieties

J. Zhang, R.-J. Xu, M.C. Elliott, and D.-F. Chen

An efficient, rapid, and reproducible system has been established for transformation of commercial varieties of rice via *Agrobacterium tumefaciens.* Calli induced from mature embryos of varieties E-yi 105, E-Wan 5, and Zhong-Shu-Wan-Geng (ZSWG) were cocultivated with the LBA4404 strain carrying the superbinary plasmid pTOK233. The frequency of transient gene expression in infected calli was as high as 100% under optimal conditions. Calli selected on hygromycin (50 mg L^{-1}) medium for 6-8 wk showed uniform *uidA* gene expression. One hundred mature embryos of E-yi 105, E-Wan *5,* and ZSWG yielded 107, 95, and *35* transgenic plantlets, respectively. Most transgenic plants of E-yi 105 reached fertile maturity in a controlled environment cabinet. Leaves, roots, immature embryos, anthers, and endosperm from these transgenic plants expressed the *uidA* gene. This rice transformation system provides a tool for introducing agronomically important genes into commercial varieties of rice and also offers the possibility of studying the mechanism of T-DNA transfer from *A. tumefaciens* into cells of monocotyledonous plants.

To fully exploit gene manipulation technology in rice, it is necessary to develop an efficient and reproducible system for transformation of commercial rice lines. E-yi 105, E-Wan 5, and Zhong-Shu-Wan-Geng (ZSWG) are elite japonica rice *(Oryza sativa* L.) varieties that are grown on more than 3.3 million, 600,000, and 600,000 ha of southern China, respectively. They yield 7.5 t ha^{-1} and are resistant to most rice diseases and pests, but are attacked by the brown planthopper.

Numerous attempts have been made to use *Agrobacterium* to transform rice plants, but only recently has the system produced transgenic plants (Chan et al 1993, Hiei et al 1994). Here, we report the successful application of this approach to commercial rice varieties.

Materials and methods

Plant materials, bacterial strain, and plasmid

Dehusked mature seeds of the three rice varieties were sterilized in 70% ethanol for 2-5 min and then transferred into 40% (v/v) 'Domestos' (Lever Bros. Ltd., UK) bleach solution for 30 min with shaking. The seeds were rinsed several times with sterile deionized water. Sterilized seeds were plated on callus induction medium [LS basal medium (Linsmaier and Skoog 1965) supplemented with 2.5 mg 2,4-D L^{-1}, 30 g maltose L^{-1}, 300 mg casein hydrolysate L^{-1}, 300 mg yeast extract L^{-1}, and 2.5 mg Gelrite L^{-1}, pH 5.8; LSI medium] at 28 °C in the dark.

A. tumefaciens strain LBA4404 harboring the superbinary vector pTOK233 was used in all experiments. The pTOK233 plasmid has been described in detail by Hiei et al (1994); it carries a neomycin phosphotransferase gene (*npt* II), a hygromycin phosphotransferase gene (*hpt*), and an intron-b-glucuronidase gene (*uidA-int*). The *hpt* and *uidA-int* genes were under the control of the CaMV35S promoter, while the *npt* II gene was fused with a *nos* promoter. Insertion of an intron into the *uidA* gene prevented expression of the gene in *A. tumefaciens* (Hiei et al 1994). The bacteria were grown in liquid AB medium (Chilton et al 1974) containing hygromycin (50 mg L^{-1}, Sigma) and kanamycin (50 mg L^{-1}, Sigma) at 28 °C for 3-5 d. For inoculation of rice calli, the bacteria were suspended at a density of 1-3 × 10^9 cells ml^{-1}.

Production of fertile transgenic rice plants

Calli of the three varieties were immersed in 10-15 ml of *A. tumefaciens* suspension at room temperature for 10-15 min. Infected calli were cultured on cocultivation medium [LSI medium with acetosyringone (AS; Aldrich Chem. Co., USA) added at various concentrations (0, 50, 100, 200 μM), without yeast extract. at pH 5.2; LSC medium]. After 5-6 d cocultivation, calli were plated on selection medium [LSI medium supplemented with hygromycin (50 mg L^{-1}) and cefotaxime (250 mg Claforan L^{-1}; Roussel Laboratories, UK), pH 5.8; LSS medium] and incubated at 28 °C in the dark for 3 wk. Hygromycin-resistant (hyg^R) calli were subcultured onto fresh LSS medium every 3 wk. The hyg^R calli were cultured on differentiation medium [LSS medium with 3 mg 6-benzylaminopurine (BAP) L^{-1} instead of 2, 4-D and 6.25 g Gelrite L^{-1}, without yeast extract, at pH 5.8; LSD medium] at 25 °C in constant light (3.0 W m^{-2}) for 3-4 wk to produce green buds. The frequency of green buds produced from the hyg^R calli was recorded after 4 wk. Green buds and/or shoots were transferred onto development medium (differentiation medium with the Gelrite concentration reduced to 2.5 g L^{-1}) for further shoot development. After being transferred to rooting medium [half strength LS medium containing hygromycin (50 mg L^{-1}) without plant growth regulators; 1/2 LSO medium], plantlets developed vigorous root systems. Robust transgenic rice plants were potted with a 3:1:1 (v:v:v) mixture of Levington M3 compost (Fisons, UK), John Innes No. 3 compost (Croxden Horticulture Products Ltd., UK), and standard Perlite (Silvaperl Products Ltd., UK). These plants were grown in a controlled environment cabinet (Fitron, Sanyo Gallenkamp PLC, UK) at 70-80%

humidity, with 10-h photoperiods of 25 W m^{-2} at 28 °C, with night temperatures of 24 °C where they matured, flowered, and set seeds.

Histochemical assay for ß- glucuronidase (GUS) activity

GUS activity was assayed histochemically by a procedure described by Jefferson (1987). Calli or plant explants were incubated at 37 °C in staining solution containing 20% methanol overnight (14-16 h). The stained materials were fixed in 70% ethanol. GUS activity was revealed by a blue color.

Results and discussion

Plant cell competence for *A. tumefaciens* transformation

When infected calli were incubated at 25 °C, the blue GUS foci were observed only in rapidly growing areas of the calli (light yellow) but not in the slow growing areas of the calli (brown yellow). Neither calli pretreated at 4 °C for 1 d before immersion in the bacterial suspension nor infected calli incubated at 4 °C showed any sign of *uidA* gene expression. It was inferred that the presence of dividing cells in the calli was an important aspect of competence of the rice cells to respond to *Agrobacterium.*

Effect of acetosyringone on T-DNA gene expression in transformed rice calli

Acetosyringone (AS) induces the transcription of the virulence genes of *A. tumefaciens* (Zupan and Zambryski 1995). The influence of AS on T-DNA gene expression in the infected rice calli was tested by adding various concentrations (0, 50, 100,200 µM) of the compound to the cocultivation medium. Without AS treatment, only 10-15 GUS spots were detected on a callus, while maximum GUS activity (400 blue foci per callus) was recorded when AS was 100 µM. This optimal concentration of 100 µM AS was, therefore, used for the transformation experiments.

Transient *uidA* gene expression in infected calli

To fully understand the time course of T-DNA transfer from *Agrobacterium* into plant cells, the GUS assay was used to test infected calli for 1-8 dafter bacterial inoculation. The number of GUS foci rose from day 1 to a peak at day 5 after infection. Therefore, it is assumed that the T-DNA transfer from bacterial cells into rice cells started on day 1 of infection and reached a maximum by day 5. Hence, infected calli were transferred onto selection medium after 5 or 6 d of infection.

Development of transformed callus

Infected calli, which had been selected on hygromycin medium for 3-4 wk, were subjected to the histochemical GUS assay. Many of the calli did not become uniformly blue. This indicated that they were still chimeric in character. When the period of selection was extended to 6-8 wk, 95% of hyg^R calli were observed to express the *uidA* gene uniformly. Clearly, exposure of infected calli to hygromycin for 6-8 wk

permitted the growth of transformed cells while inhibiting the growth of untransformed cells. This uniform *uidA* gene expression suggested that the hyg^R calli originated from single, progenitor cells. In contrast, calli from uninfected mature embryos stopped growing during 6-8 wk of hygromycin selection.

The hyg^R calli were subjected to the GUS assay after 12, 15, and 18 wk of hygromycin selection. The data showed that the longer the calli were maintained on the selection medium, the higher the frequency of uniform GUS activity obtained. But regeneration capacity of the hyg^R calli declined after long-term tissue culture (18 wk on selection medium; data not shown).

Production of fertile transgenic plants

The formation of green, compact, and embryogenic callus of the three varieties occurred within 2 wk of transferring proliferated hyg^R calli onto differentiation medium (LSD). Green buds or shoots from hyg^R calli appeared on the LSD medium after 3-4 wk. Such buds/shoots were shown to be GUS-positive. When hyg^R shoots had grown to about 0.5-1.0 cm in length, they were transferred to the development medium and cultured for another 3-4 wk to form plantlets. These plantlets produced extensive root systems after transfer to 1/2 LSO medium containing hygromycin.

To date, 9 out of 12 transgenic plants of E-yi 105, which were selected at random from several hundreds of transgenic plants, have set seeds, but the other three grew slowly. GUS activity was observed in the leaves, roots, anthers, immature embryos, and endosperms of the transgenic plants. The results indicate that the CaMV35S promoter can be efficiently recognized by the transcriptional apparatus in various organs of the rice plants. No GUS activity could be detected in control plants.

Transformation frequency of the three rice varieties

After cocultivation with the superbinary *A. tumefaciens*, transient and stable *uidA* expression was recorded in calli of all three varieties (Table 1). The frequency of transient *uidA* gene expression ranged from 86 to 100%. It is interesting to note that the number of foci of *uidA* expression 5 d after infection was dramatically higher than the number of hyg^R calli produced on the selection medium. These data indicate that our system may be exploited to determine the relationship between the events of transient gene expression after infection and the frequency of stable transformation. The stable transformation frequencies of the three varieties varied from 26 to 58% and differed from one experiment to another, even for the same genotype. One hundred mature embryos of E-yi 105, E-Wan, 5, and ZSWG yielded 107, 95, and 35 transgenic plantlets, respectively.

Conclusion

An efficient, rapid, and reproducible transformation system has been established for commercial japonica varieties of rice via *A. tumefaciens*. The rice transformation system described here based on protocols established by Hiei et al (1994) offers several benefits. First, our high frequency production of transgenic rice plants compares

Table 1. Transformation of commercial rice varieties via *A. tumefaciens* LBA4404 (pTOK233).

Plant material	Transient *uidA* gene expression		Stable transformation		
	% of GUS positive calli to total calli tested[a]	Mean no. of GUS foci (2 SD) per 3 mm × 3 mm callus[a]	% of hyg^R calli to total calli used[b]	% of buds to total hyg^R calli	No. of transgenic plants per 100 mature embryos
E-yi 105					
Exp. 1	98.6	58.2±27.8	46.5	14.5	119
Exp. 2	99.3	62.6±12.9	50.1	17.8	72
Exp. 3	100	96.9±36.4	49.8	12.8	82
Exp. 4	97.8	62.4±28.5	58.3	11.3	118
Exp. 5	92.6	56.0±30.3	42.8	10.8	145
E-Wan 5					
Exp. 1	86.7	54.4±17.3	56.4	9.2	102
Exp. 2	93.3	3a.3±20.5	37.8	9.2	89
ZSWG					
Exp. 1	100	49.7±24.1	26.5	12.3	23
Exp. 2	96.1	36.1±28.4	42.3	8.00	47

[a]Calli had been maintained on LSI medium for 4 wk. [b]These data were recorded after 6-8 wk on LSS medium.

favorably with data in previous reports of rice transformation. Second, fertile transgenic plants usually can be produced rapidly (within 7 mo). Third, somatic embryogenesis yields uniformly transformed plants rather than chimaeras. Finally, this system should facilitate the introduction of agronomically important genes into commercial varieties for rice improvement. This system is very useful for the plant breeder who requires a large population of transformants from which to select agronomically improved lines. Furthermore, the system offers opportunities for studying the mechanism of T-DNA transfer into monocotyledonous cells. We have detected transient ***uidA*** gene expression in indica rice varieties after the application of this protocol. We are optimistic about the production of fertile transgenic indica rice plants via the *Agrobacterium* system.

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Some factors influencing production of transgenic plants in polyethylene glycol-mediated transformation of rice protoplasts

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Embryo callus- and microspore callus-derived cell suspension protoplasts of three japonica rice varieties were transformed using polyethylene glycol-mediated treatment. A comparison of the efficiency of selection mediated by three different genes pointed to ammonium glufosinate mediated by the *bar* gene as the most efficient in producing resistant calli. Cotransformation of protoplasts with two separate plasmids carrying the *gus* and *bar* genes, at either a 2:1 *or* 1:1 ratio, led to resistant callus recovery frequencies of 0.8×10^{-5} and 1.6×10^{-5} and cotransformation efficiencies of 59.7 and 37.9, respectively. Mean absolute transformation frequencies ranged from 1.1 to 14.9×10^{-5} according to genotype and ammonium glufosinate resistant calli-regenerated plants with a 33.8-73% frequency, which was not affected compared with control protoplasts. Ploidy of transgenic plants ranged from n to 6n and 2n to 8n including 40 and 20% diploid plants in those regenerated from protoplasts prepared from microspore callus- and embryo callus-derived cell suspensions, respectively. Abnormal ploidy level appeared to be the major cause of infertility or low fertility of transgenic plants (which otherwise were fertile). Cotransformation frequency averaged 50%.

Rice is the only cereal where transgenic plants can be efficiently obtained using three different gene transfer techniques-chemical (Zhang and Wu 1988) and physical (Shimamoto et al 1989) protoplast treatments; microprojectile bombardment of zygotic embryos (Christou et al 1991), calli (Li et al 1993), and cell suspensions (Cao et al 1991); and more recently, *Agrobacterium* -mediated transfer of tissues (Chan et al 1993, Hiei et al 1994). Although direct gene transfer to the protoplast still remains one of the major rice transformation techniques, its efficiency is reduced by frequent infertility or low fertility of the transgenic regenerants, notably in indica varieties (Datta et al 1992, Peng et al 1992) and by undesirable variations among transgenic

progeny (Davey et al 1991), which are mainly attributed to long tissue culture procedures.

We report here our experience in producing transgenic rice plants in temperate japonica cultivars using polyethylene glycol (PEG)-mediated treatment. In cotransformation experiments, we used protoplasts having either a germinal or a somatic cell origin and several constructs bearing translational fusions between cereal gene promoters and the *gus* gene.

Materials and methods

Protoplasts were isolated from 4 to 8-mo-old cell suspensions, established from either microspore-derived or embryo-derived calli of three Mediterranean japonica rice varieties—Lido (supplied by Dr. K. Perfanov, University of Piacenza, Italy), Miara, and Ariete. Flow cytometric analysis of DNA content of protoplast nuclei prepared from cell suspensions confirmed their haploid and diploid levels, respectively, in accordance with tissue origin (data not shown). Protoplasts were treated following the protocol originally developed by Krens et al (1982) and later modified for application to indica (Peng et al 1992) and japonica (Rathore et al 1993) rice protoplasts. Unless otherwise stated, 100 µg of plasmid bearing the unselected gene and 50 µg of plasmid bearing the selected gene in 1 µg μl^{-1} solution in water were added to 1.15 ml of a 40% PEG8000 (Sigma) solution and 10^7 protoplasts previously purified and resuspended in 1 ml CPW salts (Frearson et al 1973) supplemented with 0.4 M mannitol. Protoplast culture, resistant callus production, and plant regeneration were performed as described by Chaïr et al (1995). Protoplasts were plated at a density of 10^6 ml^{-1} and membranes supporting growth of microcolonies were transferred to a selective medium (containing 7 mg ammonium glufosinate L^{-1} unless otherwise stated), 2 wk after plating. Ploidy of regenerated plants was determined by analyzing DNA content of 2.10^3 nuclei prepared from chopped leaf tissue of regenerated plants by flow cytometry following the procedure detailed in Alemanno and Guiderdoni (1994), except that a *Petunia hybrida* leaf cell nucleus preparation was used as internal standard instead of chicken red blood cells. Histochemical staining was performed according to the method described by Jefferson (1987). For controlling integration and transmission of foreign genes, genomic DNA samples isolated from leaf blades of greenhouse plants were subjected to restriction enzyme digestion, electrophoresis, Southern blot analysis, and hybridization with a ^{32}P-labeled probe consisting of the *bar* or the *gus* gene coding sequence.

Factors influencing production of transformed calli

Selected genes

Various combinations of selected marker genes/selection agents have been used to screen transformed rice tissues, including the antibiotics kanamycin (Zhang et al 1988) and hygromycin (Shimamoto et al 1989) and the herbicide phosphinothricin (Toki et al 1992). However, comparative efficiency of selection mediated by these selected

genes has not been evaluated in a single experimental system with rice. To evaluate the efficiency of selected genes, Miara haploid protoplasts were treated with 100 µg of either pGL2 (Bilang et al 1991), pRTK5 (Legavre, CIRAD, unpubl. data), or p35SAc (donated by Dr Eckes, Hoechst, Germany) constructs consisting of the CaMV35S promoter controlling the hygromycin phosphotransferase *(hph)*, neomycin phosphotransferase *(nptII)*, and phosphinothricin acetyl transferase *(bar)* genes, respectively, and followed by the CaMV35S terminator. Two-week-old protoplast-derived colonies were transferred to a protoplast culture medium supplemented with either 7 mg ammonium glufosinate L^{-1}, 50 mg hygromycin L^{-1}, or 50 mg G418 L^{-1} according to the gene used. These antibiotic or herbicide concentrations were previously found to be suitable for completely controlling the growth of untransformed colonies. In each experiment, five membranes supporting control colonies derived from protoplasts treated with no DNA were transferred to both standard and selective media.

Frequencies of recovery of resistant calli from protoplasts treated with a plasmid carrying the *hph* gene, the *nptII* gene, and the *bar* gene were 0.56±0.4, 1.1±0.36, and 3.63±1.6 × 10-5, respectively. In all three experiments, the best stable formation frequency was obtained with selection on ammonium glufosinate mediated by the *bar* gene. Use of the pGL2 plasmid combined with hygromycin selection was the least efficient, although the pGL2 plasmid has proven to be successful for repeatedly obtaining transgenic plants from rice protoplasts (Shimamoto et al 1989). In maize, the superiority of selection mediated by the *bar* gene over selection mediated by the *nptII* gene for obtaining stable transformants was also reported (Register et al 1994).

Ratio of plasmids bearing selected and unselected genes

To determine whether the concentration of plasmid carrying the reporter gene influences stable transformation efficiency, Miara haploid protoplasts were treated with 100 µg pUGCI (a 6.86-kb plasmid consisting of the 4.175 *Hin*dIII fragment of pAHC25 [Christensen et al 1992] consisting of the maize ubiquitin promoter, first intron and first exon, the gus coding sequence, and the nos terminator ligated into pUC19), and 100 µg p35SAc or 200 µg pUGCI and 100 µg p35SAc, respectively. The first treatment generated twofold more resistant calli (Table 1). On the other hand, the frequency of

Table 1. Influence of the ratio of concentrations of plasmids bearing the selected gene (p35SAc) and the unselected gene (pUGCI) on the recovery of resistant calli and frequency of calli expressing the *gus* gene and containing the *bar* gene.

Treatment	Number of calli obtained in two experiments[a]	Frequency of calli exhibiting GUS activity	Frequency of calli containing the bar gene[b]
100 µg pUGCl +100 µg p35SAc	320	37.9[c]	100
200 µg pUGCl +100 µg p35SAc	160	59.7[d]	100

[a] 10^7 protoplasts plated in each experiment. [b] 50 calli analyzed per treatment. [c] 264 calli tested. [d] 144 calli tested.

cotransformed calli—based on histochemical assays for the expression of *gus* gene and dot blot analyses for presence of *bar* gene—was twofold higher in cultured protoplasts treated with 200 µg pUGCI than those treated with 100 µg pUGCI. The fact that an increased proportion of the plasmid bearing the unselected gene appeared to favor its integration has already been reported for sorghum (Battraw and Hall 1991) and maize (Armstrong et al 1989) protoplast transformations. However, the concomitant decrease of resistant callus recovery, also noted in the latter study, would require a compromise in the ratio of plasmids bearing the selected or the unselected genes. No escape was detected in the 100 resistant calli analyzed, which demonstrates the efficacy of the selection procedure.

Genotype and tissue origin of transformed protoplasts

The results of cotransformation experiments with Miara, Lido, and Ariete protoplasts are given in Table 2. The average absolute transformation efficiencies ranged from 1.1 to 14.9 × 10^{-5} according to genotype. This parameter was found to vary between experiments in a given genotype but sample-to-sample variation was very low in a given experiment. The frequencies obtained for Miara and Ariete protoplasts fall within the range reported for producing resistant calli with the *bar* gene (Dekeyser et al 1989, Datta et al 1992, Rathore et al 1993). The frequency obtained in Lido was close to that reached in PEG-treated Nipponbare protoplasts selected on hygromycin (Hayashimoto et al 1989). In all three genotypes, regeneration frequency of selected calli was comparable with or slightly higher than that of calli derived from PEG-treated but unselected protoplast (data not shown), indicating that regeneration was not affected by ammonium glufosinate selection. Histochemical assay of leaf sections of 115 Miara plants and 120 Ariete plants revealed that the cotransformation efficiency was 47 and 50%, respectively. Southern blot analyses confirmed integration of the *bar* gene in all plants regenerated from resistant calli tested so far.

Table 2. Summary of results of PEG-mediated transformation of protoplasts of three japonica rice cultivars.

Genotype	Ploidy of protoplasts[a]	Experiments[b] (no.)	Resistant calli per 10^5 protoplasts treated		Resistant calli tested for regeneration (no.)	Frequency of regeneration	
			Mean	SD		Mean	SD
Miara	n	4	1.3	0.65	318	40.3	19.8
Ariete	n	1	1.1	–	48	72.5	–
Ariete	2n	2	5.6	0.56	541	73.0	6.2
Lido	2n	4	14.9	7.6	3646	33.8	8.1

[a]Controlled by flow cytometry. [b]0.5-5 × 10^7 treated protoplasts plated per experiment.

Table 3. Ploidy of transgenic plants regenerated from PEG-treated protoplasts prepared from 6-mo-old haploid and diploid cell suspensions.

Genotype	Ploidy of proto-plasts	Trans-genic plants analyzed (no.)	Frequency of plants exhibiting a ploidy level of					
			n	2n	3n	4n	5n	≥6n
Miara	n	111	0	40.5	21.6	35.1	2.8	0
Ariete	2n	85	0	16.5	0	71.8	0	11.7
Lido	2n	131	0	22.9	0	61.1	0	16.0

Factors affecting production of fertile transgenic plants

Frequency of albino plants ranged from 34% in cultivar Ariete to 63% in cultivar Lido. The ploidy distribution in transgenic plants regenerated from haploid protoplasts of varieties Miara and Ariete ranged from n to 5n, and included 40.5% diploid plants in Miara (Table 3). A similar range was found in plants regenerated from PEG-untreated protoplasts prepared from a previous Miara haploid cell suspension (Guiderdoni and Chaïr 1992). However, in that study, 60% of the regenerated plants were diploid. Changes of ploidy were attributed to early polyploidization events in the cultured protoplasts, confirming a phenomenon previously observed in mesophyll protoplast cultures of haploid *Nicotiana* plants (Huang and Chen 1988). The production of transgenic rice plants exhibiting higher than haploid ploidy levels is consistent with previous reports of transformation of microspore callus-derived cell suspension protoplasts of rice (Toriyama et al 1988, Datta et al 1990). Increased production of polyploids may result from protoplast fusions provoked by the PEG treatment.

Ploidy of transgenic plants regenerated from 6-mo-old cell suspension diploid protoplasts ranged from 2n to 8n including only 16.5 and 22.9% of diploid plants in varieties Ariete and Lido, respectively. Plants regenerated from either earlier experiments using the same suspensions yielded comparably high frequency of polyploid plants, as determined by morphological observations. These results are in contrast with the report of Li et al (1990) who identified 66% fertile plants among 626 transgenic regenerants from PEG-treated Nipponbare protoplasts. Tetraploid transgenic plants had thicker and taller culms, longer panicles, longer awns, and bigger grains than diploid plants and exhibited 0-20% fertility while diploid T_0 plants were 50-80% fertile. Plants of higher ploidy level were either not viable or grew under greenhouse conditions but did not flower. Ploidy of transgenic plants regenerated from PEG-treated protoplasts is rarely detailed in the rice literature in spite of the fact that sterility and morphological abnormalities were frequently reported. Our results suggest that change of ploidy level due to spontaneous polyploidization and/or fusion action of PEG is a major source of sterility.

Conclusion

PEG-mediated transformation of rice protoplasts followed by selection on ammonium glufosinate mediated by the bar gene has a high potential for producing large numbers of transgenic plants cotransformed at 50% frequency. All the japonica genotypes so far tested proved amenable to that gene transfer method. However, high frequency regeneration of polyploid plants is a major drawback of the PEG treatment tested in our conditions. Polyploidy appeared as a major cause for infertility or low fertility of transgenic plants, a phenomenon which has been frequently reported in previous studies of PEG-mediated transformation of rice protoplasts. Thorough comparison of large population of plants generated from untreated and unselected, treated but unselected, and treated and selected protoplasts is being undertaken in a single genotype to sort out the comparative impacts of protoplast culture and of the PEG treatment, and eventually of the selection procedure on the occurrence of polyploids.

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Expression patterns of RCg2 and prolamine promoters in transgenic rice

W.G. Buchholz, Y. Xu, K.C. Snowden, H. Yu, J. Ambler, J.P. Connell, D. Wallace, K. Tanaka, and T.C. Hall

The ability to produce transgenic rice plants offers real hope of utilizing novel resistance strategies to control insect pests. Expression of proteins functional against the chosen pest must be in tissues attacked by the pest, and preferably absent from other tissues. We are particularly interested in developing plants resistant to the rice water weevil (RWW), which (in its larval stages) is especially active on roots. We have isolated members of a small gene family from rice that promises to provide a promoter suitable for our RWW studies. The gene family is presently called RC (root clone) and we have shown that the RCg2 promoter is specific for the root cap and early differentiated root tissues. We have also produced and analyzed transgenic rice that expresses a reporter gene under the control of a rice 10-kDa prolamin promoter. Expression patterns and uses of these tissue-specific promoters will be discussed.

Many articles exist describing spatial patterns of expression from a variety of promoters stably integrated into dicot plants, principally tobacco. Although a growing number of instances have been recorded in which anticipated expression is absent (Murfett et al 1995) or silenced (Finnegan and McElroy 1994, Flavell 1994, Matzke and Matzke 1995), patterns and strengths of expression typically mimic those observed in the native plant. In contrast, relatively few reports exist describing expression patterns for genes stably integrated into monocot genomes and of these, expression from the viral CaMV35S promoter is the most common. The 35S promoter expresses in most tissues of rice and is generally considered a constitutive promoter. While uniform expression would be desirable for some biotechnical applications, limiting expression to a specific tissue is preferable in many instances. For example, for pests infesting roots, it seems advisable to have a promoter that drives strong expression in roots but not elsewhere. Similarly, for modification of rice grain nutritional quality, it would be advantageous to limit expression of the introduced gene(s) to only those tissues destined for human consumption.

In this paper, we describe observations obtained for the expression in transgenic rice of the ß-glucuronidase *(uid*A) gene driven by different promoters. The strongest levels of expression were found in the expected tissues, but in one case, expression in other tissues was also observed. In this case, apparently aberrant expression may result from the presence of fragmented promoters in addition to the desired, full-length promoter.

Results and discussion

We conducted an initial screening of a cDNA library prepared from rice root poly(A) RNA (Buchholz et al 1994) for sequences preferentially expressed in roots. Three different gene sequences were characterized and found to be representatives of gene families encoding root-specific proteins, cyclophilins, and triosephosphate isomerase. Although cyclophilins and triosephosphate isomerase are expressed in roots, they are also expressed in other tissues and will not be further discussed here.

The RCg2 promoter

Two different but related cDNA sequences (RCc2, RCc3) were characterized and shown by RNA blot analysis to be expressed only in rice root tissues (Xu et a1 1995). In situ hybridization experiments were performed to determine the precise site(s) of expression. RCc3 mRNA was detected only in root tips and was most abundant in cortical ground meristem cells through to the cortical cells about 1 mm back from the root apex (data not shown). This hybridization pattern is very similar to that obtained from the maize *ZRP3* gene (John et al 1992) suggesting that RCc3 is the rice homologue of ZRP3. Work is continuing to determine the site of expression of RCc2.

A genomic clone (RCg2) was isolated that contains a sequence directly corresponding to that of the cDNA, RCc2. A 5' fragment of 1656 bp was isolated from RCg2, fused to a *uid*A coding region and an *ocs* 3' element cassette and electroporated into rice as described (Battraw and Hall 1990, 1992). Two independently transformed plant lines containing full-length RCg2-*uid*A-*ocs* chimeric genes were obtained. Strong GUS expression was evident in areas of differentiation in the roots and moderate expression was detected in the root cap (Fig. la-d). Because no RCc2 RNA was detected in leaves by RNA blot analysis, we were surprised that GUS also appeared to be expressed at high levels in leaf tissue (Fig. le-f). Several reasons can be conjectured for this apparently aberrant pattern of expression. One is that each of the transformed rice lines contained rearranged, as well as full length RCg2 promoter fragments. These fragmented promoters might compete for a *trans*-acting factor(s) required to suppress expression in leaf tissue.

Another possibility is that the 1656 bp did not contain all of the 5' *cis*-acting elements necessary for correct spatial regulation. Although several hundred basepairs of promoter sequence have yielded high levels of spatially regulated expression for most plant genes studied, Nicholass et al (1995) identified an exception: whereas a 5' region of 1,412 bp yielded less than 1% of the endogenous level of polygalacturonase

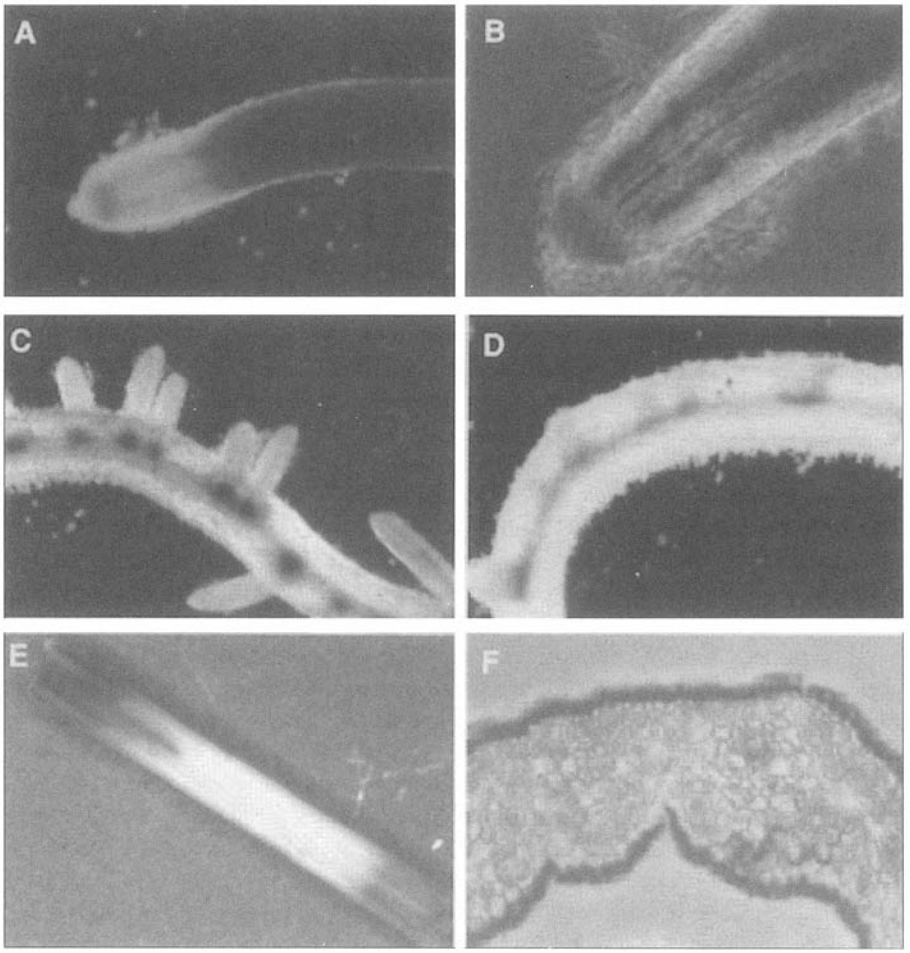

Fig. 1. Histochemical localization of GUS activity in rice transgenic for RCg2.*uid*A. a) and b) Primary root tips; note GUS activity in the root cap and zone of elongation but not in the meristem. c) and d) Primary roots with axillary roots; note lack of GUS activity in the meristematic tissue. e) Mature leaf tissue expressing GUS activity. f) Cross-section through mature leaf; note GUS expression in mesophyll and vascular regions.

mRNA in transgenic tomato, an additional element or elements in the 5' region between -4822 and -1412 was required to give high levels of tissue-specific expression.

Other possibilities for aberrant expression from the RCg2 promoter could be that a regulatory element(s) present in the normal coding or 3' region is required. Such elements have been found in the pea ferrodoxin 1 gene coding region (Elliot et al 1989) and in the 3' region of the *Brassica napus AX92* gene (Dietrich et a1 1992) and

the *Arabidopsis GL1* gene (Larkins et al 1993), raising the possibility that the 3' ocs region present in RCg2-*uid*A-*ocs* altered the spatial distribution of expression. Experiments are under way to test this possibility.

Rice prolamin promoter

Small gene families encode prolamins and globulins, the major storage proteins present in rice grains, and their promoters should be useful for expressing genes introduced to improve rice grain nutritional quality. An 885-bp fragment encoding a putative prolamin promoter and the amino terminal end of prolamin were fused to a reporter cassette composed of the *uid*A coding region and nos terminator. The fusion was in-frame and the resulting chimeric gene should produce full length b-glucuronidase with a 17-amino acid prolamine peptide attached to the amino terminal end.

To demonstrate the feasibility of transforming a single line multiple times, an embryogenic rice suspension culture was initiated from immature embryos of plant 16C1 (Battraw and Hall 1992) that had previously been cotransformed with 35S-*nptII* and 35S-*uid*A genes. The *uid*A gene in this plant is truncated at the carboxy-terminal end and nonfunctional. Protoplasts derived from this culture were cotransformed with plasmid DNA encoding the prolamin-*uid*A-*nos* chimeric gene and another plasmid encoding a ubiquitin-*bar*-*nos* chimeric gene, which confers bialaphos resistance on stably transformed rice tissue.

Plants PIPGUS 10-4 and PIPGUS 10-7, 2 of the 11 primary transformants, were shown by DNA blot analysis to contain sequences with homology to the *uid*A gene in addition to those present in the 16C1 background (Fig. 2). Plant PIPGUS 10-7 appears to have a truncated copy of the gene of interest since aberrant size fragments are produced (Fig. 2b-c). In contrast, the introduced chimeric gene appears to be intact in plant PIPGUS10-4; fragments of the predicted sizes (2.5 and 3.1 kb) were released upon digestion with two different restriction enzymes. Both plants were fertile and set large numbers of seed.

To determine if the prolamin upstream region functioned as a tissue-specific promoter, vegetative and reproductive tissues were subjected to histochemical staining with X-gluc. No staining was observed in root, culm, or leaf tissues of progeny plants (Fig. 3a-c). Following overnight imbibition in water, mature seeds of R_0 and R_1 plants were sectioned and stained. GUS activity was consistently detected in the endosperm, especially in the peripheral layers, but not in the embryo (Fig. 3d) or the aleurone layer (data not shown).

During histochemical analysis, fewer seeds appeared to stain than we predicted on the basis of normal Mendelian segregation. Therefore, segregation analysis was performed on seeds from R_0 and R_1 plants. After surface sterilization and overnight imbibition in water, the seed coats were removed and embryo rescue was performed to provide progeny for analysis. The endosperm of the seeds were sectioned and stained with X-gluc. Plants produced from embryos of those seeds that stained positive for GUS activity in the endosperm also were positive for *uid*A by polymerase chain reaction (PCR) analysis and positive for *bar* by being resistant to Herbiace. Those negative for GUS in the endosperm were negative for *uid*A by PCR and gave rise to progeny that

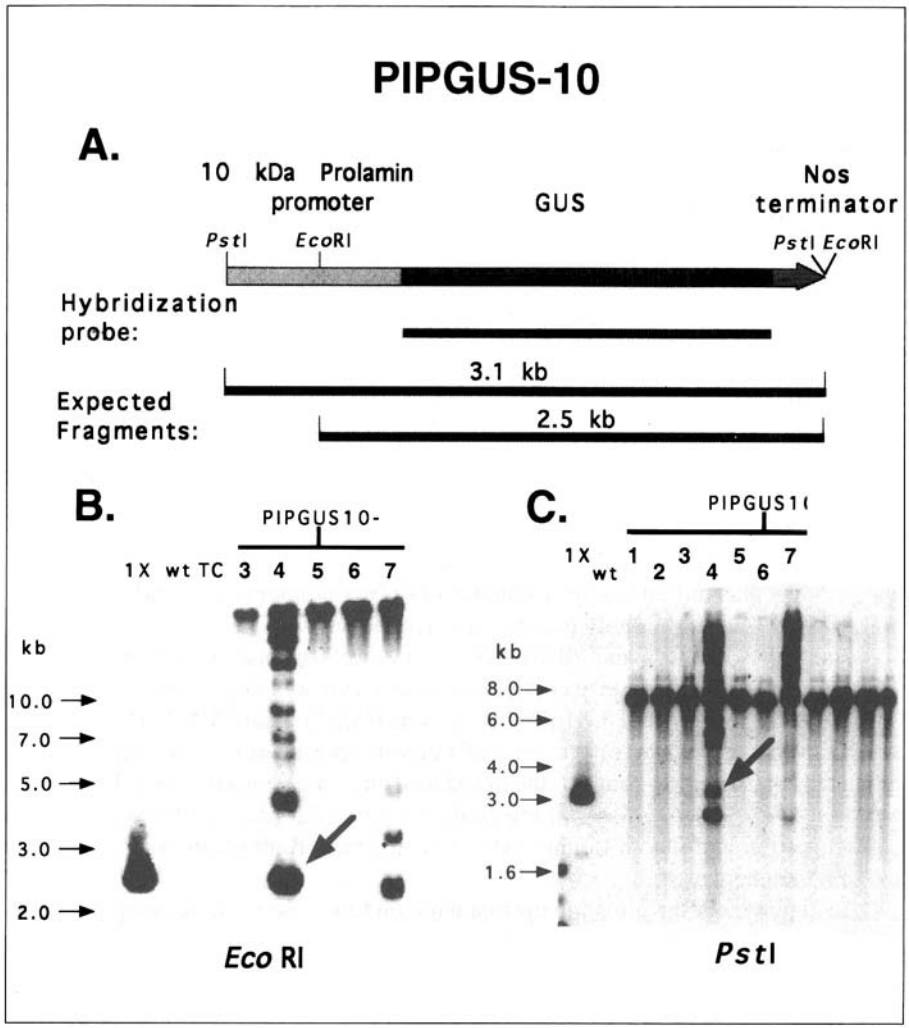

Fig. 2. Genomic blot analysis of putative PIPGUS-10 containing transgenic rice. a) Diagram showing a partial restriction map of chimeric gene, PIPGUS-10. The fragment used as a hybridization probe and those expected in the transgenic plants are indicated below the restriction map. b) and c) Genomic blots of DNA isolated from putative rice transformants after hybridization with the GUS fragment shown in (a). The DNA had been digested with (b) *Eco*RI or (c) *Pst* I. The band observed in all the transgenic lanes is due to the truncated 16cI *uid*A gene (see text). Arrows, fragments of the predicted sizes; 1X, 1 copy reconstruction; wt, wild type T309; TC, transgenic control that lacks a *uid*A gene; 1-11, transgenic plants putatively containing the PIPGUS chimeric gene.

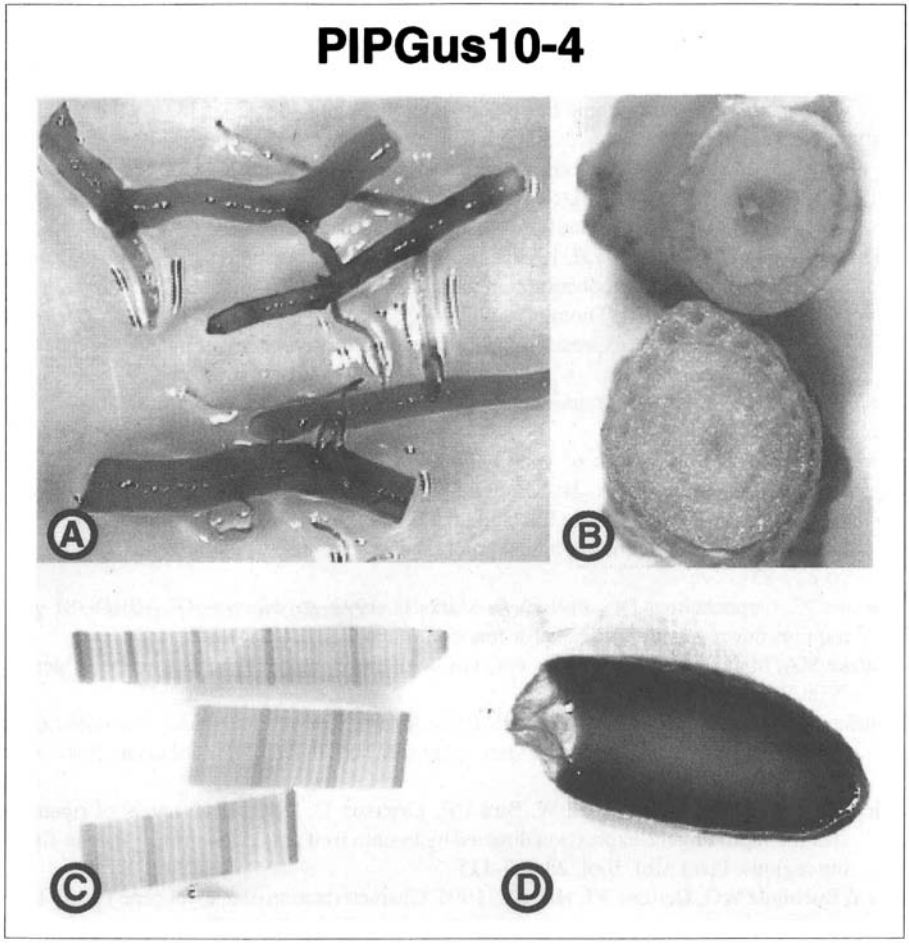

Fig. 3. Histochemical localization of GUS activity in transgenic rice plant PIPGUS10-4. a) Primary and secondary roots, b) cross-section through the culm, c) mature leaf tissue, and d) longitudinal section through a mature seed; note GUS activity is only detected in the endosperm of the seed, especially around the periphery.

were Herbiace-sensitive. These data indicate that *uid*A and *bar* genes segregate as a single locus. Furthermore they are segregating in an aberrant (1:3 to 1:4) ratio (data not shown). Taken together, these data suggest that the 885-bp prolamin promoter directs correct spatial and temporal expression of the marker gene even though both introduced genes segregate in an abnormal manner.

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Transgenic rice plants obtained via the biolistic approach

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In recent years, we have obtained a large quantity of transgenic rice plants using the polyethylene glycol-protoplast mediated system and the biolistic approach. We have investigated the genetic performance of foreign genes in transgenic rice plants, including cotransformed and tritransformed plants, and the segregation patterns of the foreign genes in the T_1 generation. We observed and analyzed the fertility in the T_0 and T_1 generations.

Growth performance and fertility of transgenic rice plants and their progeny

We compared two groups of transgenic rice plants (T_0)—one obtained by bombarding the tissues of immature embryos and the other by using embryogenic calli. No significant difference in growth performance was observed, but the average fertility of the former (19.6%) was much higher than the latter (8.3%); fertility of the check was 66.6%. However, some fertile plants were obtained from the embryogenic calli. Fertility variation ranged from 0 to 66% in both groups.

Although plants in the T_0 generation had low fertility, we were able to obtain plants in the T_1 generation. Some T_1 plants had normal fertility; fertility variation ranged from 19.5 to 99.1%. Southern blot analysis proved that the foreign genes were present in the genome of these plants. The low fertility might be due to the effect of prolonged tissue culture rather than to the inserted foreign genes. Research is under way to attempt shortening the tissue culture duration to decrease the fertility variation.

Segregation patterns of transferred foreign genes in the T_1 generation

Segregation patterns of 13 lines in the T_1 generation showed a Mendelian segregation ratio of 3:1. Segregation patterns in five other lines showed a 1:1 ratio possibly due to the passage of the transgene through one kind of gamete, either the sperm or the egg. Further research is needed to elucidate the process in detail.

Homozygous transgenic plant lines with the *Hph, GUS, RTBV CP,* and *Bt* genes were isolated in the T_2 generation. A pure line with the *Bt* gene showed high resistance to pink stem borer (*Sesamia inferens* [Walker]). This line could be used as parental material for breeding insect-resistant rice varieties.

Cosegregation of two cotransformed genes

The *Hph* and *GUS* genes, located on different plasmids, were mixed and transferred into target tissues via the biolistic approach. The frequency of cotransformation was about 30%.

Three cotransformants were investigated in more detail. Southern blot analysis showed that the *Hph* and *GUS* genes were present in the DNA. Hygromycin resistance and X-Gluc staining showed that these two genes were expressed in the T_0 plants. Segregation of either gene in the T_1 generation showed a 3:1 Mendelian ratio. Inheritance of the two genes was closely linked. One cotransformant (N-12) showed complete linkage inheritance and all hygromycin-resistant T_1 plants gave a positive response to X-Gluc staining. The other two cotransformants (K4-39, K4-40) showed close linkage segregation and most of the hygromycin-resistant T_1 plants had a positive response to X-Gluc staining. The mechanism for producing the above linkage inheritance may be due to a linked insertion of two genes at a "hot point" on the chromosome.

Tritransformed rice plants

We have obtained transgenic rice plants, which were transformed by three genes (*Hph, GUS,* and *RTBV CP*) located on different plasmids. We will conduct molecular demonstrations to confirm this tritransformation. However, our initial results show that the biolistic approach may be used to transfer multiple genes or a series of genes into plants.

Notes

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Transgenic fertile rice plants obtained through biolistic transformation using reporter genes and the TR promoter

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The purpose of this study was to develop a highly embryogenic suspension culture for biolistic transformation with a genotype commonly used in Hungary to introduce the TR promoter into rice and compare its function with the 35S promoter. Japonica rice Unggi 9 was selected for its embryogenic character. Rigorously selected immature embryo-derived embryogenic calli were used to develop the suspension culture. In transformation experiments, gold microcarriers coated with plasmid DNA (pGSGLUC1, pRT99GUS) were accelerated at high velocity using a biolistic particle delivery system (PDS-1000). For selection after bombardment, agar medium at each stage was supplemented with 75-100 mg G-418 L^{-1}. Isolated calli were tested for NPT II and only the positive calli were used in regeneration experiments. Frequency of regeneration was dependent on plasmid molecule used for the transformation experiment. The pRT99GUS-bombarded and G-418-selected calli showed significantly lower regeneration than the pGSGLUC1-shot calli. The presence and expression of introduced genes in R_0 plants and their offspring were confirmed by NPT II testing, Southern blot analysis, and fluorometric GUS assay. The marker genes were inherited by R_1 and R_2 plants. Among the progeny, significant segregation ratios were obtained. The introduced marker genes were found to be sexually transmittable into nontransgenic plants.

In rice, several transformation techniques have been developed to deliver foreign DNA into the rice genome. Particle bombardment of regenerable tissue or suspension cultures has been found to be suitable for obtaining transgenic rice at high efficiency. We evaluated the offspring generations for *gus* gene expression by histochemical staining and studied the segregation of the *npt II* gene in the R_1 and R_2 generations.

Materials and methods

Plant materials

From seven different rice genotypes, a previously cultivated japonica variety, Unggi 9, was selected for its embryogenic character on Gelrite solidified MS medium (Murashige and Skoog 1962) supplemented with 2 mg 2,4-D L^{-1}, 3% sucrose, and 10 mM L-proline. Rigorously selected immature embryo-derived embryogenic calli were used for the development of suspension culture in G1 medium (Chen 1986) supplemented with 1 mg 2,4-D L^{-1} and 3% sucrose.

Plasmid DNA transformation and selection

In transformation experiments, fine embryogenic suspension was plated on filter paper over a selective medium and then bombarded. Gold microcarriers (0.4-1.2 µm, Heraeus) coated with plasmid DNA (pGSGLUC1, pRT99GUS) were accelerated at high velocity using the biolistic particle delivery system (PDS-1000) according to Sanford et al (1993). Plasmids pGSGLUC1 and pRT99GUS contain *uid*A (coding for ß-glucuronidase, GUS) and *npt II* (coding for neomycin phosphotransferase II) genes both driven by the TR promoter in pGSGLUC1 plasmid and by the CaMV35S promoter in pRT99GUS.

For selection after bombardment, the agar medium at each stage was supplemented with 75-100 mg G-418 L^{-1}. Isolated calli were tested for NPT II and only the positive ones were used in regeneration experiments.

Enzyme assays

GUS activity was determined histochemically with 0.5% X-Gluc as substrate in a 0.1-M sodium phosphate buffer (pH 7.0) and 1% Triton X-100. Plant material was incubated at 37 °C for 24 h. Quantitative determination of GUS activity was assayed fluorimetrically with 4-MUG as the substrate according to Jefferson (1987).

Results and discussion

Transformation and regeneration

After consistent selection for highly embryogenic character from six different rice genotypes (Panda, B42, Unggi 9, Ching Hsi, Sandora, 258), Unggi 9 was found to be highly responsive and subsequently used for suspension culture development.

Unggi 9 kept its regeneration capacity in suspension culture for a long time (6-12 mo). Fine cell suspension of Unggi 9 was used in biolistic transformation. As a result of the bombardment of suspension-derived callus cultures with pGSGLUC1 and pRT99GUS plasmids, 64 and 89 colonies, respectively, were isolated under selective condition using G-418. Only NPT II-positive calli were used in regeneration experiments. Following the bombardment with pGSGLUC1 and pRT99GUS plasmid molecules of suspension culture, 56 and 3 putative transformants were regenerated, respectively, and transplanted into normal soil in the greenhouse. From the two different groups of putative transformants, 56 and 1 were NPT-positive, respectively. Total

genomic DNA was isolated from 57 NPT II-positive putative transformants and analyzed by Southern hybridization. In all cases, hybridization with a radioactively labeled probe specific for the protein coding region of *npt II* demonstrated that this gene had integrated into the rice genome.

Comparing the two different plasmid-transformed calli, in the case of pRT99GUS-bombarded calli, regeneration (4.7% regeneration frequency) was strongly hindered, whereas the pGSGLUC1-contained calli regenerated plantlets in a significantly higher percentage (63%).

Three plants originating from the pRT99GUS transformation experiment were sterile and among the 56 pGSGLUC1-transformed plants, 15 were fertile and 41 were sterile. Among R_0 plants producing seeds, fertility (total seeds/fertile seeds) varied from 0.3 to 25.1%; control plants (Unggi 9) displayed 89.2% fertility. Among R_1 plants producing seeds, fertility varied from 21.8 to 65%; Unggi 9 plants displayed 80.9% fertility.

Rigorous safety measures were followed in the greenhouse with our transgenic plants.

Expression of *GUS*

R_0, R_1, and R_2 plants expressing GUS were normal and healthy, and they produced seeds. The pGSGLUC1 plasmid construct with the TR promoter expressed the GUS reporter gene in the R_0, R_1, and R_2 generations. Our data confirmed the results of Langridge et al (1989) obtained with dicotyledonous tobacco. This promoter has been preferably used in studies on the expression of chimeric genes in both transient

Table 1. Segregation of the *npt II* gene in the R_1, R_2 generations under G-418 selective conditions at the seedling stage.

Genotype	Segregation of *npt II* gene in offspring	
	R_1 seeds R^+/R^-	R_2 seeds R^+/R^-
Unggi 9 (control)	R^-	R^-
BS1	7:1	7:1
BS3	16:1	
BS4	5:1	
BS5	11:1	4:1
BS6	3:1	3:1
BS14	6:1	R^+
BS19	8:1	
BS23	14:1	41:1
BS29	11:1	
BS30	3: 1	36:1
BS35	14: 1	40:1
BS47	6:1	
BS48	4:1	1:8
BS49	5:1	7:1

expressions and transgenic plants. It is assumed that its transcriptional activity is constitutive.

Inheritance of the *npt II* gene and its sexual transfer

The presence of the introduced *npt II* gene in R_0 plants and their offspring was confirmed by NPT II testing and Southern blot analysis. The *npt II* gene was inherited by the R_1 and R_2 progeny. Table 1 summarizes segregation of the R_1 and R_2 generations according to G-418 resistance and sensitivity. The R^+:R ratios varied in the R_1 generation from 3:1 to 16:1. In the R_2 generation, all seedlings of the BS-14 transgenic line were found to be resistant in the G-418 test (75 mg L^{-1}). The segregation ratios of other lines varied from 1:8 to 41:1 (testing of the R_2 generation is not yet complete).

The introduced marker genes were transmittable into nontransgenic plants by artificial pollination as well.

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Identification and use of specific promoters and synthetic *Bacillus thuringiensis* toxin genes in rice biotechnology

R. Sardana and I. Altosaar

Tissue-specific gene promoters are required in rice biotechnology. Our work is aimed at the identification and isolation of genes that are unique to leaf sheath, blade, stem, and auricle/collar regions of the rice plant. Such genes and their promoters may be used for some biotechnological applications such as insect resistance. We are using the differential display techniques to identify cDNAs that are tissue-specific. Working with IRRI, we are developing strategies to deploy plant codon-optimized *Bacillus thuringiensis* toxin genes in a tissue-specific manner to deter yellow stem borer and other insect pests.

To fully realize the yield potential of rice, there is a need to identify and isolate genes and promoters that are specific to different tissues and cell types (such as pollen, stem, sheath, blade, and the auricle/collar region). Availability of such types of genes and promoters will provide novel opportunities to genetically engineer rice for superior agronomic performance.

Novel tissue-specific expression genes

Our research has been aimed at the identification of tissue-specific genes/promoters in rice. One of the potential applications for such tissue-specific expression promoters is the deployment of insecticidal genes in a tissue-specific manner. In a broad sense, these genetic tools can help provide durable resistance against the yellow stem borer (YSB), which is a major insect pest of rice. Constitutive promoters may be harmful in the sense that they lead to production of toxin proteins in even those plant parts that are not eaten by the pest, thus increasing unnecessarily the "environmental load" of the selective agent. To prevent the possibility of pest populations developing resistance against *Bacillus thuringiensis* (*Bt*), the toxic protein should only be made in those plant tissues that are consumed by the insects.

The target tissues that should deploy *Bt* toxins against the YSB have been identified in consultation with IRRI scientists. These include the rice stem, sheath, auricle/collar region, blade, and pollen. The goal is to produce a test series of transgenic rice plants with the *Bt* toxin synthesized in each tissue in separate plants and then study the feeding behavior of the YSB larvae on these plants. These experiments should indicate which promoter confers the most environmentally efficacious strategy.

The access to genes and promoters that are unique to the above target tissues could help achieve many different objectives. For example, gene promoters that are unique to the rice stem could be used not only to deploy insecticidal genes (such as *Bt* toxin) in a tissue-specific manner, but also to engineer increased lodging resistance (sturdy stems) in rice. Similarly, the genes that are predominant and/or unique to leaf sheath can be used to understand the salt-adaptive response in rice where the absorbed salts are preferentially accumulated in sheath compared with the blade; thus allowing the most photosynthetically active parts of the leaf to be protected from the damaging effects of salt (Yeo and Flowers 1982). An understanding of this process could lead to the engineering of enhanced salt resistance in rice.

To identify genes that are specific to these target tissues, we have been using the differential mRNA display technique (Liang and Pardee 1992; Liang et al 1993, 1994). This technique is particularly useful in speeding up the work aimed at identification of tissue-specific and induced genes. It is technically simpler than the routinely used subtractive hybridization and differential plaque hybridization. It also requires very little starting material and is less biased against rare messages. In other laboratories, the same technique has been used to isolate three different fruit-specific genes from strawberry (Wilkinson et al 1995) and dormancy-associated genes from oat (Johnson et al 1995). In addition, a gibberellin-induced gene was identified from stem sections excised from 12-wk-old deepwater rice plants (Knaap and Kende 1995).

In our laboratory, we have been using the differential display technique to identify genes whose mRNAs are present in the target tissues (such as stem, auricle/collar region, and sheath) but absent from the leaf blade. During the course of this work, we have identified several cDNA sequences that are unique to the stem, auricle/collar region, blade, and sheath.

Methods

We have used the differential mRNA display technique to identify tissue-specific transcripts. With this method, partial cDNA sequences are amplified from subsets of mRNAs by reverse transcription using oligo-dT primers anchored to the beginning of the poly(A) tail. This is followed by the polymerase chain reaction (PCR) that includes a second arbitrary primer. The amplified short sequences are then displayed on a sequencing gel. A schematic representation of the steps in differential display is shown in Figure 1. The bands of interest are located from these gels by orienting the autoradiogram (of the sequencing gel) with the original gel itself. These bands are excised from the dried gel and the eluted DNA is subjected to another round of PCR

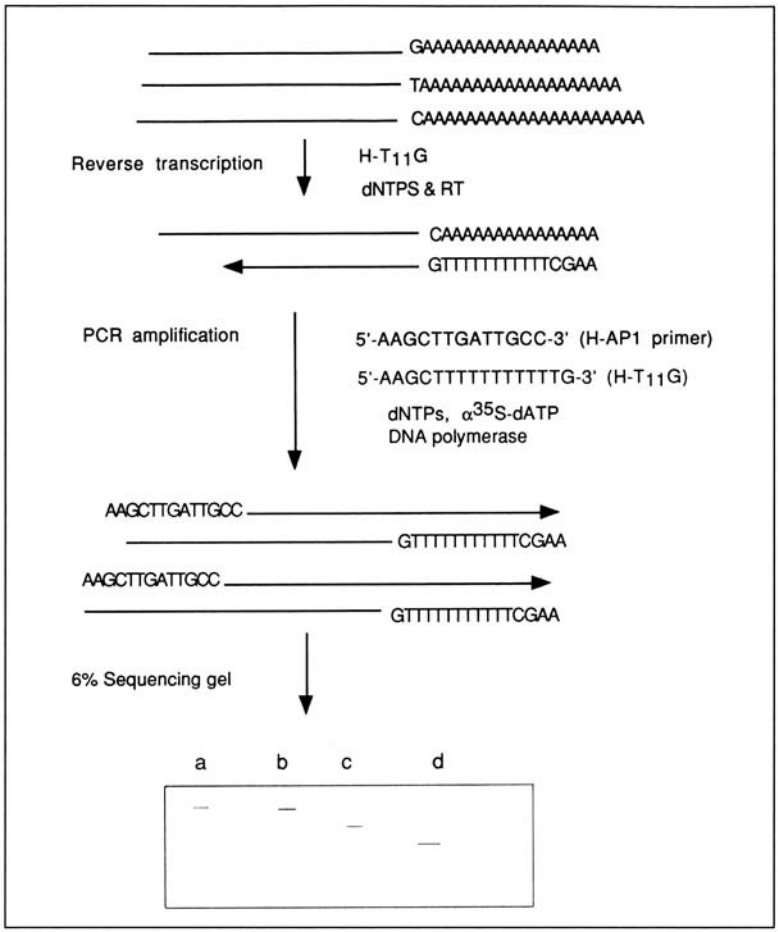

Fig. 1. An outline of steps in differential display.

using the specific primers. The PCR products are then used in Northern blots to confirm their tissue specificity.

We have used this technique to simultaneously display mRNAs from the target tissues, i.e., sheath, blade, stem, and the collar/auricle region. For our work, we isolated total RNA (DNA-free) from the target tissues of the rice variety Nipponbare. It was grown in a greenhouse and tissues were collected after the panicle initiation stage. The RNA was isolated using the Trizol kit from Life Technologies Gibco BRL Corp. These RNAs from the above tissues were subjected to reverse transcription using three different oligo-dT primers (H-T_{11}G, H-T_{11}A, and H-T_{11}C; GenHunter)—a total of 12 reactions. The products of each reverse transcription reaction were subjected to PCR amplification using eight different arbitrary primers (H-AP1 to H-AP8;

GenHunter). The conditions used for the amplification were 94 °C for 30 s, 40 °C for 2 min, and 72 °C for 30 s. There were 40 cycles for the whole reaction. In addition, one final step of 5 min at 72 °C was also used. In total, 96 different PCR reactions were performed. These PCR products were separated on 6% sequencing gels.

Results

Tissue-specific transcripts and clones

After testing all the above primer combinations on RNAs from four different tissues simultaneously, we succeeded in identifying fragments on the sequencing gels that were specific to the target tissues. To confirm that these fragments are truly unique to the target tissues, the differential display work was repeated at different times. The unique fragments thus identified were excised from the sequencing gels and amplified using the particular primers.The products of each amplification reaction were run in duplicate on 1% agarose gels. To confirm the tissue specificity (in relation to leaf blade tissue), the duplicate blots from these gels were probed with total cDNA probes made from target tissues (^{32}P-labeled probes complementary to mRNAs; single stranded). For making probes, a total of 2µg of mRNA was used from each tissue. The DNA fragments that hybridized to probes from target tissues and not to the probe from the blade tissue (or the ones that only weakly hybridized to the blade probe) were selected for cloning. These unique cDNA fragments were cloned into the pCR-TRAP plasmid vector (GenHunter).

To further confirm the tissue specificity (presence or absence of message in the target tissue compared with the blade), the inserts were amplified using the primer set (Lgh and Rgh primers) flanking the cloning site of the pCR-TRAP vector. The inserts were then subjected to gel electrophoresis. The duplicate blots were probed with the total cDNA probes (^{32}P-labeled as before) from different tissues. The results are shown in Figures 2a-d.

In Figure 2a, the clones A/4-1, A/4-2, A/4-3, A/4-4, A/4-5 (A/4 set), C/2-1, and C/2-3 (C/2 set) show hybridization only to the probe from auricle/collar tissue and not at all to the one from the blade tissue. This indicates that these clones are unique to the auricle/collar region. The two other clones hybridized to the probe from both the blade and auricle/collar tissues.

In Figure 2b, a clone A/8-3-4 hybridizes only to the probe from stem tissue and not to the blade probe, indicating that it is a clone that is unique to the stem tissue. All other clones showed hybridization to both the probes.

Figure 2c shows that the clones A/7-1-1, A/7-1-3, A/7-1-4, A/7-1-5, A/7-1-6, and A/7-1-7 (A/7-1 set) give strong hybridization signals with the probe from the sheath tissue. However, the hybridization signals from these clones are several fold lower with the blade probe, indicating that they are expressed at low levels in the leaf blade.

Furthermore, in Figure 2d, a clone A/7-3-3 shows hybridization to the blade probe and gives almost no signal with the probe from the sheath tissue, indicating that the clone is unique to the leaf blade (Fig. 2d). In the same figure, a clone A/7-2-5 gives

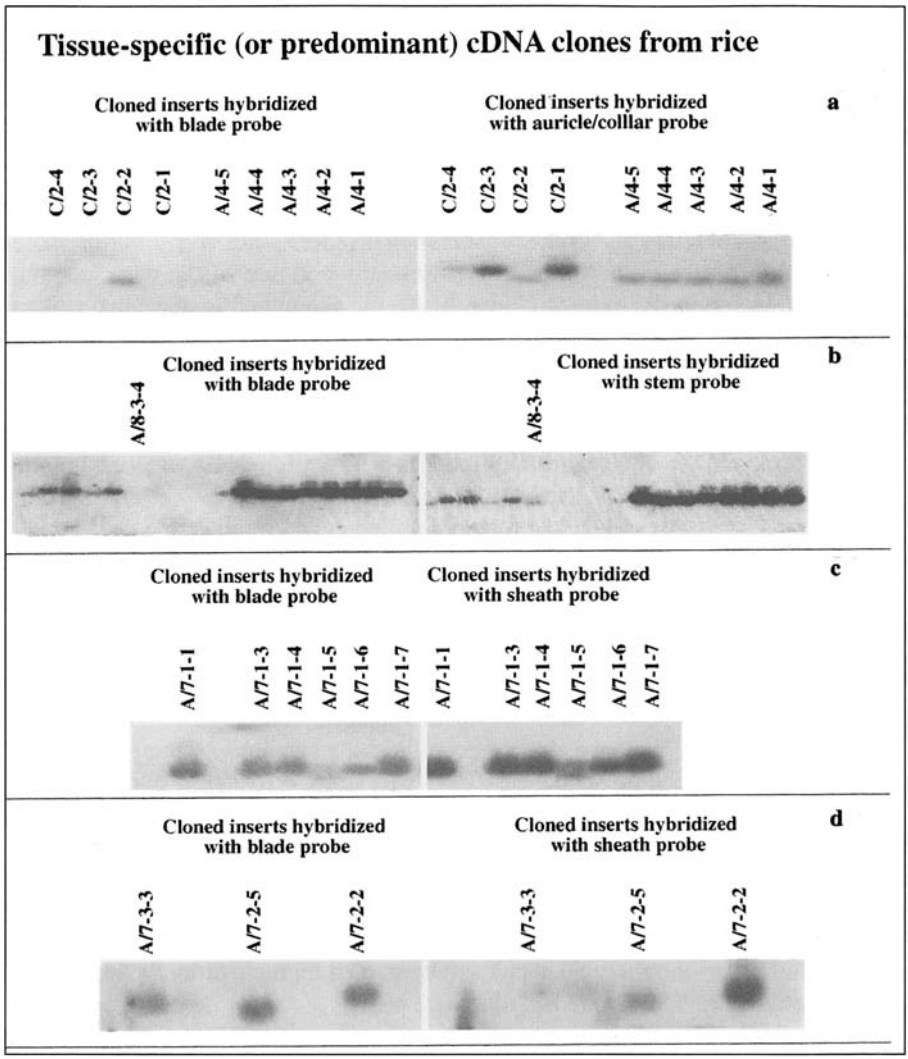

Fig. 2. (a, b, c, and d). For the figures shown here, the cloned inserts (cDNAs identified through differential display) were amplified by PCR. The amplified insert bands were subjected to Southern analysis and hybridized with the labeled total cDNA probes as described in the text and indicated here.

stronger signal with the blade probe compared with the sheath probe. In contrast to A/7-2-5, the clone A/7-2-2 shows more expression in the leaf sheath.

To summarize, we have identified and cloned the cDNA sequences that are unique to three different tissues, i.e., auricle/collar region, stem, sheath (in comparison with

blade tissue). In addition, we have also cloned a cDNA that is predominantly expressed in the blade tissue and shows very low expression in leaf sheath.

Further characterization of cloned inserts and cloning of genomic fragments containing the gene promoters

The desired cloned insert fragments are presently being sequenced. In parallel, we are also using the cloned inserts as probes on Northern blots to determine expression levels and transcript sizes and to check expression in other tissues, if any. Furthermore, Southern blots are being performed to determine the nature of the corresponding genomic DNA fragments to identify possible single genomic fragments that contain the promoter sequences.

To speed up the promoter cloning work, we have also acquired a rice genomic library in the form of bacterial artificial chromosomes (BAC, courtesy of P. Ronald, University of California) and have started screening it. The rice BAC library contains three haploid genome equivalents and there is a 95% probability of it having any specific single-copy genes (Wang et al 1995).

The stage is now set where the required promoters presently being isolated will be used to initially make tissue-specific expression (TSE) cassettes. The novel TSE vectors for genetic engineering of rice should allow us to attempt radically novel experiments, wherein coding sequences for enzymes, receptors, toxins, structural proteins, etc., are expressed in specific tissues to alter the metabolism, morphology, or agronomic properties of the rice plant. Our parallel concomitant work with *Bt* toxin genes will proceed with the deployment of plant codon-optimized *Bt* sequences (Sardana et al 1996). We have already completed the synthesis of the plant codon-optimized *Bt* toxin coding sequences [CryIA(b) and CryIA(c)] in our laboratory. Consequently, we should be able to construct the following classes of TSE cassettes: pollen promoter—CryIA(b), pollen promoter—CryIA(c), sheath promoter—CryIA(b), sheath promoter—CryIA(c), stem promoter—CryIA(b), stem promoter—CryIA(c), blade promoter—CryIA(b), blade promoter—CryIA(c), auricle/collar promoter—CryIA(b), and auricle/collar promoter—CryIA(c).

The full series of transgenic plants will be studied for their various effects on the feeding behavior of YSB larvae and other rice pests.

The unique gene sequences reported here may have many potential applications in rice genetics and biotechnology. The isolated genes can be used in rice genome mapping and linkage studies. They can also be used to learn more about tissue-specific gene expression in rice. The further study of these genes would empower us with the necessary tools and ability to target rice cells/tissues for desired genetic modifications aimed at improving its agronomic performance. All this, in turn, will help create opportunities to design, engineer, and breed novel rice plant types/land or breeding lines for the different agroclimatic regions.

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Notes

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Transformation of indica rice with *Bt* pesticidal genes

S. Riazuddin, T. Husnain, E. Khan, S. Karim, F. Khanum, R. Makhdoom, and I. Altosaar

A large number of isolates of *Bacillus thuringiensis* separated from different ecological environments in Pakistan were characterized for *Cry* gene composition and entomocidal activity against rice yellow stem borer and leaffolder. Isolates harboring combinations of *CrylA(c)* and *Cry*IIA genes were found most effective against the target larvae. To improve expression of the genes in plants, a synthetic *CrylA(c)* gene was placed under a constitutively expressing ubiquitin promoter and used in rice transformation experiments. Mature embryos of indica rice (cultivar Basmati 370) were bombarded with tungsten particles coated with DNA harboring synthetic *CrylA(c)* gene. Plants derived from the meristem tissues were transferred into the soil under greenhouse conditions where they grew to maturity bearing viable seeds. Three plants exhibited the presence of *CrylA(c)* gene in the plant genome as evidenced by Southern blotting. Further, laboratory bioassays confirmed expression of the *Cry* gene in the transformed plants. Two of these plants showed preferential resistance to leaffolder while one plant exhibited repellant properties. Transformed rice plants in the R_1 progeny are being tested for stable inheritance and expression of the *Cry* gene.

Traditional breeding for insect resistance in rice has been a dismal failure, with many resources having been expended for little return. Consequently, farmers have resorted to the use of chemical insecticides. Such man-made chemicals have indeed been effective in reducing crop damage by insects; nevertheless, the indiscriminate use of chemical insecticides has led to environmental and health issues that have raised the need to develop environmentally safe biological control agents.

It has been demonstrated that *Bacillus thuringiensis* (*Bt*) spores contain biological pesticides that can be used to control various plant pests either directly as a bioinsecticide or indirectly through transgenic plant technology. However, worldwide use of biological insecticides has remained limited because of inefficiency of

insecticidal action, target specificity, requirement for ingestion, inadequate field persistence, and high cost of production. These drawbacks can be overcome partly, if not completely, through the application of transgenic plant technology whereby bacterial toxin genes can be transferred and expressed in plants, yielding transgenic crops with inherent capabilities to resist insect attack.

To exploit the potential of transgenic plant technology to breed insect resistance in rice, several laboratories are working to search for new and novel *Bt* pesticidal genes, change codon usage to optimize expression in monocots and clone potentially efficacious genes in plant expression vectors, develop rice transformation procedures and apply them to incorporate and stably express *Bt* genes in selected rice cultivars, and study the inherent potential of transgenic plants to resist agronomically important insects.

This paper describes the studies being carried out as part of the International Program on Rice Biotechnology sponsored by the Rockefeller Foundation at the National Centre of Excellence in Molecular Biology, University of the Punjab, Lahore, Pakistan.

Search for new isolates of *Bt*

The 476 samples collected from different ecological environments in Pakistan were analyzed to yield 1000 *Bt* isolates. In all, 373 isolates were characterized for gene content by DNA-DNA hybridization studies, enzyme-linked immunosorbent assay and sodium dodecyl sulfate polyacrylamide gel electrophoresis analyses (Fig. 1). Selected isolates were studied in biotoxicity assays against rice stem borer (*Tryporyza incertulas*) and rice leaffolder (*Cnaphalocrocis medinalis*) (Table 1).

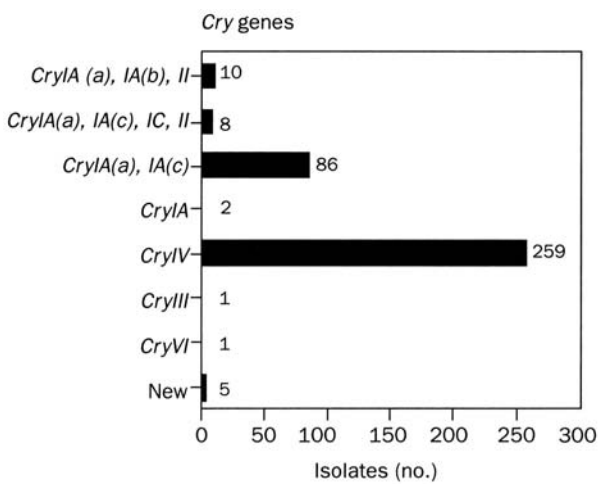

Fig. 1. Abundance of *Cry* genes in *Bt* isolates separated from Pakistani environment.

Table 1. Larvicidal activity of *Bt* isolates against yellow stem borer *(Scirpophaga incertulas)* and leaffolder *(Cnaphalocrocis medinalis).*

Isolate	Yellow stem borer LC_{50}[a] (µg ml^{-1})±SE	Leaffolder LC_{50}(µg ml^{-1})+SE	*Cry* gene composition
IS2 (Btk)	632.06±1.40	3.36±4.71	IA(a)/IA(b)/IA(c)/IIA/IIB
D1.1	509.81±0.45	3.00±4.88	IA(a)/IA(c)/IC
C1.4	>800	3.65±4.80	IA(a)/IA(c)/IC/II
D4.10	568.59±0.60	>800	Not determined
KC17.11	446.65±0.30	>800	IA(a)/IA(c)/IC
F4.21	730.63±1.23	>800	IA(a)/IA(c)/IC
RC1.5	>800	6.90±4.31	IA(a)/IA(c)
KM7.5	1034.96±2.23	>800	IA(a)/IA(b)/II
KM7.3	>800	>800	IA(a)/IA(b)/II
BC3b6	413.31±1.84	>800	IA(a)/IA(b)/II
KM7.6	>800	13.13±3.83	iA(a)/IA(b)/II
KC14.1	150.86±3.10	>800	Not determined
CEMB 1	>800	0.48±.27	Novel
BC14A8	>800	14.15±4.44	IA(a)/IA(b)/II
KM3.1	846.29±3.21	>800	IA(a)/IA(b)/II
RC1.4	498.22±1.75	15.12±3.71	Not determined
SB2.3a	>800	24.47±3.95	IA(a)/IA(b)/II
D4.16	478.93±2.30	>800	Not determined
D3.11	531.55±2.90	>800	Not determined
D4.4	1720.84±2.31	>800	IA(a)/IA(c)/IC/II
KM9.5	73.16±1.50	3.13±4.99	IA(a)/IA(b)/II
PR17.4	30.66±1.64	2.48±5.08	IA(a)/IA(b)/IA(c)
JR6.3	10.31±0.93	8.79±4.11	IA(a)/IA(c)
ABBOT (Bti)	>800	>800	Multiple IV

[a] LC_{50} values were determined with solubilized and trypsin-activated crystal protein. Isolates giving LC_{50} values above 800 µg ml^{-1} were considered nontoxic.

Changes in codon usage of *Bt* gene to optimize expressions

Plant-codon-optimized *CryIA(c)* gene consisting of 1845 bp was synthesized using an improved polymerase chain reaction (PCR) procedure based on recessive principles. The synthetic *CryIA(c)* gene was put under the control of a maize ubiquitin promoter in a pGEM-4Z-based vector (Fig. 2) and the construct cotransformed with a *hpt* gene containing plasmid.

Transformation of Basmati 370

The ß-glucuronidase gene was introduced into leaf bases and mature embryos for transient expression, while a selectable marker gene, *hph,* that confers hygromycin resistance, was used for stable transformation. Leaf bases isolated from 5-d-old etiolated seedlings of rice were kept on petri plates containing MS medium solidified with 1% agar and bombarded with DNA-coated tungsten particles using a home-made particle acceleration gun. About 70% of the leaf bases showed transient expression.

Mature embryos were bombarded and kept on MS medium for 5 d when they were transferred to selection medium containing hygromycin at a concentration of 50

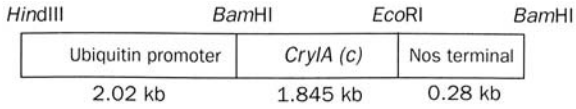

Fig. 2. Plant codon-optimized *CrylA(c)* gene under the maize ubiquitin promoter in a pGEM-4z-based vector.

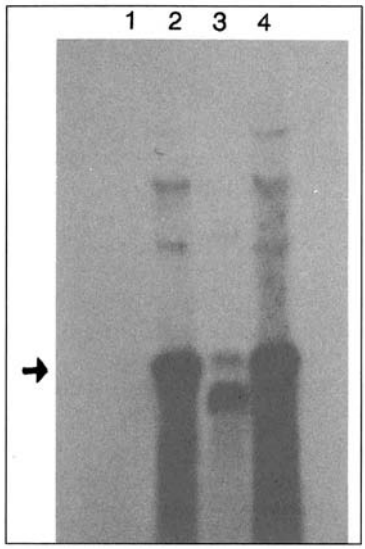

Fig. 3. Southern blot analysis of transgenic rice plants (Bas 370). lane 1: *Bam*HI-digested DNA of untransformed plant. Lanes 2, 3, and 4: *Bam*HI-digested DNA of three independent transformed plants, CAMB2, 10 and 11. The probe was DIG-labeled *Bam*HI fragment of the Ubi:*CrylA(c)* vector. The arrow indicates the predicted position of the *Bam*HI fragment expected for the unrearranged gene.

μg ml^{-1}. DNA from plasmid pROB5 (hygromycin gene) and ubi-*CryIA(c)* was mixed in a 1:3 ratio to coat tungsten particles. Leaf bases (2 mm) were excised from the resultant plants and cultured on a medium containing hygromycin (50μg ml^{-1}). Putative transformants were multiplied in vitro and transferred into pots containing a 1:1 mixture of peat moss and clay where they grew to maturity and bore viable seeds. Green fast-growing plants were subjected to Southern blot analyses to study the presence of the marker gene.

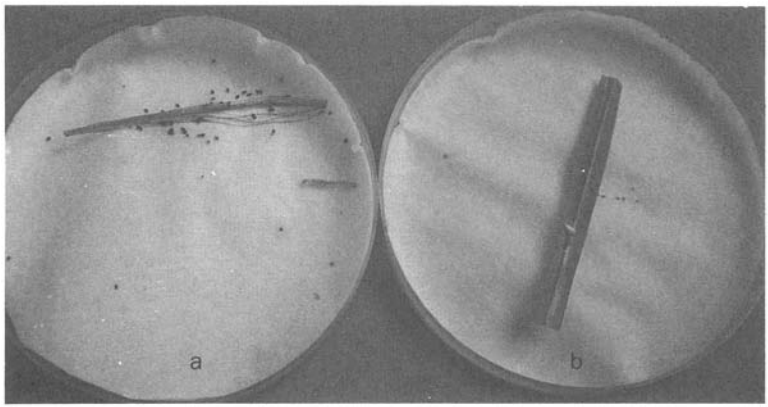

Fig. 4. Insect feeding assay with *Cnaphalocrosis medinalis* (rice leaffolder) on transformed rice plants. a) Leaf of control plant bombarded with tungsten; b) leaf of plant no.2 showing mortality (dead, black larvae).

Studies on transformed Basmati 370 rice plants

Dot blot analysis of 30 plants (40-120 d old), selected on hygromycin-containing medium, identified 10 plants to contain *CryIA(c)* sequences. Southern blot analysis of *CryIA(c)* containing two plants revealed several copies of the intact gene (Fig. 3). Seven independent transgenic lines have been selfed to obtain R_1 plants.

Fresh leaves from 3-6-mo-old transformed plants were fed with 10 second-instar larvae. After 3 d, the number of dead insects and the weight of surviving larvae were recorded. During the 3-d time period, 3-7 larvae feeding on transformed leaves died whereas there was no adverse effect on larvae fed with untransformed leaves (Fig. 4).

Notes

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Transfer of yellow stem borer resistance genes to indica rice cultivars

P. Nayak, D. Basu, S. Das, D. Ghosh, A. Nandi, A. Basu, and S.K. Sen

Production of transgenic crop plants with the endotoxin gene of *Bacillus thuringiensis* has been a proven potential strategy to reduce yield losses attributed to rice-feeding insects such as yellow stem borer (*Scirpophaga incertulas* [Walker]). Feeding and binding assays revealed that CrylA(b) protein was the most effective toxin in terms of unit activity, followed by the CrylA(c) and CryllA proteins. Reconstruction of the *crylA(c)* gene was completed first and then transferred to indica rice cultivars and two exotic aromatic indica rice strains. The chimeric construct of the *crylA(c)* gene was developed in pUC19 with the ubiquitin promoter along with the *ubiquitin intron* 1 at the 5' end and the nos terminator at the 3' end. The gene was cotransformed with the *bar* gene conferring resistance to the herbicide bialaphos as the selectable marker. A total of 106 bialaphos-resistant plants were obtained, of which 76 showed the *cry* gene, as determined through polymerase chain reaction analysis.

Yellow stem borer (*Scirpophaga incertulas* [Walker]) is a major pest of cultivated rice. Damaging larvae, which feed on the soft plant tissues, remain inside these tissues often beyond the reach of chemical pesticides. Production of transgenic crop plants with the endotoxin gene of *Bacillus thuringiensis (Bt)* appears to be a potential alternative to using pesticides. Among monocots, such a strategy has proven useful in maize and japonica rice. Feeding assays of the first- and second-instar larvae as well as binding assays of the brush border membrane vesicle (BBMV) receptors of the larval epithelial gut cells revealed that CryIA(b) protein was the most effective toxin (i.e., lethal in terms of unit activity), followed by CryIA(c) and CryIIA proteins.

At the first phase of our program for the development of transgenic indica rice, we transferred the truncated *Bt* gene driven by different plant promoters. The maximum expression that we could get at the R_0 generation was approximately 90 ng mg^{-1} of soluble protein from leaf tissue. The chimeric gene construct that had been used in

such a case contained a CaMV35S promoter, Shrunken 1 intron of maize, a post-transcriptional enhancer from TMV, an initiatory kozak sequence at the 5' end of the gene, and the nos terminator at the 3' end. The progeny of such transgenic plants are now in the field and being tested for insect resistance.

Realizing that the capacity for expression of the bacterial *Bt* gene in plants is usually poor, a simultaneous attempt was initiated in 1993 to develop synthetically reconstructed *cryIA(b)* and *cryIA(c)* genes in the laboratory. Reconstruction of the *cryIA(c)* gene was completed first. It was transferred to elite indica cultivars IR64 and IR72 and to two exotic aromatic indica strains Kalonunia and Gayabhog. The results of our progress with IR64 are presented.

The chimeric construct of the *cryIA(c)* gene was developed in pUC19 with the ubiquitin promoter along with the ubiquitin intron 1 at the 5' end and the nos terminator at the 3' end. The gene was cotransformed with the bar gene that confers resistance to the herbicide bialaphos as the selectable marker. Both genes were delivered through microprojectile bombardment using the BioRad PDS-1000/He particle gun. A total of 106 bialaphos-resistant plants were obtained; of these, 76 plants showed the presence of the *cry* gene, as confirmed by polymerase chain reaction analysis. Enzyme-linked immunosorbent assay and Western analyses identified six individual transformants that showed high expression of the toxin peptide. These plants were fully fertile and produced seeds. When selfed, the seeds of the individual transgenic plants produced R_1 plants that expressed the *Bt* toxin at either high or low levels. Genetic analysis revealed that more than one chromosome contained the transgene in most of the primary transgenic plants.

Experimental trials on insect bioassays further revealed that the plants that expressed the toxin peptide retained their entomocidal property.

Fifty-four transgenic IR64 plants with the *cryIA(b)* gene were subsequently produced. The chimeric gene construct in this case contained a CAMV35S promoter with the Sh 1 intron at the 5' end and the nos terminator at the 3' end. The expression level of the CryIA(b) toxin peptide in these plants is being monitored.

The homozygous lines derived from the transgenic plants showing high expression of the toxin peptide with genes *cryIA(c)* and *cryIA(b)* will be field tested for their capacity to resist insect damage.

Notes

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Synthetic *Bacillus thuringiensis (Bt)* toxin genes for insect resistance and their rapid bioassay using maize endosperm suspension cultures

I. Altosaar, R. Sardana, S. Dukiandjiev, M. Giband, and X. Cheng

Two plant codon-optimized genes, *CrylA(b)* and *CrylA(c),* each consisting of 1,845 bp, have been synthesized using polymerase chain reaction techniques. A maize ubiquitin promoter was used to rapidly test the synthetic *CrylA(c)* gene. This construct was tested in a maize endosperm-derived suspension culture system. The use of maize endosperm culture in combination with a maize ubiquitin promoter has been found to be a quick and efficient system for testing the activity of synthetic genes.

Bacillus thuringiensis (*Bt*) produces crystal proteins called d-endotoxins that are highly toxic to many insects (Hofte and Whiteley 1989). The native genes encoding the crystal proteins are poorly expressed in plants (Barton et al 1987, Vaeck et al 1987). In recent years, the modified genes for these proteins have been introduced in plants (Perlak et al 1990, 1991; Sutton et al 1992; Adang et al 1993; Fujimoto et al 1993; Koziel et al 1993; van der Salm et al 1994). These modifications were aimed at potential plant intron-like sequences, polyadenylation signals, and codons that are infrequently used in plants. The engineered plants showed significantly increased expression of the modified genes and were insect-resistant.

Our work has been focused on building the 1,845-bp sequences (truncated) of *CryIA(b)* and *CryIA(c)* genes that have been previously modified (Perlak et al 1990, 1991). Though the expression-related work on these modified genes has been reported, the detailed procedures for their complete synthesis have never been published. To our knowledge, this is the first detailed report on the totally polymerase chain reaction (PCR)-directed synthesis of two large synthetic modified *CryIA(b)* and *CryIA(c)* toxin genes.

We also report on the use of maize endosperm-derived suspension culture as an effective way to test the expression of modified *Bt* toxin genes. In earlier work, electroporated protoplasts from endosperm cultures have been shown to be capable of expressing genes (Quayle et al 1991, Ueda and Messing 1991).

Results and discussion

The two genes were assembled from blocks, referred to as E, MI, MII, Sb, and Sc (Fig. 1). Blocks E, MI, and MII were common to the two genes. Blocks Sb and Sc represent the specific parts unique to the *CryIA(b)* and *CrylA(c)* genes, respectively.

Gene synthesis by PCR

The first phase PCR reaction was performed in a 100-µl reaction volume using Vent polymerase. In total, 30 cycles were used (the detailed reaction conditions are described in Sardana et al 1996). For the second phase, 5 µl of the first phase PCR reaction were used as the template and amplification was done by adding 30 picomoles of outer oligos, which served as the primers. The outer oligos used contained convenient restriction sites to facilitate cloning into plasmid vectors. The products of the second phase PCR are shown in Figure 2. The expected bands of sizes 732, 461, 414, 534, and 534 bp belonging to blocks E, MI, MII, Sb, and Sc, respectively, are marked by arrows.

Final assembly of the synthetic *CrylA(b)* and *CrylA(c)* genes

The fragments of sizes 732, 461, 414, 534, and 534 bp were excised from the gel shown in Figure 2B. They were gene-cleaned and digested with desired restriction endonucleases to give blocks of required sizes. These blocks were cloned and sequenced (^{T7}Sequencing™ Kit, Pharmacia). The point mutations were repaired by site-directed mutagenesis (U.S.E. Mutagenesis Kit, Pharmacia) and/or PCR. The standard cloning procedures (Sambrook et al 1989) were used for the assembly of the two genes.

Expression of synthetic *CryIA(c)* gene in the maize endosperm suspension culture

For expression, we placed the synthetic *CryIA(c)* gene under the control of a maize ubiquitin promoter (Christensen et al 1992). The construct, named Ubi-CryIA(c), is shown in Figure 3. This construct along with the pAHC25, a GUS plasmid also under the control of the same ubiquitin promoter (courtesy of Dr. Peter Quail), was used for

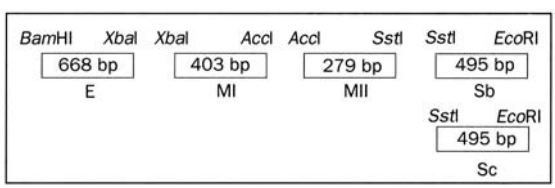

Fig. 1. The blocks used for synthesis and assembly of *CryIA(b)* and *CryIA(c)* truncated and modified genes of endotoxins from *Bacillus thuringiensis.* The blocks E, MI, MII, Sb, and Sc of sizes 668, 403, 279, 495, and 495 bp, respectively, are shown here along with the desired restriction sites.

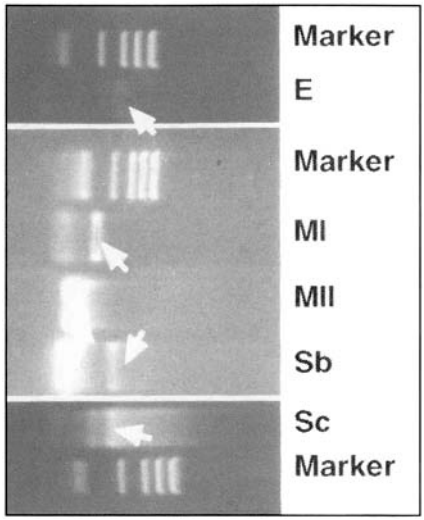

Fig. 2. The second phase PCR products. For the blocks E, MI, MII, Sb, and Sc, the oligos were chosen and mixed to give products of 732, 461, 414, 534, and 534 bp, respectively. The second phase PCR products for the five blocks were electrophoresed in 1% agarose gel and stained in ethidium bromide. øX174 DNA digested with *Hae*III enzyme was used as a marker. The arrows indicate the expected size bands for the blocks.

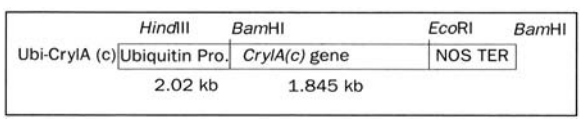

Fig. 3. The Ubi-CrylA(c) construct. The plasmid Ubi-CrylA(c) contains the coding region of the synthetic *CrylA(c)* gene under the maize ubiquitin promoter (Christensen et al 1992). The polyadenylation signal (NOS TER) is from the nopaline synthase gene. The vector is pGEM-4Z (Promega)-based.

cobombarding (Klein et al 1987) clumps of collected suspension cells of maize endosperm-derived suspension culture (Shannon and Liu 1977, Shannon 1982). For this, we used a PDS-1000/He particle delivery system (BIO-RAD). The bombarded samples were stained with X-Gluc (Jefferson et al 1987). The total protein was extracted from the pooled blue-colored cellular clumps as previously described (Koziel et al 1993). Briefly, 100 mg of the pooled tissue was ground in 100 µl of the extraction

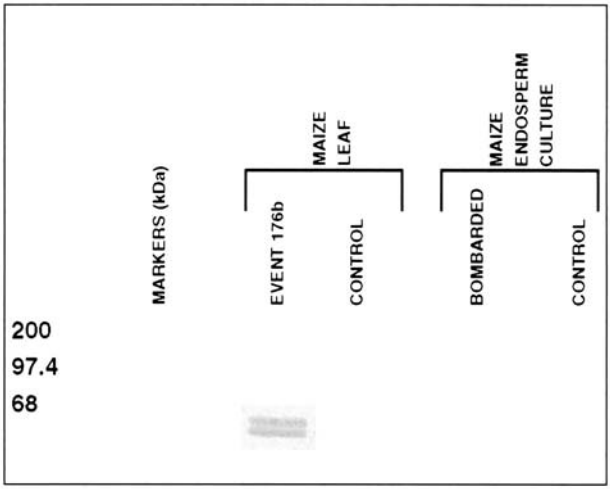

Fig. 4. Western blot analysis. The nontransformed maize leaf and endosperm material represent the controls. Note that a protein of size, which corresponds to the standard *Bt* toxin protein in the maize leaf sample, is produced in the bombarded endosperm sample.

buffer. A 10-µl aliquot was subjected to Western blot analysis (Fig. 4) as described by Sardana et al (1996). A major protein band of the correct size was detected only in the sample bombarded with the Ubi-CryIA(c) construct. These results suggest that the synthetic *CryIA(c)* gene with G+C content of 47.7% is actively expressed and its mRNA is efficiently translated in the maize endosperm tissue. The fact that we could easily detect the *Bt* gene product on Western blot is important. Relatively, only a fraction of the cells is transiently transformed per bombardment. The observation that the levels of Bt protein in the bombarded sample are comparable with the transgenic maize leaf sample (where every cell is stably transformed) shows the effectiveness of our approach. This can be explained mainly by 1) the modified *CryIA(c)* gene itself—the same modified *CryIA(c)* gene sequence—being 100-fold more expressed in dicots compared with the unmodified wild-type gene (Perlak et al 1990, 1991) and 2) the ubiquitin promoter being very active in its native host. We have found that the ubiquitin promoter is much stronger than the CaMV35S promoter when tested in maize endosperm-derived culture (data not shown). Furthermore, with this reported accumulation of Bt protein in the bombarded endosperm sample, it is feasible to conduct experiments where the bombarded material is fed directly to the insect larvae for biological assays (it can also be mixed with the insect diet).

Conclusions

The transient assay procedure discussed here and used for testing the synthetic toxin gene is technically simpler. It does not require the isolation of protoplasts. The results

demonstrate the usefulness of maize endosperm-derived suspension culture as an alternative system for testing the expression of synthetic and mutant *Bt* toxin genes.

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Potato proteinase inhibitor II gene that confers insect resistance in transgenic rice plants

X. Duan, X. Li, Q. Xue, M. Abo-El-Saad, D. Xu, and R. Wu

A potato proteinase inhibitor II (PINII) gene (*Pin2*) was introduced into several japonica rice varieties, and a large number of transgenic rice plants were regenerated. Wound-inducible expression of the *Pin2* gene, driven by its own promoter, together with the first intron of the rice actin 1 gene *(Act1),* resulted in high-level accumulation of the PINII protein in transgenic rice plants. Protein extracts from the transgenics exhibited a strong inhibitory activity against bovine trypsin. The introduced *Pin2* gene was stably inherited in the second, third, and fourth generations, as shown by molecular analyses such as DNA blot hybridization and proteinase inhibitor activity assay. Based on data of molecular analyses, many homozygous transgenic lines were obtained. Small-scale tests for insect resistance in an isolated experimental field of the fourth generation transgenic rice plants showed that these plants had significantly increased resistance to two major rice insect pests: striped stem borer *(Chilo supressalis)* and pink stem borer *(Sesamia inferens).* Thus, introduction of insecticidal proteinase inhibitor genes into cereal plants might be used as a general strategy for control of insect pests.

Natural or modified *Bacillus thuringiensis (Bt)* crystal protein genes have been introduced into a number of dicot plants and two monocot plants to develop insect-resistant crops. For genetic engineering of insect resistance, additional resistance genes are needed. Plant-derived proteinase inhibitors are of particular interest because they are part of the natural plant defense system against insect predation (Ryan 1990). Many insect species possess serine-type proteinases, such as trypsin and chymotrypsin, in their digestive systems for food protein digestion. When foreign plant proteinase inhibitor genes were introduced into tobacco plants, the production of proteinase inhibitors at relatively high levels made the transgenic tobacco plants resistant to typical tobacco insect pests (Hilder et al 1987, Johnson et al 1989). These studies demon-

strated the feasibility of genetic engineering of insect resistance by introducing proteinase inhibitor genes into crops.

Significant progress in gene transfer technologies for rice makes it possible to produce new rice cultivars by introducing agronomically useful genes into rice. We have introduced the potato proteinase inhibitor II gene (*Pin2*) into several rice varieties. Based on extensive molecular analyses, the *Pin2* gene was shown to be stably inherited in four successive generations of the transgenic rice plants (Duan et al 1996). More importantly, in a preliminary small-scale field test, transgenic rice plants showed significantly increased resistance to two major rice insect pests.

Results and discussion

Production of fertile transgenic rice plants

Plasmid pTW contains the *Pin2* gene regulated by its own promoter and 3' terminator sequence. The first intron of the rice *Act1* gene was inserted between the *Pin2* promoter and the *Pin2* coding region. In our previous study, the combination of the *Pin2* promoter and the *Act1* intron was demonstrated to confer high-level, wound-inducible expression of foreign genes in transgenic rice plants (Xu et al 1993). Plasmid pTW also contains the bacterial *bar* gene joined to the CaMV35S promoter, which serves as the selectable marker in rice transformation.

Plasmid pTW was introduced into suspension cells of three rice varieties using the biolistic method (Cao et al 1992). From three transformation experiments, 65 independent lines (more than 200 plants) were regenerated and grown in a greenhouse. Plants from more than 50 lines were analyzed by DNA blot hybridization; 73% of them were shown to contain the *Pin2* gene. More than 70% of the transgenic plants were fertile (Table 1).

Digestion of plasmid pTW or rice genomic DNA from transgenic plants with *Bam*HI releases the 1.5-kb fragment containing the *Pin2* coding and 3' regions. Figure 1 shows that all nine R_0 transgenic plants gave the 1.5-kb hybridization band as the strongest band. The presence of weak hybridization bands of larger molecular sizes may be due to partial digestion, and bands of smaller sizes in several samples

Table 1. Summary of transformation experiments.

Rice variety	Plates of cells bombarded (no.)	Resistant calli selected (no.)	Lines of plants regenerated (no.)	Transgenic lines shown by DNA blot (no.)	Fertile lines (no.)
Nipponbare	8	52	14		
Tainung 67	4	34	11		
Pi4	8	149	40		
Total	20	235	65	37 (73)[a]	27 (73)[b]

[a]The number in parentheses is the percentage of DNA blot-positive plants compared with the total number of plants analyzed. Of 51 plants analyzed, 37 plants gave positive signals in DNA blot hybridization. [b]The number in parentheses is the percentage of fertile transgenic plants compared with the total number of transgenic plants. Of 37 transgenic plants analyzed, 27 plants were fertile.

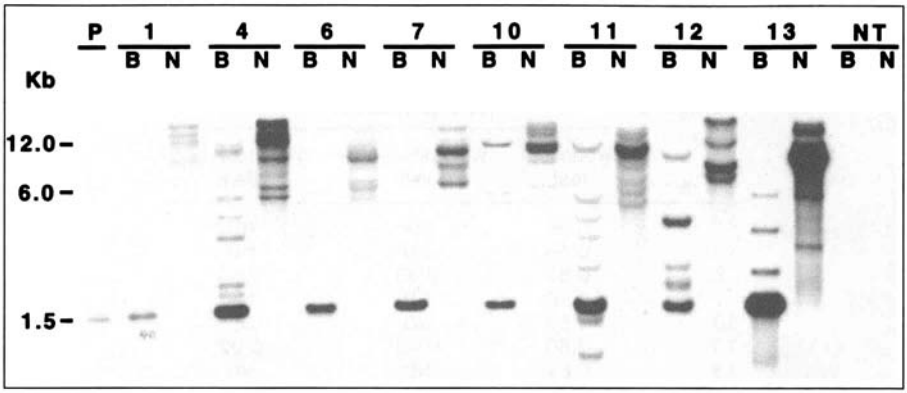

Fig. 1. DNA blot analysis of eight R_0 transgenic plants. For each sample, *Bam*HI-digested (left lane) or *Nco*I-digested (right lane) genomic DNA was separated in a 1% agarose gel. DNA blot hybridization was carried out using a ^{32}P-labeled, 1.5-kb DNA fragment containing the same *Pin2* coding and 3' regions as the probe. The identification numbers of R_0 transgenic plants are marked on the top. P, plasmid pTW digested with *Bam*HI. NT, nontransformed wild-type control plant. The DNA molecular size markers are indicated on the left-hand side. The predicted 1.5-kb hybridization band is marked with an arrow.

may be due to rearrangement of the plasmid DNA sequence. In lanes with undigested genomic DNA, hybridization signals appeared only in the high molecular DNA region, suggesting that the transferred genes were indeed integrated into the rice genome.

Analysis of wound-inducible production of the PINII protein in R_0 transgenic rice plants

The PINII protein levels were estimated, based on the percentage of inhibition of a fixed amount of bovine trypsin, by increasing amounts of tissue protein extracts. Purified PINII protein was used as the standard in the inhibition assays. In nonwounded plants, a low level (0.05-0.2% of total soluble proteins) was detected in leaves, stem, and roots. In wounded leaves, analysis made 20 h after wounding showed that PINII protein levels increased 5- to 10-fold to 0.5-2.0% of total soluble proteins (Table 2). Variations in the PINII protein level among different transgenic lines might be due to the difference in copy number of the *Pin2* gene or to an integration position effect. Increased levels of PINII protein were also detected in nonwounded upper leaves (wound-induced leaves) adjacent to the wounded leaves and in the young stem tissue (wound-induced stem) of a wounded tiller (Table 2).

Inheritance of the transferred *Pin2* gene in the second (R_1) and third (R_2) generations of transgenic rice plants

Among 60 analyzed R_1 plants from seven transgenic lines, 45 R_1 plants (75%) contained the *Pin2* gene, as shown by DNA blot hybridization. A number of R_1 plants from each of two representative transgenic lines, No. 6 and No. 12, were analyzed by

Table 2. Levels of PINII protein in the first-generation (R_0) transgenic rice plants.

Transgenic line	PINII protein level (%total soluble proteins)		
	Wounded leaf	Wound-induced leaf	Wound-induced stem
1	0.96	ND[a]	ND
4	0.65	ND	ND
6	0.52	0.35	0.55
7	1.90	ND	ND
10	0.60	ND	ND
12	0.60	0.43	0.92
13	0.67	ND	ND

[a]ND = not determined.

DNA blot hybridization. Among 14 analyzed R_1 plants from line No. 6, 10 still contained the *pin2* gene. Among 12 analyzed R_1 plants from line No. 12, 10 still contained the *Pin2* gene. In each line, all those positive R_1 plants showed the same hybridization pattern as their R_0 parent (data not shown).

PINII protein levels in R_1 plants after wounding were also analyzed. The PINII protein level in the R_0 plant of line No. 6 was estimated to be 0.5% of total leaf-soluble proteins. All 10 R_1 plants of this line containing the *Pin2* gene also accumulated PINII protein in leaves in response to wounding. PINII protein levels in some R_1 plants are similar to those of the R_0 plants, and in some R_1 plants, PINII protein levels are 2- to 3-fold higher than those of the R_0 plants. Similar results were obtained with R_1 plants from line No. 12.

Approximately 25% of R_1 plants are expected to be homozygous transgenic plants with respect to the transferred gene. We reasoned that those R_1 plants with the same DNA blot hybridization pattern that produced at least twice the amount of PINII protein, as compared with their R_0 parents, are potential homozygous transgenic plants. To confirm this and to obtain homozygous transgenic lines, we selected two such R_1 plants, No. 6-8 from line No. 6 and No. 12-3 from line No. 12, for further analysis of their R_2 plants. All 14 R_2 plants from R_1 plant No. 6-8 contained the *Pin2* gene and showed a DNA hybridization pattern as did their R_1 and R_0 parents (data not shown). All these R_2 plants accumulated at least equally high levels of PINII protein as in their R_1 parent No. 6-8 (Fig. 2a). Similarly, all 14 R_2 plants from R_1 plant No. 12-3 contained the *Pin2* gene and showed the same DNA hybridization pattern as did their R_1 and R_0 parents (data not shown). All these R_2 plants accumulated at least equally high levels of PINII protein as in their R_1 parent No. 12-3 (Fig. 2b). Based on these data, we conclude that R_1 plants No. 6-8 and No. 12-3, and all their R_2 progeny, are homozygous transgenic plants.

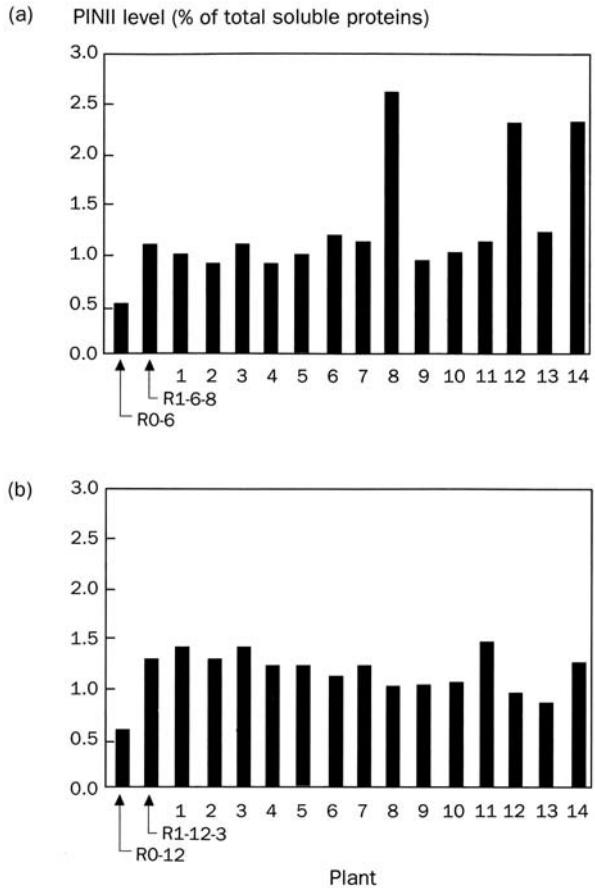

Fig. 2. PINII protein levels in three generations, R_0, R_1, and R_2, of two transgenic lines, No. 6 and No. 12. a) PINII protein levels in the R_0 plant of line No. 6, one R_1 plant (No. 68) from this line, and 14 R_2 plants from R_1 plant No. 6-8. b) PINII protein levels in the R_0 plant of line No. 12, one R_1 plant (No. 12-3) from this line, and 14 R_2 plants from R_1 plant No. 12-3.

Conclusion

In this study, we introduced into rice a well-characterized, serine-type proteinase inhibitor gene from potato. A large number of independent transgenic lines were produced, and more than 70% of the transgenic rice plants were fertile. Based on molecular analyses, several homozygous transgenic lines have been identified. Preliminary small-scale tests in an isolated experimental field showed that transgenic rice plants expressing the potato proteinase inhibitor gene had increased resistance to two

species of rice stem borers (for details see Xue et al 1996). Our results suggest that introducing proteinase inhibitor genes into rice is a useful strategy to control certain rice insect pests.

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Resistance management strategies for *Bt* rice: what have we learned so far?

M.B. Cohen, R.M. Aguda, A.M. Romena, G.K. Roderick, and F.L. Gould

Rice genetically engineered with toxin genes from *Bacillus thuringiensis (Bt)* can provide safe and effective control of selected insect pests, but will not provide durable resistance unless *Bt* rice plants are carefully designed and deployed. Based on modeling and experimental studies of numerous pest systems, maintaining spatial refuges (plant parts, plants within fields, or entire fields left untreated with *Bt* toxins) is, in general, the most reliable approach to resistance management. Using tissue-specific gene promoters, it will likely be possible to produce rice in which toxin genes are expressed only in selected tissues. However, it is not clear what pattern of tissue-specific expression will establish a suitable refuge for stem borers. Within-field mixtures of *Bt* and non-*Bt* plants may not be an effective strategy for stem borers because larvae move from plant to plant during development. Combining *Bt* toxins within plants is unlikely to provide long-term suppression of resistance because some insect mutations can confer cross-resistance to multiple toxins. Combinations of *Bt* toxins with unrelated toxins, such as proteinase inhibitors, will probably be more durable. However, toxin combinations will be most effective if used in association with refuges.

Genetic engineering of rice with toxin genes from *Bacillus thuringiensis (Bt)* can provide highly effective and environmentally safe control of various insect pests. However, the major weakness of *Bt* technology is its potential lack of durability. More than 15 species of insects have now been selected for resistance to *Bt* strains or individual toxins (Tabashnik 1994). Numerous strategies have been proposed to retard and stabilize the evolution of pest resistance to *Bt* toxins in transgenic plants. We have initiated a set of research projects to determine which of these approaches will work best for *Bt* rice. Our principal target insects are stem borers, the most important of

which are the yellow stem borer (YSB), *Scirpophaga incertulas,* in tropical rice, and the striped stem borer (SSB), *Chilo suppressalis,* in temperate regions.

Spatial refuges

Experiments and modeling studies indicate that maintaining spatial refuges (plant parts, plants, or fields in which toxins are not present) is, in general, an effective approach to retarding the evolution of pest resistance to insecticides and resistant plants (Tabashnik 1994). The underlying principle of spatial refuges is to maintain, within each generation, a proportion of the pest population that is not subject to selection by toxins. Within the unselected subset of the population, the frequency of alleles conferring resistance to these toxins will not increase. If extensive mating occurs between insects from the unselected subset of the population and the small number of toxin-resistant individuals that successfully complete development on toxic plants, then the proportion of resistant individuals in the subsequent generation will remain small for a long period of time.

It is particularly effective to use spatial refuges in combination with plants that produce levels of toxin high enough to kill all homozygous susceptible and heterozygous individuals (Gould 1994). In many cases of insecticide resistance, heterozygotes have levels of resistance intermediate to that of the two homozygous genotypes. As mortality of heterozygotes approaches 100%, additive genetic variance in a population with a low frequency of resistance alleles approaches zero and the rate of evolution of resistance in the population approaches zero. Note, however, that in the absence of a refuge, high dose plants will lead to rapid evolution of resistance.

Tissue-specific expression

The locations within a plant in which *Bt* genes are expressed can be controlled by use of tissue-specific gene promoters, but it is not clear which tissues are best for selective expression of *Bt* toxin genes directed against stem borers. Leaf sheaths and growing shoots are the preferred feeding sites of young larvae on vegetative and reproductive stage plants, while almost all larvae on reproductive stage plants eventually move to the internodes of the stem (V.F. Magalit and D.G. Bottrell, unpubl. data). Thus, expression of *Bt* genes in leaf sheaths, growing shoots, or stems will probably not maintain a large enough refuge of unselected larvae. On plants infested by YSB larvae at the reproductive stage, up to 30% of young larvae were found feeding on the spikelets of developing panicles (V.F. Magalit and D.G. Bottrell, unpubl. data). Bt gene constructs with a pollen-specific promoter, such as those developed by Altosaar et al (1996), would be useful in targeting these larvae. In this case, however, the refuge of unselected larvae may be too large to provide adequate levels of stem borer control.

Movement of larvae among tissues during development can undermine the effectiveness of refuges based on tissue-specific expression. In species with this type of behavior, such as stem borers, larvae may ingest sublethal doses of *Bt* toxin as they

move from tissue to tissue, increasing the survival of heterozygotes and therefore the additive genetic variance of the resistance trait (Gould 1994).

Within-field mixtures of *Bt* and non-*Bt* rice plants

Within-field mixtures can be conveniently established by mixing seeds of *Bt* and non-*Bt* rice in the right proportion before sowing. The two seed types would be identical except for the presence of the *Bt* gene. Movement of larvae among *Bt* and non-*Bt* plants can reduce the effectiveness of within-field mixtures, just as movement of larvae among tissues presents difficulties for tissue-specific expression (discussed above). Consequently, we have conducted a series of experiments to quantify the dispersal pattern of YSB and SSB larvae in plantings of rice (A.M. Romena et al, unpubl. data). Both species move among plants and feed on more than one plant during larval development. Almost all SSB larvae begin to feed on the plant on which they hatch, while many YSB larvae leave the plant on which they hatch before feeding, particularly if the plants are at vegetative stage. We intend to repeat our experiments in plantings of *Bt* rice, to determine if larvae show increased dispersal from *Bt* plants and if larvae that feed on *Bt* plants and then leave are able to establish themselves on non-*Bt* plants and survive to maturity.

Field-to-field mixtures of *Bt* and non- *BT* rice

The success of field-to-field or region-to-region mixtures of *Bt* and non-*Bt* plants requires sufficient migration of adult insects among fields or regions prior to mating. We have used allozyme electrophoresis to quantify the genetic structure of YSB and SSB populations within the Philippines (G.K. Roderick et al, unpubl. data). Our studies indicate that levels of gene flow among YSB and SSB populations are very low in comparison with other species of order Lepidoptera. Migration of moths from regions where *Bt* rice is not planted is unlikely to be sufficient to reduce resistance buildup in regions where *Bt* rice is planted extensively. Studies of more localized gene flow are under way to evaluate the potential of mixtures at smaller spatial scales, e.g., field-to-field mixtures within villages.

Combinations of *Bt* toxins within plants

The use of multiple toxins within *Bt* plants is an often cited approach to resistance management because it is relatively straightforward to implement. Reduced *Bt* toxin binding to midgut receptors is the most common mechanism of *Bt* resistance identified to date. If *Bt* toxins are used in combination, then it will be best to combine toxins that bind to different receptors so that a mutation in one receptor will not confer resistance to all toxins in the mixture. In collaboration with D. Dean's laboratory at The Ohio State University, we have found that in both YSB and SSB, CryIA(a) and CryIA(c) bind to a receptor that is different from that recognized by CryIIA or CryIC (M.K. Lee et al, unpubl. data).

However, not all cases of *Bt* resistance are attributable to changes in receptors and, in some cases, selection with one *Bt* toxin has been shown to generate cross

resistance to other (even distantly related) *Bt* toxins. In addition, some insects have evolved resistance to *Bt* strains that produce as many as five different toxins (Tabashnik 1994). Combinations of *Bt* toxins with unrelated insecticidal proteins, such as proteinase inhibitors, may provide more durable resistance, as a single mutation is unlikely to confer cross resistance to both classes of toxins. Toxin combinations, however, will be most successful if they are used in association with a refuge.

Release of *Bt* rice to farmers

The genetic engineering of *Bt* rice varieties is proceeding faster than the development of resistance management strategies to prolong their effectiveness. Rice plants containing single *Bt* toxin genes have now been produced by several research groups. Additional breeding with these plants is under way and some *Bt* genes may soon be in agronomic backgrounds suitable for use by farmers. If released in areas with severe stem borer infestations, these *Bt* rice varieties may be eagerly adopted by most farmers, leaving few fields to serve as non-*Bt* refuges and consequently leading to the rapid buildup of pest resistance. Short-term benefits will need to be weighed against the possible long-term loss of *Bt* effectiveness. However, in many rice-growing areas, current levels of stem borer infestation are low, and may not justify the release of *Bt* rice until resistance management programs have been developed.

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Mechanism of mannose-binding snowdrop lectin for use against brown planthopper in rice

K.S. Powell and J.A. Gatehouse

Mannose-binding snowdrop lectin (*Galanthus nivalis;* GNA), an insecticidal gene product, has been shown to be toxic to brown planthopper (BPH; *Nilaparvata lugens*). Two approaches were taken to further elucidate the mechanisms of action of GNA against BPH using electrophysiological and immunohistochemical techniques. The electrical penetration graph (EPG) method was used in in vitro studies of BPH feeding behavior. Four clearly distinguishable EPG patterns were observed: nonpenetration, probing, active ingestion, and passive ingestion. Insects fed with the GNA diet experienced an eightfold decrease in diet ingestion duration compared with insects fed with the control diet. The percentage of insects ingesting on the GNA diet was also reduced. These results support the hypothesis that GNA has an antifeedant effect on BPH. Immunohistochemical studies indicate that GNA not only binds to the midgut epithelium tissues of adult rice BPH, but is also transported across the gut, and can be found in the haemolymph and surrounding tissues. The results suggest that GNA has a multimechanistic effect against BPH.

Genetically engineered rice lines expressing insecticidal gene products are currently being investigated as a novel control method for controlling brown planthopper (BPH) *Nilaparvata lugens.* Our approach in identifying gene products, which could be effective against BPH, has been to screen such products using an artificial diet bioassay system (Powell et al 1993) and to examine the mechanism of action using honeydew excretion analysis (Powell et al 1995). Using these techniques, the mannose-binding snowdrop lectin (Galanthus nivalis [GNA]) was shown to have significant toxic and antifeedant effects against BPH. To further elucidate on the mechanism of GNA action on BPH, an electrophysiological technique (electrical penetration graph [EPG]) was used to examine the detailed feeding behavior of adult rice BPH when exposed to GNA in vitro. Preliminary immunohistochemical studies were also carried out at the light microscope level to determine what became of GNA once it was ingested.

Materials and methods

Electrical penetration recording

A strand of flexible gold wire (length 5 cm; diameter 25 µm) was connected to the dorsum of adult female brachypterous BPHs using electrically conductive silver paint (RS Components, Corby, UK). Each tethered insect was then connected to a recording electrode and attached to the input terminal of a 10^9 Wimpedance GIGA-4 (4-channel) DC amplifier. A second electrode, connected to the voltage supply of the amplifier, was placed inside a parafilm feeding sachet containing 200 µl of 20% sucrose with and without the diet treatment. To minimize any possible stress effect due to tethering and to allow adaptation to the diet, the insects were exposed to the control diet for 1 h before EPG signals were recorded. Both controls and treatments were placed under continuous illumination at 25±2 °C at 60% relative humidity. The amplified electronic signals were recorded directly onto the hard disk of a Macintosh computer, using an analog-digital data conversion and acquisition card and Quicklog data acquisition software (Strawberry Tree Computers Inc., California, USA). The stored data were analyzed using the MacStylet software package (Febvay et al 1992).

GNA was supplied by Vector Laboratories, Peterborough, UK, and all other reagents were supplied by Sigma Chemical Co., Dorset, UK.

Immunohistochemical studies

The BPHs were allowed to feed on the artificial diet (MMD-1, Mitsuhashi 1974) containing 0.02 mM GNA or the control diet for a 5-d period. Insects were then placed in Karnovsky fixative, dehydrated, and embedded in LR resin (Powell 1993). Resin sections 1 µm in thickness were stained immunohistochemically for the presence of GNA by the Avidin-Biotin technique using avectastain ABC Kit (Vector Laboratories, Peterborough, UK) and examined using a light microscope.

Results

Feeding behavior on artificial diet

Preliminary observation allowed a correlation to be made between insect activity and EPG pattern. Figure 1 shows typical EPG traces observed for insects feeding on the GNA and control diets. The four patterns observed are described below.

Pattern 1 (nonpenetration). This pattern is indicated by a zero voltage base line, showing there is no contact between the insect labium and the feeding membrane. This was observed when insects were seen either walking on the feeding sachet or settled but with no stylet insertion in the feeding sachet.

Pattern 2 (stylet probing). This is a complex signal of varying amplitude and frequencies and is correlated with labium contact with, and exploratory probing into, the feeding sachet. Probing events were associated with brief bursts of negative voltage in the signal. Visual observations confirmed that the insects were settled with stylets intermittently in contact with the feeding sachet when this waveform was recorded.

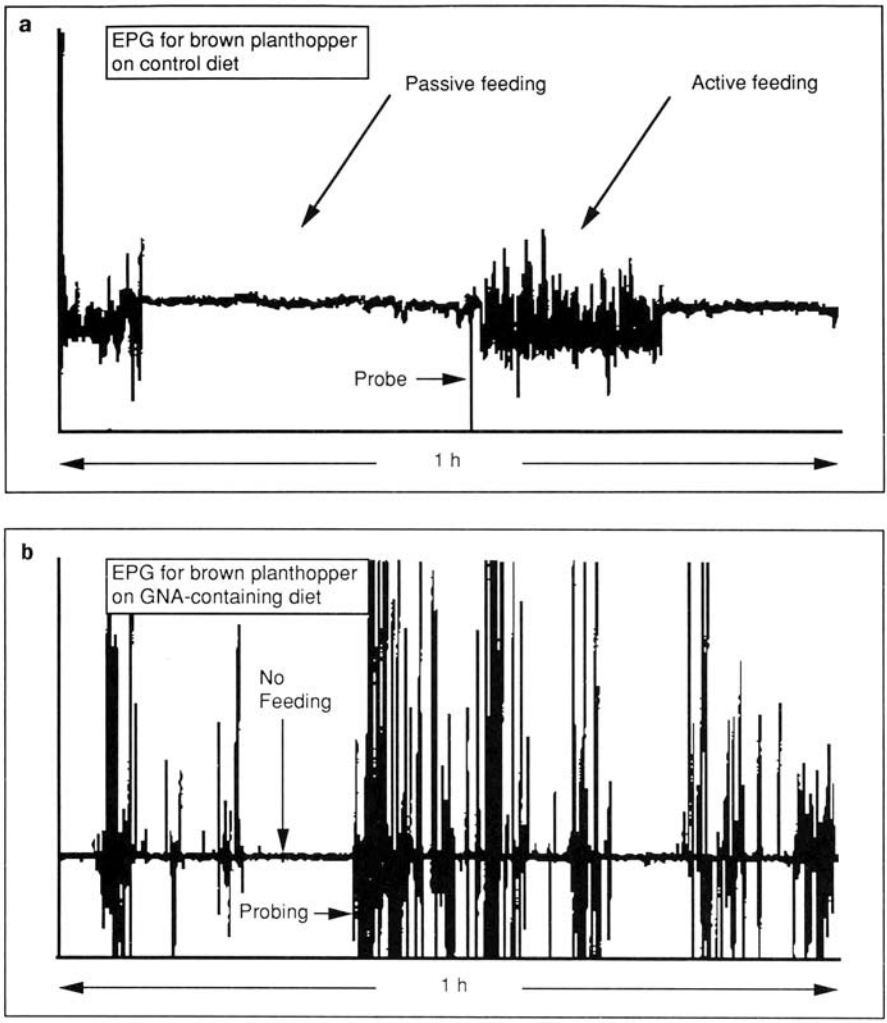

Fig. 1. Typical EPG breeding behavior patterns observed from adult rice BPH feeding during 1-h period on diets containing a) 20% sucrose and b) 0.02 mM GNA.

Pattern 3 (*active diet ingestion*). This has a regular frequency of 5-15 Hz, with amplitudes between 5 and 40 mv, higher in amplitude than pattern 4. Visual observations of insect behavior indicated settled insects with stylets inserted in the diet and honeydew produced.

Pattern 4 (*passive diet ingestion*). This has a relatively low amplitude, typically less than 4 mV with a regular frequency between 0.13 and 0.2 Hz. No honeydew production was observed when this pattern was evident, even though the insects' stylets were inserted in the diet and insects were settled.

Effects of GNA on feeding behavior

When the GNA diet was fed to the insects over a 2-h period, the mean duration of probing was significantly increased ($P<0.05$), when compared with insects fed with the control diet, with GNA-fed insects spending approximately 50% more time probing than control insects. The mean duration of ingestion was significantly decreased ($P<0.05$) approximately eightfold in GNA-fed insects; passive ingestion was decreased approximately threefold, but active ingestion was reduced ($P<0.01$) approximately thirtyfold. The effect of GNA on ingestion was also shown in the number of insects feeding—only 9% of GNA-fed insects ingested any diet at all, compared with 58% of the control insects (data not presented).

Immunohistochemical studies

Abdominal transverse sections of the insects were treated using the Avidin-Biotin immunohistochemical technique and incubated with both primary (anti-GNA) and secondary antibodies. In control insects, no staining was observed in the midgut region, haemolymph, ovarioles, or oocytes, but yeastlike symbionts did stain darker than the surrounding section indicating the presence of some endogenous peroxidases in the symbiont walls. In sections of GNA-fed insects, staining was clearly evident throughout the abdominal region. Intense staining, indicating lectin presence, was seen on the microvilli brush border of the midgut while less intense staining was seen within midgut epithelial cells, ovarioles, eggs, and the haemolymph. The degree of staining in the yeastlike symbionts was higher than in sections of the control insects (data not presented).

Discussion

The feeding behavior of BPH on artificial diet as shown in this EPG study cannot be compared directly with previous EPG studies. Pattern 2 (probing) includes both labial exploratory contact with the feeding membrane and exploratory probing, as it is difficult to distinguish between the two forms of behavior. Pattern 3 (active ingestion) is a regular waveform and of a higher frequency than pattern 4. This may relate to the fact that the dorsal canal of the maxillary stylet connects to the sucking pump of the insect (Sogawa 1977), thus pattern 3 was considered to be due to active ingestion. During sustained ingestion, the insect excretes a large amount of honeydew; this was evident in the present study during ingestion by insects feeding on the control diet, but was not quantified. Pattern 4 with a lower frequency waveform has been correlated with passive ingestion; it could also indicate that the stylet is merely in contact with the diet and that there is no net flow of diet into the insect. The waveforms observed bear similarities with in planta EPG waveforms observed by other workers.

Presence of the lectin in the GNA diet significantly affected the BPH's feeding behavior as measured by increased probing activity and reduced duration of ingestion. It also greatly decreased the number of insects that showed any ingestion at all. Using analysis of honeydew production, Powell et al (1995) presented evidence on GNA's antifeedant effect on BPH over a 48-h bioassay period. The results in the present

study suggest that the effect is even more immediate since the antifeedant effect is expressed over the 2-h EPG assay period.

The mechanism of GNA's antifeedant action may be attributed to interference with chemosensory receptors located in the labium, which may be involved in the initial probing activity (Foster et al 1983a), or with receptors located in the stylet and cibarium, which may be involved with sap ingestion and/or stylet movement within the plant (Foster et al 1983b). However, immunohistochemical evidence presented here suggests that GNA not only has an antifeedant effect, but may also bind to oligosaccharides linked to proteins in the midgut epithelium of BPH, as has been suggested for lectins affecting other homopteran groups (Sauvion 1995). GNA also can pass through the midgut by a mechanism as yet undetermined, possibly by disruption of midgut epithelial cells, to reach other regions including the haemolymph, ovarial organs, oocytes, and symbionts. GNA thus appears to have a multimechanistic action against BPH.

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Evaluation of indica rice variety IR58 after its transformation with a synthetic *cryIA(b)* gene from *Bacillus thuringiensis*

J. Wünn, A. Klöti, P.K. Burkhardt, G.C. Ghosh Biswas, S. Bieri, R. Terada, and I. Potrykus

We transformed indica rice breeding line IR58 with a synthetic version of a truncated *crylA(b)* gene from *Bacillus thuringiensis* via particle bombardment into immature zygotic embryos. Stable integration of the transgene could be shown by Southern analysis of R_0, R_1, and R_2 generation plants. DNA dot-blot analysis revealed a segregation ratio close to 3:1, indicating the insertion of the *crylA(b)* gene in a single locus on one chromosome. Protein analysis showed the activity of the transgene in all three generations analyzed so far. The insecticidal effect of the transgenic IR58 plants could be shown by insect bioassays. Neonate larvae of yellow stem borer *(Scirpophaga incertulas)* and striped stem borer *(Chilo suppressalis),* two of the most destructive insect pests of rice in Asia, showed mortality levels of up to 100% with feeding for 2-4 d on *crylA(b)*- expressing tissue. Feeding damage on transformed leaves was consistently limited. Larvae feeding on nontransgenic material caused high feeding damage and showed normal larval development, with mortality levels of 5%. Preliminary results also showed an effect of the *crylA(b)*-expressing plants against two rice leaffolder species, *Cnaphalocrocis medinalis* and *Marasmia patnalis.*

Worldwide rice production reached 530 million t in 1994 and more than 80% of it is derived from indica rice cultivars (IRRI 1989). It is estimated that the annual yield losses caused by insect pests, diseases, and weeds can reach up to 50% (Grayson et al 1990). Among the 100 insect species affecting rice plants in Asia, 20 are known to cause significant economic damage. The lepidopteran stem borers are generally considered the most destructive insect pests (Pathak and Khan 1994). The yellow stem borer (*Scirpophaga incertulas*) is the most important rice pest in tropical Asia, whereas the striped stem borer (*Chilo suppressalis*) is predominantly found in more temperate climates. Together they account for an annual yield loss of 5-10%, with occasional outbreaks of up to 60-95% (Yambao et al 1993, Pathak and Khan 1994).

Therefore, rice breeders worked for many years to produce stem borer-resistant rice varieties. However, despite screening more than 30,000 rice varieties for stem borer resistance, sufficient levels of resistance have not been found and none of the rice varieties developed so far has more than a moderate level of resistance (Khan et al 1991).

The entomocidal soil bacterium *Bacillus thuringiensis* (*Bt*) offers a promising variety of so-called *cry* genes, which encode for specific endotoxins with insecticidal activity. We transformed indica rice breeding line IR58 with a synthetic version of a truncated *cryIA(b)* gene under the control of the CaMV35S promoter. This gene has been modified for expression in monocotyledonous plants and the CryIA(b) protein has been shown to be effective against lepidopterous insect pests (Koziel et al 1993). For transformation, we used a particle inflow gun, constructed according to Finer et al (1992). The scutellum side of 700 zygotic immature embryos was bombarded. From one transformation event, 11 highly fertile plants were regenerated. Southern analysis of R_0, R_1, and R_2 generation plants showed the stable integration of the transgene in the genome of variety IR58. Besides the expected band corresponding to the *cryIA(b)* expression cassette, five additional bands were detected, indicating the presence of rearranged copies of the transgene in the rice genome.

Out of 48 selfed R_1 plants analyzed by DNA dot-blot analysis, 35 showed the presence of the transgene, whereas 13 plants did not give any signal. This segregation ratio close to 3:1 indicates the integration of the transgene in a single locus on one chromosome.

The expression of the *cryIA(b)* gene was analyzed by enzyme-linked immunosorbent assay. A maximum of 84 ng *Bt* protein mg^{-1} total soluble protein was found in leaves of 12- to 4-wk-old plants of the R_1 generation. The presence of the *Bt* protein in transgenic plants was further demonstrated by Western blot analysis, where a protein with the expected size of 60 kDa was found in all R_1 plants tested.

The insecticidal activity of the CryIA(b) protein produced in the transgenic IR58 plants could be shown in insect bioassays. Neonate larvae of different lepidopteran rice pests were fed with leaves of the transgenic rice plants in a petri dish assay. For the yellow stem borer and the striped stem borer, up to 100% mortality was found after feeding for 2-4 d on *Bt*-containing leaf tissue. The feeding damage in these dishes was limited and larvae did not reach further larval stages, indicating an early cessation of feeding by the larvae. Larvae feeding on nontransformed control leaves of variety IR58 reached second- and third-instar stages and heavily damaged the leaves. Mortality in control dishes was 5%.

In collaboration with the International Rice Research Institute (IRRI), the effect of the *cryIA(b)*-expressing plants against two of the most abundant rice leaffolder species, *Cnaphalocrocis medinalis* and *Marasmia patnalis*, was tested. Preliminary results also showed feeding inhibition against these defoliator species. R_2 plants are currently being analyzed at IRRI, where whole plants or even small plant populations can be analyzed under more natural conditions.

The constitutive expression of one *Bt* gene in a widely grown crop plant could lead to the development of resistant insect populations. We are therefore focusing our

future research on the investigation of the potential of stage- or tissue-specific promoters, which would direct the expression of the *Bt* gene exclusively to the target tissue or would allow the production of the *Bt* protein only during certain growth stages of the plants. Both approaches would reduce the selection pressure in the field, minimizing the risk of the buildup of resistant insect populations.

The combination of *Bt* genes, which are known to bind to different receptor sites in the midgut of lepidopteran insect pests (e.g., *cryIA(c)* or *cryIIA*) and other genes with insecticidal activity (e.g., proteinase inhibitors) in the same plant, should help in developing a durable stem borer resistance. To identify the most suitable constructs or construct combinations, we are currently transforming them to rice variety TP309, for which an efficient transformation system is established in our laboratory. This will allow the production of a large number of independent transgenic plants in a relatively short time. Insect feeding studies will reveal the most promising constructs for a long-term insect resistance strategy. These constructs will then be transformed to other IRRI breeding lines.

With the transfer of a synthetic *cryIA(b)* gene into the germplasm of an elite indica rice cultivar, the stem borer resistance trait is available now for improvement of other modem rice varieties by conventional breeding.

Such *Bt*-expressing indica rice plants could be used in association with integrated pest management (IPM) programs, providing alternatives to the ecological disadvantages of the widespread use of chemical insecticides. In combination with other IPM strategies, *Bt*-expressing indica rice plants have the potential to save a significant part of the yield loss caused by stem borer infestations every year.

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Toward genetically engineered resistance to tungro virus

A. Klöti, J. Fütterer, R. Terada, S. Bieri, J. Wünn, P.K. Burkhardt, G. Chen, Th. Hohn, G.C. Ghosh Biswas, and I. Potrykus

We have produced 93 fertile, transgenic lines (var. Taipei 309 or Kinuhikari) containing genes that code for complete or mutated rice tungro bacilliform virus (RTBV) proteins. Southern blot analysis confirmed that these plants contain the RTBV open reading frames (ORFs) 1, 3, or 4, respectively, subfragments thereof. The respective genes are under the control of a CaMV35S promoter or the corresponding RTBV promoter. The activities of the promoters were enhanced by the presence of the first exon and parts of the intron of the RTBV ORF 4 mRNA. Promoter activity was verified by the analysis of transgenic plants containing a ß-glucuronidase gene under the control of the respective promoters. For screening the R_1 generation of the fertile lines for resistance to rice tungro disease, the first 20 lines are currently being tested under natural conditions in the C4-containment facilities of the International Rice Research Institute (IRRI).

Tungro is probably the most important viral disease of rice in Southeast Asia. No natural, durable resistance genes have been found in the rice gene pool. Genetically engineered resistance against a large number of plant RNA viruses has been achieved in recent years. In most cases, resistance was obtained by simply expressing a normal or mutated, single viral gene product in transgenic plants (Beachy 1993). So far, efficient, engineered resistance has not been described against a plant pararetrovirus such as rice tungro bacilliform virus (RTBV), the component responsible for the severe symptoms of tungro disease. The differences in the viral life cycle between the RNA viruses and the pararetrovirus RTBV—particularly the presence of RTBV as DNA copy in the nucleus in contrast to the more labile cytoplasmic RNA copies of the RNA viruses—may demand different strategies. However, a similar approach should be feasible and it is to be expected that for RTBV, expression of functional viral proteins at the onset of virus infection could interfere with an ordered progression through the

viral life cycle, and that expression of mutated viral proteins might interfere with the function of normal viral proteins by competition.

We have produced a large number of fertile, transgenic rice plants (var. Taipei 309 or Kinuhikari) containing genes that code for complete or mutated RTBV proteins 1, 3, and 4 (Table 1). Protein 3 is a precursor from which the viral coat protein, the replicase, a protease, and probably a variety of other proteins are generated by proteolytic processing. A variety of constructs for direct expression of the coat protein

Table 1. RTBV sequences introduced into rice plants.

	DNA sequence[a]	Amino acids[b]	Description[c]
ORF1	99-664	12-199	Complete ORF1, ATT start codon mutated to ATG
ORF3	997-5957	2-1655	Complete ORF3
	2375-5957	462-1655	Coat protein+polymerase;
	3313-3354[d]	774-787[d]	RNA-binding motif
	5014-5019[d]	1341-1342[d]	mutated to GG; DD in active site of the reverse transcriptase mutated to EE
	3394-5957	801-1655	Polymerase, including putative DNA-binding motif at 5' end
	3747-5957	919-1655	Polymerase
	3747-5957	919-1655	Polymerase; DD in active
	5014-5019[d]	1341-1342[d]	site of the reverse transcriptase mutated to EE
	3747-5010	919-1339	5' half of polymerase
	5020-5957	1343-1655	3' half of polymerase
	2375-3453	462-821	Putative coat protein sequence
	2375-3298	462-768	Putative coat protein sequence, lacking RNA-binding motif at 3' end
ORF4	5973-7186	1-409	Complete ORF4, 1st and 2nd exon
	5973-6677	1-237	5' half of ORF4
	6483-7186	174-409	3' half of ORF4
Leader	7859-7408		Part of the leader sequence of the pregenomic RNA, inserted in antisense orientation into the 3' untranslated region of an *aph4* gene.

[a]DNA sequences according to Hay et al (1991). [b]Amino acids of each open reading frame (ORF) according to Hay et al (991). [c]Amino acids are given in one-letter code. [d]Mutated DNA or protein sequences, respectively.

or the reverse transcriptase have been prepared on the basis of sequence homologies to related viruses. To create mutant proteins that might act as competitive inhibitors for critical viral functions, mutations have been introduced into the coat protein and the reverse transcriptase. In the coat protein region, a sequence motif containing three invariable cysteines and one histidine is conserved between almost all viruses using reverse transcriptase in their replication cycle (retro and plant pararetroviruses; Covey 1986). This motif, which is involved in several RNA-binding steps, has been mutated to glycine in the RTBV coat protein, since such a mutation has been shown to preserve the structure (and thus part of the function) of the coat protein but abolishes the infectivity of a retrovirus (DeRocquigny et al 1992, Morellet et al 1992).

In the reverse transcriptase region, a mutation was introduced into a highly conserved sequence motif containing two aspartates (Argos 1988). In addition, subfragments of the polyfunctional reverse transcriptase have been cloned in an attempt to repeat successes obtained with some RNA viruses where subfragments of the polymerase gene produced high levels of protection (Wilson 1993). Since functions for the remaining RTBV proteins are unknown, changes are difficult to design. In protein 4, we localized a leucine zipper similar to those that are involved in protein-protein interactions (Gruissem 1990).

In a yeast system (Fields and Song 1989), we found that protein 4 indeed has the capacity to dimerize but the dimerization domain resides outside the leucine zipper, which may, however, interact with another protein. We have cloned subfragments of the protein 4 coding region containing either the leucine zipper or the dimerization domain to express proteins that lack one of the putative interaction domains. They probably will not have a complete function but will still interact with one of the original partners, thereby acting as a competitive inhibitor.

In addition to these approaches involving expression of a protein, we also have introduced a construct expressing antisense RNA against the leader sequence of the RTBV pregenomic RNA. Antisense RNAs had little effect on RNA viruses (Wilson 1993) but viruses like RTBV with a nuclear phase might be more susceptible.

The respective genes are under the control of a CaMV35S promoter or the corresponding RTBV promoter (Fig. 1). The activities of the promoters were enhanced by the presence of the first exon and parts of the intron of the RTBV ORF 4 mRNA (Fütterer et al 1994). Promoter activity was verified by the analysis of transgenic plants containing a ß-glucuronidase gene under the control of the respective promoters.

Fig. 1. General form of the constructs used. CaMV35S or RTBV promoter; DNA sequences according to Hay et al (1991), describing the RTBV-intron; variable RTBV sequences fused to tag-sequence; CaMV35S terminator: hygromycin^r gene with CaMV35S promoter and terminator.

At the 3'-ends, all the genes are fused to the same sequence, encoding a small tag-peptide, which is recognized by a commercially available antibody (Kolodziej and Young 1990).

For transformation, we used a particle inflow gun, constructed according to Finer et al (1992). Six-day-precultured immature embryos or embryogenic suspension cultures of the varieties Taipei 309 and Kinuhikari were bombarded and resistant clones were selected on media containing hygromycin B. Among the 376 regenerated transgenic plants, 128 different lines could be identified by Southern analysis. Ninety-three (73%) of these lines proved to be fertile. In Southern analysis, 31 (33%) of the fertile lines showed a correct single or multiple gene integration pattern.

For screening the R_1 generation of the fertile lines for resistance to rice tungro disease, the first 20 lines are currently being tested under natural conditions in the C4-containment facilities at IRRI.

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Biological screening and analysis of transgenic rice lines expressing coat protein gene(s) of rice tungro spherical virus

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For a growing number of positive-strand RNA viruses, it has been demonstrated that transformation of host plants with the viral coat protein gene confers resistance to the corresponding virus. In a similar approach, we have attempted to demonstrate coat protein-mediated resistance to rice tungro spherical virus. Earlier, we reported the successful transformation of rice varieties with three-coat protein gene cassettes. The offspring of the stably transformed rice lines were inoculated with viruliferous green leafhoppers in greenhouse trials. Among many lines tested by double antibody sandwich-enzyme-linked immunosorbent assay and Northern blot analysis, a representative example, which has each of the coat protein genes and which showed significant disease resistance, is reported here.

Tungro disease in rice is caused by a complex of two viruses (Hibino et a1 1978): rice tungro bacilliform virus (RTBV), a double-stranded DNA virus, and rice tungro spherical virus (RTSV), a positive, single-stranded RNA virus. These two viruses are transmitted in a semipersistent manner by the green leafhopper (GLH), *Nephotettix virescens* (Cabauatan and Hibino 1985). RTBV alone causes severe symptoms, such as stunting, yellowing of leaves, etc., whereas RTSV alone causes mild or no symptoms. Double infection by both viruses causes severe symptoms. It has been demonstrated that *Agrobacterium* inoculation of an infectious clone in rice plants causes systemic spread of the viruses. It has also been suggested that RTBV transmission is dependent on a helper function(s) associated with RTSV infection for the entry of RTBV into rice plants (Dasgupta et al 1991).

These findings offer a good hope of controlling tungro disease in rice by preventing the spread of RTSV. Our aim in this study is to 1) further examine the role of RTSV in tungro disease in rice by coat protein-mediated resistance, and 2) study the possibility of controlling tungro by eliminating RTSV, which is reported to be important for the transmission of RTBV.

Shen et al (1993) reported that there are at least three capsid protein subunit cistrons present near the N terminus of the large open reading frame of the RTSV genome and designated them as CP1, CP2, and CP3. In an effort to achieve coat protein-mediated resistance to RTSV in rice, we have earlier reported transformation of chimeric cDNA clones of these individual coat protein genes in rice varieties (Sivamani et al 1994). In this report, we present data on biological screening with viruliferous (RTSV) GLH inoculations and analysis of the transgenic rice lines in greenhouse trials.

Materials and methods

To obtain homozygotes for screening, the seeds of R_0 lines were imported to the Malaysian Agricultural Research and Development Institute, Malaysia, and germinated and analyzed for the presence and segregation of the gene of interest (GOI). Among many lines tested, the most promising lines that showed evidence of protection against RTSV infection are reported here.

Results and discussion

Screening of a transgenic TN1 line transformed with the *CP1* gene of RTSV

Only one line of the transgenic variety TN1 was obtained with a gene cassette and subjected to screening experiments. In the Southern blot analysis, the *CP1* gene seems to have integrated in one locus of the genome and, based on the intensity of the bands on the blot, it is assumed that a single copy of the gene may be present. In the Northern analysis of total RNA, good expression was evidenced by the presence of a correct sized message corresponding to the cassette introduced. Offspring up to the R_3 generation have been inoculated with RTSV viruliferous GLH (Table I). The data suggest that the line is still segregating the GOI even in the R_3 generation at a 3:1 ratio. Based on the percentage of resistant plants obtained in the three generations, it is inconclusive that this coat protein gene may confer resistance to RTSV infection. To prove this observation, a homozygous offspring in the line must be achieved for further screening.

Screening of lines transformed with the *CP2* gene of RTSV

The TP309 line with the RTSV *CP2* gene, when analyzed with Southern blot, confirmed the presence of the GOI located in at least two loci with two or three copies per genome. The expression of the GOI in Northern blot analysis of total RNA was good. Seeds from six siblings of the R_1 generation were raised to give R_2 offspring, which were challenged with RTSV through GLH inoculations (Table 1). From one of the six lines (E6), a sample of 12 plants was screened in polymerase chain reaction (PCR) for the presence of RTSV *CP2*. All 12 plants were PCR-positive for the gene, which probably suggests that it is homozygous. The percentage of resistant plants of the R_2 offspring of this line obtained after two inoculations (up to 50%) suggests that RTSV *CP2* may be conferring resistance to the virus. Northern blot analysis is in progress to analyze resistant plants from the offspring obtained in this line.

Table 1. Details of transgenic rice lines subjected to biological screening with RTSV.

Rice variety	Line	Generation	Segregation ratio of gene of interest	Lines screened (no.) (coat protein gene-positive)	Resistant plants (no.) by DAS-ELISA after 2 inoculations	Resistant plants after 2 inoculations (%)
TN1	B[a]	R_1 (heterozygous)	3:1	8	3/8	37.5
TN1	L[a]	R_1 (heterozygous)	3:1	23	6/23	26
TN1	B.1[a]	R_2 (homozygous)[b]		7	0/7	0
TN1	B.5[a]	R_2 (homozygous)[b]		3	0/3	0
TN1	B.8[a]	R_2 (homozygous)[b]		3	0/3	0
TN1	B.2.3[a]	R_3 (heterozygous)	3:1	7	1/7	14.2
TN1 (Hygromycin control)		R_2 (heterozygous)	1:1	10	0/10	0
TP309	E3[c]	R_2 (homozygous)[b]		15	2/15	13.3
TP309	E6[c]	R_2 (homozygous)[b]		73	31/73	42.4
TP309	E7[c]	R_2 (homozygous)[b]		9	1/9	11.11
TP309	E9[c]	R_2 (homozygous)[b]		13	1/13	7.6
TP309	E14[c]	R_2 (homozygous)[b]		12	6/12	50
TP309	E16[c]	R_2 (homozygous)[b]		25	9/25	36
TP309	J[d]	R_1 (heterozygous)	2:17	2	0/2	0
TP309	J4[d]	R_2 (heterozygous)	3:1	18	2/18	11.11
TP309	J17[d]	R_2 (heterozygous)	1:1	19	7/19	36.8
TP309 (Hygromycin control)		R_2 (homozygous)		14	0/14	0

[a] Offspring from one R_0 line transformed with the RTSV *CP1* gene cassette. [b] PCR analysis to be completed in all offspring to confirm status. [c] Offspring from one R_0 line transformed with the RTSV *CP2* gene cassette. [d] Offspring from one R_0 line transformed with the RTSV *CP3* gene cassette.

Screening of a transgenic TP309 line with *CP3* gene of RTSV

Among three lines obtained, line J with the RTSV *CP3,* under a maize ubiquitin promoter, yielded two coat protein-positive siblings out of 17 plants tested in the R_1 generation. Southern blot analysis of these two siblings showed the presence of the GOI in at least one locus and about one copy per genome. The expression of the GOI was found good in the Northern analysis of the total RNA from these siblings. Both siblings were susceptible to the viral inoculations in the R_1 generation. Seeds from these two siblings (J4 and J17) were collected and 24 and 36 offspring, respectively, were tested in PCR for the presence of GOI. Certainly the ratio of segregation of the GOI is on the increase (Table 1) when compared with the results from R_1 progeny. This gives an indication that some of these siblings may form homozygotes in the R_3 generation. The percentage of resistant plants obtained after two inoculations (up to 36%) gives hope that the RTSV *CP3* may also provide resistance to the viral infection. From these offspring, a pure homozygous line is to be achieved to support this evidence.

Conclusion

The results from these screening experiments are encouraging, with special reference to the two homozygous TP309 lines expressing RTSV *CP2* and/or RTSV *CP3*. The role of RTSV *CP1* in coat protein-mediated resistance for RTSV is still being studied. Most of the promising results obtained in this study were from homozygous or near-homozygous plants, suggesting the importance of homozygous lines in screening experiments.

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Rice ragged stunt virus synthetic resistance genes and japonica rice transformation

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Coding region sequences of rice ragged stunt virus (RRSV) genome segments 7 and 10, which encode nonstructural proteins, were used to prepare synthetic resistance gene constructs. CaMV35S, rolC, and act1 promoters were used to drive expression of both sense and antisense sequences. Calli derived from mature or immature embryos of japonica rice cultivar Taipei 309 were bombarded with gold particles coated with the plasmid DNA containing a gene *(hpt)* encoding hygromycin resistance driven by CaMV35 promoter and the plasmid DNA of one of the above gene constructs. The regeneration efficiency of calli, after codelivery of different RRSV genes and the *hpt* gene, and selection on hygromycin ranged from 0.6 to 21%. Polymerase chain reaction and Southern blot analysis of putative transgenic lines revealed 25-90% cotransformation of RRSV genes with the selectable marker gene. Copy number of transgenes varied from 1 to 13. Analysis of R_1 progeny (around 20 plants line^{-1}) of four transgenic lines containing *hpt* and *rolC*-S7 sense or *rolC*-S7 antisense genes revealed cosegregation of these transgenes with an average segregation ratio of 2.4:1. The progeny plants of three transgenic lines containing only the *hpt* gene segregated in a 37:1 ratio, suggesting multiple independent transgene locations.

Rice ragged stunt virus (RRSV) is transmitted exclusively by the brown planthopper *Nilaparvata lugens* (Hibino et al 1977) and is the second most important viral disease of rice in South and Southeast Asia (Boccardo and Milne 1984). Some rice cultivars are resistant to the brown planthopper and therefore are protected against RRSV. However, there seems to be no resistance to RRSV itself in rice cultivars (IRRI 1994). Over the past 5 yr, there has been considerable success in engineering virus resistance by incorporation and expression of virus-derived genes in host plants (Wilson 1993). Rice cultivars have been transformed using methods such as electroporation (Shimamoto et al 1989), polyethylene glycol-mediated DNA uptake (Zhang et al 1988),

biolistics or microprojectile bombardment (Cao et al 1992), and more recently by *Agrobacterium* (Chan et al 1992). Our approach has been to use biolistics to transform rice cultivar Taipei 309 with RRSV-derived genes and test their effectiveness in protecting plants from RRSV infection.

Materials and methods

RRSV gene constructs

Coding region sequences of RRSV genome segments 7 and 10, which encode two nonstructural proteins, were cloned into plant expression vector pJ35SN (Walker et al 1987) in both sense and antisense orientations. For the segment 7 gene, similar constructs were also made with rolC (Sugaya et al 1989) and rice act1 promoters (Zhang et al 1991). Selectable marker gene constructs used were either pMON410 (provided by the Monsanto Company) or pTRA151 (Zheng et al 1991; provided by N. Murai), both of which contain CaMV35S promoters driving the *hpt* gene.

Tissue culture and transformation

The japonica rice cultivar Taipei 309 mature or immature seeds (10-15 d after pollination) were used according to Thompson et al (1986) for callus induction with NB solid media (Li et al 1993), which contained N6 macronutrients (Chu et al 1975), B5 micronutrients and vitamins (Gamborg et al 1968), proline (500 mg L^{-1}), enzymatic casein hydrolysate (300 mg L^{-1}), glutamine (500 mg L^{-1}), MS Fe-EDTA (Murashige and Skoog 1962), sucrose (30 g L^{-1}), phytagel (2.5 g L^{-1}, Sigma), and 2,4-D (2 mg L^{-1}). Embryogenic calli (1-2 mm size) from not more than four subcultures (2 wk each) were used as target tissue for particle bombardment.

The Biolistic PDS-1000/He particle delivery system (BIO-RAD) was used according to manufacturer's instructions. Sixty to 100 calli (1-2 mm in size) were arranged in a circle (about 2 cm in diameter) in the center of an NB media plate also containing 30 g L^{-1} each of mannitol and sorbitol as osmotins, 4 h prior to bombardment. Two milligrams each of 1 µm and 5 µm gold and 5 µg of DNA comprising the selectable marker plasmid and the gene of interest in a molar ratio of 1:4 were used. DNA coating was performed according to the manufacturer's instruction except that spermidine was added prior to addition of $CaCl_2$. Shooting conditions were as follows: 1100-psi He pressure, 26-in Hg chamber vacuum, 20-cm gap (2nd slot) between the rupture disk and macrocarrier, and 60-cm gap (4th slot) between macrocarrier and the target tissue. Calli containing plates were kept in the dark (27 °C) for 16 h.

Selection and analysis of transgenic plants

Sixteen hours after shooting, calli were placed in the selection medium, NH30 (NB with 30 mg hygromycin B L^{-1}, Boehringer Mannheim) and incubated in the dark at 27 °C. Hygromycin-resistant clusters were excised after 2-3 wk and subcultured with 50 mg hygromycin selection L^{-1} (NH50). Hygromycin-resistant calli were then transferred after 2-3 wk to the preregeneration medium, PRH50 (NB containing 2 mg benzylaminopurine L^{-1}; 1 mg naphthalene acetic acid L^{-1}; and 5 mg abscisic acid L^{-1}

instead of 2,4-D). After 10-15 d, calli were transferred to the regeneration medium, RNH50 (NB containing 3 mg benzylaminopurine L^{-1} and 0.5 mg naphthalene acetic acid L^{-1} instead of 2,4-D) and plantlets from RNH50 plates were transferred to half-strength MS medium with 10 g sucrose L^{-1} and 0.05 mg naphthalene acetic acid L^{-1} without hygromycin and maintained at 22-26 °C with 16 h light (20-40 µE m^{-2} s^{-1} initially and 130 µE m^{-2} s^{-1} after 1 wk). Regenerated plantlets were planted in pots containing a mixture of soil, perlite, sand, and peat moss (50:25:15:10 v/v) and grown in a glasshouse with 28 °C day and 20 °C night temperatures.

Genomic DNA was isolated from plants as described by Drapper and Scott (1988). Transformants and their progeny were screened for the presence of the selectable marker gene and the gene of interest by polymerase chain reaction (PCR). Based on the published sequences of CaMV35, rolC, and act1 promoters and unpublished sequences of RRSV segments 7 and 10 primers (unpubl. data) were designed to amplify a portion of promoter region spanning into the adjacent sense or antisense strand DNA of the inserted DNA. Genomic DNA, digested with required restriction enzymes, was electrophoresed on 0.8% agarose and subjected to Southern blotting (Hybond N+) according to the manufacturer's instructions. Radiolabeled probes (^{32}P) were prepared by random priming (Feinberg and Vogelstein 1983) and hybridization was conducted according to Khandjian (1987). Membranes were washed with required stringency and hybridizing bands visualized by autoradiography using phosphor screens (Molecular Dynamics). Proteins produced by transgenes were detected by Western blot analysis according to established methods (Sambrook et al 1989) using antibodies raised against segment-specific fusion proteins.

Results and discussion

Transformation efficiency

Calli derived from immature or mature embryos were successfully used as target material in the biolistic method of DNA delivery. For each construct used, two to eight different shootings were done with the total calli ranging from 100 to 774. With the regime of selection and regeneration employed on bombarded calli, regeneration frequencies of from 0.65 to 21% were achieved depending on the gene construct DNA delivered along with the selectable marker gene DNA (Table 1).

Transgene analysis

The PCR and Southern blot hybridization revealed that all regenerated plants contained the hygromycin (*hpt*) gene and that cotransformation of *S7* or *S10* genes with the *hpt* gene ranged from 25 to 90% among different constructs used (Table 1). To estimate the number of transgene copies in each of the transgenic lines, genomic DNA was digested with a restriction enzyme, which has a single recognition site in the transgene and then probed with radiolabeled coding region sequences. Such analysis indicated that the copy numbers ranged from 1 to 13. Transgene proteins were detected by Western blot analysis only in plants having the *S7* gene driven by the act1 promoter.

Table 1. Analysis of transgenic Taipei 309 plants produced by biolistics and segregation of transgenes in R_1 progeny.[a]

Construct	rolC-S7-S	rolC-S7-AS	35S-S7-S	35S-S7AS	act1-S7-S	act1-S7-AS	35S-S10-S	35S-S10-AS
Shootings	8	8	4	4	2	2	7	8
Calli shot	426	442	407	380	100	100	614	774
Lines regenerated	9	8	5	12	12	21	4	9
Plants regenerated	20	21	13	27	15	25	10	16
Regeneration frequency (%)	2.10	1.81	1.23	3.16	12.00	21.00	0.65	1.16
hpt +ve lines by PCR	9	8	5	12	12	21	3	9
GOI +ve lines by PCR	7	2	3	8	10	19	3	4
GOI +ve lines by Southern blot	5+2?	2	2+2?	8+3?[b]	10	13+6?	0+4?	2+4?[c]
GOI copy number	2-7	1-3	2-8	2-13	1-8	2-12	1·3	2-4
Cotransformation (%)	78	25	40	66	83	90	75	44
GOI +ve by Western	nd	na	nd	na	5/5	na	nd	na
Fertile lines (total)	3	5	1	2	5	6	1	4
GOI +ve fertile lines	2	2	1	2	5	4	1	0
GOI or *hpt* segregation in R_1 progeny[d]	55:24 (L1) 20:12 (L2)	17:2 (L1) 41:17 (L2)	nd	nd	nd	nd	nd	nd
hpt segregation in R_1 progeny of GOI -ve lines	nd	35:0 25:1 14:1	nd	nd	nd	nd	nd	nd

[a]na = not applicable, nd = not done, S = sense, AS = antisense, S7 or S10 = RRSV genome segment 7 or 10, rolC, 35S, and act1 = rolC, CaMV35S, and rice actin1 promoters. [b]Two independent transformants from a single callus.[c]Same banding pattern between two lines.[d]GOI and *hpt* cosegregated in these progeny.

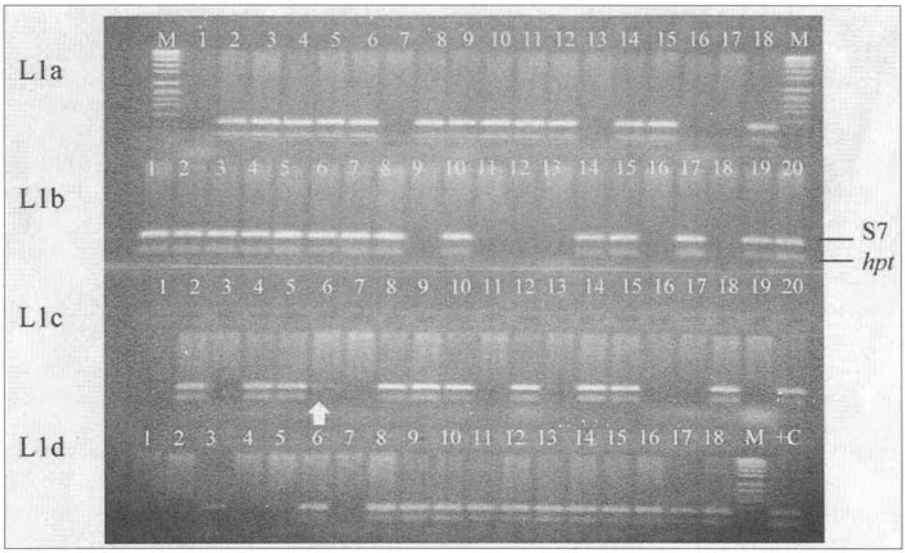

Fig. 1. Segregation of the *S7* and *hpt* genes in R_1 progeny of transgenic Taipei 309 plants. Genomic DNA was isolated from R_1 plants and used in coamplification of *S7* and *hpt* gene-specific DNA fragments by PCR using primers for respective promoter sequences and for the 5' regions of the *S7* and *hpt* genes. L1a, L1b, L1c, and L1d represent R_1 progeny of four plants from the same transgenic line. The arrow indicates occasional absence of a particular PCR fragment possibly due to deletion of a region of the transgene in the segregating population.

R_1 progeny analysis

Because of the cooler season coinciding with flowering time and also possibly due to tissue culture-induced variations, fertility of these transgenic lines was poor (20-50%). Even with the fertile plants, there was poor seed set (5-30%). The first available R_1 seeds from transgenic plants with the rolC-driven *S7* sense and antisense genes along with other control seeds were germinated and plants were analyzed by PCR for transgene segregation. In lines analyzed so far, the *hpt* and *S7* genes (sense or antisense) cosegregated, suggesting that both genes have integrated into a single locus in the rice genome (Fig. 1). However, occasionally, there seems to be a loss of one of the genes possibly due to deletions (Fig. 1). Transgenic lines having only the *hpt* gene segregated as multiloci genes as the segregation ratios in three lines were 35:0, 25:1, and 14:1 (Table 1).

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Genetic transformation of rice with viral genes for novel resistance to rice hoja blanca virus

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Rice hoja blanca virus (RHBV) is present throughout much of tropical America. When epidemics occur, yield losses may reach 100%. Most Latin American varieties have the same resistance gene. This project aims to provide new sources of resistance to minimize the possibility of an outbreak of the disease. RHBV is a member of the tenuivirus group. Coat-protein cross protection and antisense-gene down regulation of the major nonstructural protein NS4 are being attempted. The antisense-gene strategy for the expression of RNA4 aims to determine the function of the major NS4 protein and to evaluate the potential for a different and complementary method of producing virus-resistant plants. Immature embryos or immature panicle-derived calli from three tropical irrigated Latin American indica varieties and two tropical upland japonica lines are used as targets. Direct gene transfer is performed using the PDS-1000/He system. Constructs containing the RHBV-CP or the RHBV-RNA4, and the 35S CaMV-*hph* gene are being tested. Previous molecular and inheritance analyses indicate that about half of the regenerants have stable integration of the *hph* gene. Preliminary Southern and Northern analyses show that some of the *hph*-resistant plants also contain and express one of the RHBV genes.

Rice hoja blanca virus (RHBV) causes severe recurrent epidemics in the Andean, Central American, and Caribbean countries of tropical Latin America (Morales and Niessen 1983). The planthopper insect, *Tagosodes oryzicola* (Muir), is a serious pest of rice that causes direct damage and is also the vector of RHBV. This virus has the potential to cause severe yield losses (up to 100%) since there is limited distribution of varieties that are resistant to both the vector and the virus. Some very popular varieties are vector-resistant but are virus-susceptible. The uncertainty of epidemics induces farmers to spray up to 5-6 times to control this planthopper vector of RHBV. There is a need to incorporate additional sources of resistance into improved germplasm

to ensure stable and durable resistance, since the resistance is from a single resistant source. Moreover, the source of resistance does not confer immunity on most commercial varieties. Therefore, farmers start applying insecticides even when only 10% of the plants from resistant cultivars start showing RHBV symptoms.

RHBV is a member of the tenuivirus group that consists of 3-8 nm-wide filaments that may adopt helical or loose configurations (Morales and Niessen 1985). The genome of RHBV consists of four ssRNA species (Ramírez et al 1992). The nucleotide sequence of RHBV RNA4 is known and the genome encodes two genes in an antisense manner (Ramírez et al 1993). The major NS4 protein is encoded by the viral (v) RNA strand. The molecular characterization of RHBV has led to the design of novel virus-resistant strategies to genetically engineer commercially grown rice cultivars. Two different strategies are being attempted: a) the nucleocapside (NC) cross protection and b) the antisense-gene down regulation of the major NS4 protein. The NC-mediated cross protection has been successful for the tenuivirus rice stripe virus (Hayakawa et al 1992). The strategies for the expression of RNA4 aim to determine the function of the major NS4 protein and to study the potential for a different method of producing virus-resistant plants.

Results and discussion

The direct delivery of genes into immature embryos or immature panicle-derived calli is conducted using DNA-coated gold particles accelerated by the PDS-l000/He system. The tropical irrigated Latin American indica varieties Oryzica 1, Cica 8, and Inti and the tropical upland japonica lines CT6241-17-1-5-1 and Oryzica Sabana 6 were used as targets. To optimize the conditions for gene delivery into these genotypes, cotransformation experiments were conducted using equal amounts of the pAct1D construct (kindly provided by Dr. R. Wu, Cornell University) containing the *GUS* reporter gene under the control of the rice actin-1 promoter-actin-1 intron, and a construct containing the ***hph*** selective gene encoding for hygromycin resistance (Hyg^r) driven by the 35SCaMV promoter. The putative transgenic events were recovered using a stepwise selection on culture medium containing 30 mg hygromycin B (hyg b) L^{-1} followed by 50 mg hyg B L^{-1} throughout plant regeneration (Li et al 1993). Four to 85% of the bombarded explants developed Hyg^r clusters after 2 wk of selection on 30 mg hyg B L^{-1} containing medium. Between 64 and 96% of these resistant clusters show Hyg^r at 50 mg hyg B L^{l}, giving an average of 0.2-2.2 Hyg^r cell clusters per original bombarded explant. Evaluations of the ß-glucuronidase expression of 156 Hyg^r-calli recovered from 50 mg hyg B L^{-1} containing medium indicated that 46.4 calli ± 3.9 were also cotransformed with the *GUS* gene.

About 27-100% of the Hyg^r calli regenerated at least one Hyg^r plant on medium containing 50 mg hyg B L^{-1}. These results indicate that the efficiency of recovering putative transgenic plants depends highly on the genotype. One Hyg^r plant line might be recovered from 2 to 33 explants initially bombarded. Some of the putative transgenic plants were evaluated by Southern blot analysis to confirm the integration of the ***hph*** gene into the rice genome. Results suggest that 3 of 5 Cica 8 (IM) plants and 3 of 4

CT6241-17-1-5-1 (CM) plants analyzed have the *hph* gene. Single or multiple copies of the *hph* gene were noted. Only plants recovered from 50 mg hyg B L^{-1} regeneration medium contain the *hph* gene.

The segregation of the Hyg^r trait among offspring of the transgenic plants was demonstrated by germinating R_1 seeds on a medium containing 50 mg hyg B L^{-1} (Table 1). A 3:1 segregation among offspring of four transgenic plants was noted (Table l), indicating Mendelian inheritance from a single genetic locus of a functional *hph* gene. On the other hand, six transgenic plants showed a skewed segregation pattern, in which the number of Hyg^r offspring was significantly lower than the expected 3:1 (Table 1). Similar results had been reported in transgenic rice. Several possible interpretations of these results may include the linkage of the transgene with semidominant or dominant lethal mutations, inactivation of the transgene by methylation, and/or excision of the transgene from the genome (Hayakawa et al 1992). The inheritance of the *GUS* gene was also evaluated on selfed-progeny from three transgenic plants showing high level of *GUS* expression (Table 2). In this case, only one plant (CM12) showed a lower number of offspring GUS^+ than expected and the b-glucuronidase expression was diminished with respect to the original transgenic plant, probably indicating inactivation of the *GUS* gene. Plants CM12 and CM17 were cotransformed with both genes (Tables 1 and 2).

Direct gene transfer of the RHBV genes was initiated. Constructs containing the RHBV-NC or the antisense RHBV-NS4 genes driven by the 35SCaMV promoter were used. The 35SCaMV-*hph* gene was used as the selective marker. After the complete stepwise selection process throughout plant regeneration on 50 mg hyg B L^{-1}, 165 plants from the antisense RHBV-NS4 and 187 plants from the RHBV-NC bombardments had been recovered.

Preliminary analyses by Southern blot of genomic DNA and Northern blot of 38 plants recovered from the antisense RHBV-NS4 bombardments indicated that two of these plants (5.3%) contain and express the antisense-RNA4 gene. The identification of transgenic plants that express the RHBV antisense allows for the analysis of the effect of the major nonstructural gene and determines the down regulation this virus

Table 1. Inheritance of Hyg^r in the R_1 generation.

Transgenic R_0 rice line	R_1 seeds germinated (no.)	Well-grown seedlings (no.)	Dead seedlings (no.)	Ratio	c^2	Probability
CM1	47	35	12	3:1	0	1.00
CM3	32	19	13	2:1	0.55	0.46
CM11	25	10	15	1:1	0.64	0.42
CM12	21	14	7	3:1	1.05	0.31
CM17	26	19	7	3:1	0.21	0.64
IM1	363	155	208	1:1.3	0.10	0.75
IM2	175	58	117	1:2	0	1.00
IM3	70	44	26	2:1	0.58	0.45
IM5	275	194	81	3:1	0.07	0.79
IM7	363	155	208	1:1.3	0.10	0.75

Table 2. Inheritance of the GUS gene in the R_1 generation.

Transgenic R_0 rice line	R_1 seeds germinated (no.)	Well-grown seedlings (no.)	Dead seedlings (no.)	Ratio	c^2	Probability
CM12	45	20[+a]	25	1:1	0.56	0.46
CM16	16	14[++]	2	3:1	1.33	0.25
CM17	25	25[+++]	0	1:0	0	1.00

[a]b-glucuronidase expression: [+] faint blue, [++] light blue, and [+++] dark blue, respectively, was noted on roots, seed endosperm, and primary leaves.

gene confers on resistance to RHBV. Twenty-one of 31 plants analyzed from RHBV-NC experiments contain the RHBV gene. In all cases, NC fragments larger than expected were visualized on the Southern blots. Apparently, a variety of integration patterns had been obtained in other works when circular plasmid is used (Hayakawa et al 1992). Therefore, future experiments will include the linearization of the expression vector before bombardment. These plants are being subjected to Northern and Western blot analyses to determine if the NC gene is being expressed correctly.

Inheritance studies of progeny from the recovered plants is in progress to confirm stability of the integrative transformation and expression for the RHBV-NC and the antisense RHBV-NS4 genes. Following local regulations, the progeny from transgenic plants will be tested in a greenhouse under biosafety conditions for resistance to RHBV using viruliferous planthoppers.

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Genetic engineering of rice for resistance to sheath blight and other agronomic characters

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Two methods, one involving protoplasts and the other a biolistic approach, are being used for transferring genes to rice. Several cultivars have been transformed with selectable marker genes, hygromycin phosphotransferase (*HPT*) and *bar* and the reporter gene, b-glucuronidase (*GUS*), driven by the 35S promoter of cauliflower mosaic virus (CaMV) or the ubiquitin promoter. The protoplast system provides a reproducible method for obtaining transformants when embryogenic suspensions are available. A comparative study of both systems (protoplast and biolistic) and approaches for optimization has been elaborated. We are utilizing the transformation system to introduce genes of interest: the *Bacillus thuringiensis (Bt)* toxin gene, coat protein gene for rice ragged stunt virus, genes for submergence tolerance, genes for producing cytoplasmic male sterile lines, *M. sexta* chitinase, and serine proteinase inhibitor genes. Constitutive expression of chitinase in cereal plants may have the potential for improving resistance against the attack of fungal pathogens. A 1.1-kb rice genomic DNA fragment containing a chitinase gene under the control of CaMV35S promoter was cloned into the rice transformation vector pGL2. After transformation of rice protoplasts in the presence of polyethylene glycol, plants were regenerated. The presence of the chimeric chitinase gene in T_0 and T_1 transgenic rice plants was detected by Southern blot analysis. HPT assay demonstrated the segregation of the *HPT* gene among the T_1 progeny predominantly in a 3:1 ratio. Western blot analysis of transgenic plants and their progeny revealed the presence of two proteins with apparent molecular weights of 30 and 35 kDa that reacted with a chitinase antibody. Progeny from the chitinase-positive plants were tested for their resistance to the sheath blight pathogen, *Rhizoctonia solani.* The degree of resistance displayed by the transgenic plants to this pathogen was correlated with the level of expression of chitinase.

Since the first reported success on the amenability of the rice genome to foreign gene insertion, rice transformation has progressed considerably in less than a decade. A number of gene transfer methods have likewise been developed, but two of the most popular methods are protoplast-mediated and biolistic transformation (Christou et al 1991). Each method has its advantages and constraints, but, at the present time, protoplast transformation appears to be the most efficient and reliable, provided an embryogenic cell suspension is available (Datta et al 1992, Peng et al 1992, Fujimoto et al 1993, Uchimiya et al 1993, Alam et al 1996). As these two methods vary in the advantages they offer, both are currently used in IRRI's rice transformation program.

Sheath blight, caused by *Rhizoctonia solani*, is one of the major rice diseases that cause significant yield losses (Khush and Toenniessen 1991). In response to infection, the plant synthesizes an assortment of new proteins as a defense mechanism. One of such proteins is chitinase, an enzyme that hydrolyzes chitin, a major component of the fungal cell wall. Chitinase genes can come from a wide variety of biological sources. We report the production of transgenic sheath blight-resistant rice from protoplast-mediated transformation.

Materials and methods

Genetic engineering of rice

Two of the available methods of gene transfer—protoplast-mediated and biolistic transformation—are the most preferred and are being used at IRRI. The scheme for the delivery of plasmids using these techniques is shown in Figure 1. Datta (1995) outlined the details on the protoplast transformation procedure.

For biolistic transformation, embryogenic calli, immature embryos, and embryogenic cell suspensions were used as explants. Embryogenic calli were produced in MS (Murashige and Skoog 1992) basal medium with 2 mg 2,4-D L^{-1} from immature embryos or mature seeds. Immature embryos used for bombardment were collected at 9-12 d after anthesis and sterilized following standard laboratory procedures. Embryogenic cell suspensions used for protoplast isolation were also bombarded. The calli and immature embryos were selected in 50 µg hygromycin B or 25 µg PPT ml^{-1} for at least 6 wk, while embryogenic cell suspensions were selected at 100 or 200 µg ml^{-1}. Plants were regenerated either in the presence or absence of hygromycin or PPT.

The genes of interest utilized in our transformation studies include *Bacillus thuringiensis* toxin gene (pCIB4418), coding region sequences of rice ragged stunt virus genome segment, genes for submergence tolerance (*pDC, pTC*), seven *Menduca sexta* chitinase (*pMSc*) and serine proteinase inhibitor genes for fungal disease resistance, and the Amal (*pSB*) gene from *Amaranthus hypochondriacus* for protein improvement.

Sheath blight resistance

Embryogenic cell suspension and protoplast transformation of the indica cultivar Chinsurah Boro II were done following the method used in IR72 rice transformation

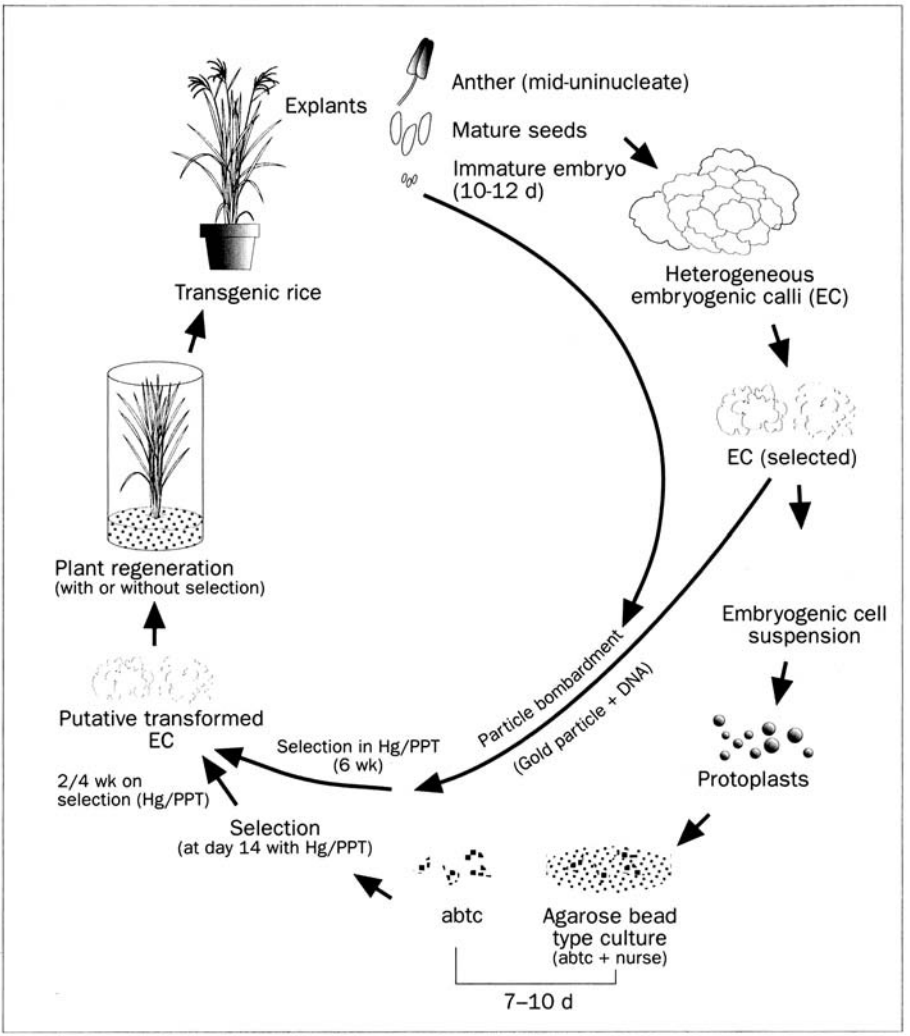

Fig. 1. Production of fertile transgenic rice plants from protoplast and biolistic systems (modified from Datta 1995).

(Datta et al 1992). A 1.1 -kb DNA fragment from a rice genomic chitinase gene driven by a CaMV35S promoter was cloned into the vector pGL2 to form the chimeric plasmid pGL2 (CaMV-CHIII) used in transformation (Huang et al 1991, Lin et al 1995).

Various tests including HPT and chitinase activities, Southern and Western blot analyses, and bioassays to assess the integration and expression of genes were performed on selected T_0, and T_1 plants (Datta et al 1990, Lin et al 1995).

Results and discussion

Gene transfer

Japonica as well as indica varieties are being used in our transformation studies, with emphasis on the indicas. Various genes have been incorporated into rice using the protoplast system. The most promising is the incorporation of sheath blight resistance into an indica cultivar. As the biolistic approach has been used much later, most of the putative transformants are yet to be analyzed. It appears that the success of this technique depends highly on the ability of the cultivars to regenerate plants.

Japonica rice (i.e., Taipei 309), which can be transformed by the protoplast system (Zhang et a1 1988), also works well in producing efficient transgenic plants by the biolistic method. Southern blot analysis showed the evidence of integration of several genes: *pDC, PTC, hph, GUS,* and *p13A.* Analysis is in the process for other genes, including *Bt* genes.

Sheath blight resistance

Analysis of transgenic plants based on Southern and Western blot analyses. From 1,044 resistant calli, 202 green plants were regenerated, of which 28 were fertile. Most of the plants (94%) exhibited HPT activity and 36% showed chitinase activity, suggesting the presence of a functional chitinase transgene in some of the transformants. Western blot analysis of 11 independent primary transformants (T_0) revealed four regenerants having various amounts of chitinase (Fig. 2). Progenies of two chitinase-positive transformants likewise expressed the chitinase and *HPT* genes with a 3:1 segregation in most cases for both loci. However, the segregation ratios for the HPT gene were not identical to the chitinase gene in all cases, indicating that the loci containing active forms of these genes are not necessarily linked in the transgenic plants. Presumably, different integration events can result in the inactivation of one gene without affecting the other.

Six plants, including the four that were positive in the Western blot analysis, underwent Southern blot analysis (Fig. 3). Genomic DNA was digested with *Hin*dIII and probed with CHIII chitinase fragments. The 1.5-kb band was detected in all four T_0 plants that gave a positive reaction in the Western blot analysis; the other two failed to express this band. Estimates of the maximum number of chitinase genes inserted into the rice genome can be obtained by counting the number of autoradiographic bands unique to the transgenic plants in digests of genomic DNA with *Eco*RV. This number ranged from two (#178 and #358) to five copies (#105 and #354).

Expression of chitinase in different tissues in control and infected transgenic rice plants. Two representative T_1 plants having high and low levels of chitinase expression and control plants were analyzed (Fig. 4). No chitinases were detected in extracts of leaves and sheaths of uninfected nontransgenic plants while chitinase was present constitutively in leaves, roots, and sheaths of transgenic plants, with the levels increasing upon fungal infection, probably due to induction of host chitinase genes by pathogen infection just as in control plants.

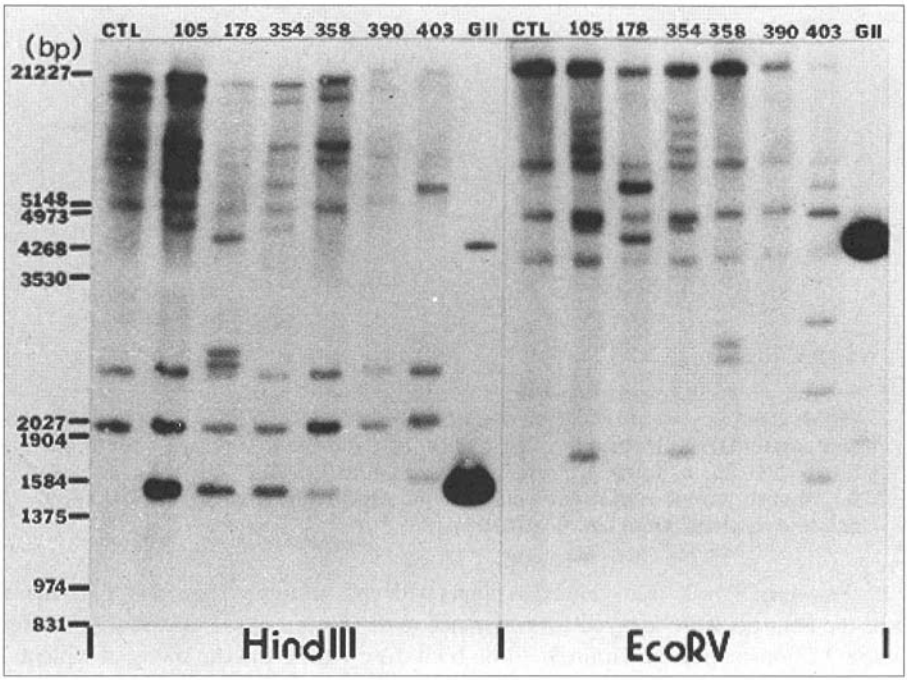

Fig. 2. Western blot analysis of protein extracts of primary regenerants (T_0). M = markers whose sizes in kilodaltons (kD) are indicated on the left; IE = extracts from sheaths infected with *R. solani* for 1 wk; CTL = transformed with pGL2 vector (from Lin et al 1995).

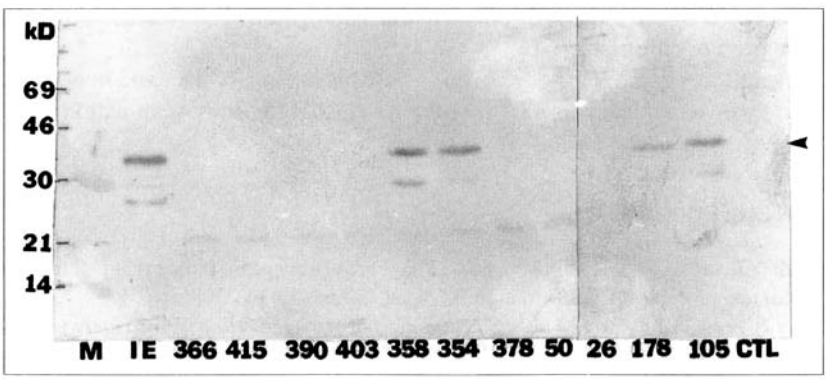

Fig. 3. Southern blot analysis of the T_0 transgenic rice plants. CTL = untransformed control; G11 = pGL2 (CaMV-CHIII) DNA (from Lin et al 1995).

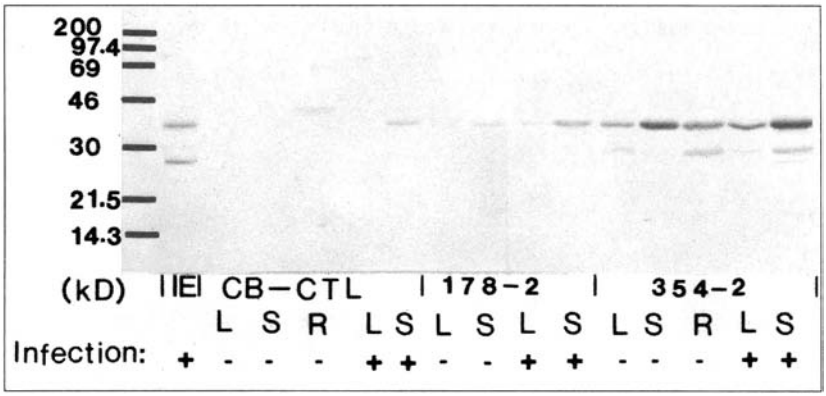

Fig. 4. Western blot analysis of leaf, sheath, and root extracts from infected and uninfected T_1 transgenic rice plants. IE = infected IR58 rice sheath extract; CB-CTL = control plant transformed with pGL2 vector. L = leaf, S = sheath, and R = root extracts. + = Infected with *R. solani* for 7 d. — = unInfected plant (from Lin et al 1995).

Bioassay. Two T_1 transgenic rice plants with constitutive expression for chitinase and the controls were assayed for resistance to the fungus at the maximum tillering stage. Lesions appeared within 3-4 d on both the controls and the transgenic plants, however, the number and size of lesions in the transgenic plants were smaller than those of the control plants. Three weeks after inoculation, the lesions had spread to the upper half of the control plants, while the lesions were confined to the lower half of the sheaths in the transgenic plants. Western blot analysis of the transgenic plants and their progeny revealed the presence of two proteins with apparent molecular weights of 30 and 35 kDa that reacted with a chitinase antibody. The degree of resistance displayed by the transgenic plants to this pathogen was correlated with the level of expression of chitinase.

Several transgenic lines have been selected based on T_1 data and now T_2 plants are being grown in transgenic CL4 greenhouse at IRRI for further evaluation of sheath blight resistance.

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Antibacterial effects of *cecropin B* gene-transformed rice plants

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Cecropin B is a small basic polypeptide isolated from a giant silkworm moth, which shows broad antibacterial activity in vitro. Using recombinant DNA techniques, a plasmid for plant transformation was constructed harboring a synthetic *cecropin B* gene driven by the actin 1 gene promoter and a selectable marker gene *(bar)* driven by the 35S promoter. Plasmid DNA was introduced into scutellum cells of immature embryos of two rice varieties using the biolistic method. After selection on Basta-containing medium and regeneration, three putative transgenic plants were obtained. Basta spraying indicated that all three plants were highly resistant to this herbicide. Polymerase chain reaction amplification and Southern blot analysis demonstrated that both the *bar* and *cecropin B* genes had been integrated into the rice genome. Disease resistance testing showed one transgenic plant with high resistance to rice bacterial streak and another with improved resistance to both rice bacterial streak and rice bacterial blight; a third plant was susceptible to both diseases.

Cecropin B, a member of an insecticidal polypeptide family (Boman and Hultmark 1987), consists of 36 amino acid residues. According to the amino acid sequence, a *cecropin* B gene was synthesized and cloned into M13mp19/AB (Q. Wang, unpubl. data). In this paper, we report the results in producing antibacterial transgenic rice plants using the *cecropin* B gene.

Materials

Immature embryos (12 d after pollination) of two japonica rice varieties, Zhongbai 4 (ZB4) and Jingying 119 (JY119), were used as target tissues for biolistic transformation. The starting materials for plasmid construction were pBY505 (Wang and Wu 1995) and M13mpl9/AB.

Methods

One week after bombardment with a PDS-1000/He (BioRad) particle gun, the embryos were selected on callus growth medium containing Basta (20 mg glufosinate ammonium L^{-1}). Proliferating small calli, which were derived from different transformation events, were excised and transferred for further selection (20-40 mg glufosinate ammonium L^{-1}). The resistant calli were regenerated into plantlets.

The basic medium included N6 macrosalts, B5 microsalts and vitamins, 30 g sucrose L^{-1}, 3 g phytagel L^{-1}, pH 5.8, and 2 mg 2,4-D L^{-1} in callus growth medium; 3 mg BAP L^{-1} and 0.5 mg NAA L^{-1} in regeneration medium.

For Basta resistance testing, a 0.25% Basta aqueous solution was sprayed on plants and damage was scored 1 wk later. For polymerase chain reaction (PCR) amplification, the two primers were synthesized as follows: 5'-ATGCAGCCTCGTGCGGA-3' and 5'-GAACTAGTGGATCCGGA-3'.

The Southern blot analysis procedure (Sambrook et a1 (1989) was followed. In the bioassay of rice bacterial streak (BS) resistance, rice leaves were wounded with pins and inoculated with a prevalent BS strain (S-98 11). BS symptoms were scored 25 d later according to IRRI's standard evaluation system for rice. In the bioassay of rice bacterial blight (BB) resistance, six leaves of each plant were sheared and the wounded edges were immersed in a BB strain (25173) bacterial suspension. Thirty days later, symptoms were scored according to international standard, and the disease index was calculated.

Results and discussion

Construction of plasmid for transformation of rice, regeneration of transgenic plants, and Basta resistance testing

Plasmid pBY505 was cut with *Sma*I and *Hin*dIII. The larger fragment, which has one cohesive end (*Hin*dIII) and one blunt end, was collected. The *cecropin B*-harboring M13mpl9/AB DNA was cut with *Bam*HI and treated with Klenow enzyme to make a blunt end. Then the DNA was cut with *Hin*dIII to make the other end cohesive. This fragment, which is 141-bp long and contains *cecropin* B gene, was ligated with the larger fragment of pBY505 by T4 ligase. The resulting plasmid (PCB 1) is a construct harboring the *cecropin B* gene driven by the actin 1 gene promoter and a selectable marker gene (*bar*) driven by 35s promoter was produced. DNA sequencing of the adjacent regions confirmed the presence of an intact *cecropin B* gene with no change in open reading frame.

Putative transgenic rice plants were obtained after transformation, selection, regeneration, and transplanting. Six plants were regenerated, but only three survived: JY119 B1-3, JY119 B1-4, and ZB4 B1-1. All three of these transgenic rice plants expressed high resistance to Basta after spraying, while control plants died 1 wk after treatment.

PCR amplification of *cecropin 5* gene

PCR amplification indicated that these three plants all contained the pCB1 fragment. This fragment is about 0.19 kb in size and contains the entire sequence of *cecropin B* and part of the actin 1 gene promoter.

Southern blot analysis

Southern blot analysis of the genomic DNA from JY119 B1-3 and JY119 B1-4 showed hybridization signals in the high molecular weight regions in the uncut lanes. When genomic DNA was digested with *Hin*dIII and *Xho*I, different band patterns appeared, as expected, based on the structure of the plasmid pCB1 used in transformation. No signal could be found in the control lane, indicating that the *cecropin B* gene was integrated into the rice genome of the plants. Southern blot analyses of the genome DNA of ZB4 B1-1 indicated the presence of the *cecropin B* gene.

Southern blot analysis of these three plants, using the *bar* gene as the probe, also demonstrated the integration of the *bar* gene into the rice genome.

Bioassay of disease resistance

Bacterial streak. Twenty-five days after inoculation with a prevalent strain BS (S-98 II), plant ZB4 B1-1 showed high resistance to BS. It scored 1 (highest resistance) while the ZB4 control scored 7. JY119 B1-3 showed improved resistance to BS (score: 5) while JY119 B1-4 showed no resistance.

Bacterial blight. In resistance testing with BB strain ZJ173, plant JY119 B1-3 displayed improved resistance to BB (disease index 4). Plant JY119 B1-4 was very susceptible (disease index 8), as was the control plant JY119 (disease index 12). Plant ZB4 B1-1 was not tested because it was too immature at the time.

These bioassay results indicate that different transgenic plants may have different expression levels of the foreign gene due to a position effect or copy number. The results also indicate that, since cecropins have broad antibacterial activities, cecropin-containing transgenic plants may give similar responses to the infections by different pathogenic bacteria.

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Transformation of rice with agronomically useful genes toward production of insect-resistant and water stress-tolerant plants

D. Xu, X. Duan, Q.-Z. Xue, B. Wang, and R. Wu

A cowpea trypsin inhibitor gene, *CpTi,* was introduced into rice. Expression of the *CpTi* gene driven by the rice actin 1 *(Act1)* promoter led to high-level accumulation of the CpTl protein in transgenic rice plants. Protein extracts from transgenic rice plants exhibit a strong inhibitory activity against bovine trypsin. Small-scale tests in an isolated experimental field showed that the transgenic rice plants expressing the *CpTi* gene had significantly increased resistance to two species of rice stem borers: striped stem borer *(Chilo suppressalis)* and pink stem borer *(Sesamia inferens).* Thus, introduction of insecticidal proteinase inhibitor genes into cereal plants might be used as a general strategy for control of insect pests. A late embryogenesis abundant (LEA) protein gene, *HVA1,* from barley was introduced into rice. Expression of the barley *HVA1* gene regulated by the rice *Act1* promoter led to high-level, constitutive accumulation of the HVA1 protein in transgenic rice plants. Second-generation transgenic rice plants showed significantly increased tolerance to water stress and salt stress as compared with nontransformed control plants. Under stress conditions, transgenic plants maintained higher growth rates and showed delayed development of damage symptoms. Upon removal of stress conditions, transgenic plants showed better recovery. The extent of increased stress tolerance correlated with the level of the HVA1 protein accumulated in different transgenic rice plants.

Production of insect-resistant transgenic rice plants

To test the general usefulness of plant proteinase inhibitor genes (Ryan 1990) for the genetic engineering of insect resistance in rice, we set out to introduce several proteinase inhibitor genes into rice using our established rice transformation systems. Insect-resistant transgenic rice plants have been produced by introducing an insecticidal cowpea trypsin inhibitor gene (Hilder et al 1987) into rice.

Production of CpTi-transgenic rice plants

Two plasmids were used to cotransform rice (*Oryza sativa* L. cv Taipei 309) protoplast followed by plant regeneration. Plasmid pDM402 contains the cowpea trypsin inhibitor gene (*CpTi*) coding region flanked by the rice actin gene 1 (*Act1*) promoter and the nopaline synthase gene (nos) 3' terminator. Plasmid pDM307 contains the bacterial phosphinothricin acetyl transferase gene (*bar*) joined to the CaMV35S promoter, and it was used as the selectable marker in rice transformation.

From four transformation experiments, 211 plants, which represent 74 independent lines, were regenerated. Under greenhouse conditions, 24% of the plants were fertile. More than 60 plants were tested for resistance to herbicide, and all of them showed various degrees of resistance to 1% Basta® (a commercial herbicide containing 162 g ammonium glufosinate L^{-1}), whereas nontransformed control plants were highly sensitive to the herbicide. Forty-seven herbicide-resistant plants were analyzed by DNA blot hybridization using the *CpTi* gene coding region as a probe. Twenty-one plants were shown to contain the *CpTi* gene. Thus, the average cotransformation efficiency of the *CpTi* gene construct relative to the selectable marker gene was 45%.

Analysis of CpTI production in transgenic rice plants

Production of the CpTI protein in transgenic rice plants was analyzed by slot-blot immunoassay. As shown in Figure 1a, leaf protein extracts from six transgenic plants contained various levels of the CpTI protein. whereas CpTI protein was not detected in nontransformed (NT) control plants. Protein extracts from transgenic plants No. 2 and No. 10 showed strong inhibitory activity against commercial bovine trypsin. Protein extracts from NT control plants showed little such inhibitory activity (Fig. 1b). The levels of CpTI protein produced in transgenic rice plant tissues were estimated based on the inhibitory activity of CpTI against bovine trypsin using purified CpTI protein as the standard in inhibition assays. The levels of CpTI protein in the leaf tissue of nine transgenic rice plants were estimated to be between 0.3 and 2.7% of the total leaf soluble proteins (Fig. 1c).

Field test of transgenic rice plants for insect resistance

The R_2 generation progeny of one transgenic line, line No. 2, were tested for resistance to natural infestation by two species of rice stem borers: striped borer (*Chilo supressalis*) and pink stem borer (*Sesamia inferens*). The R_0 transgenic plant of this line produced a large number of seeds and also produced a high level of CpTI protein (1.3% of total soluble proteins) in both leaf and stem tissue. R_1 seeds from the R_0 plant were used to raise the R_2 seeds.

Small-scale tests conducted in an isolated experimental field are described in detail by Xue et al (1996). The results showed that all NT control plants were severely damaged by insect attack and became almost completely lodged. Most of the shoots of these control plants produced dead panicles (commonly known as whitehead) with no seeds. On the contrary, transgenic plants showed significantly increased resistance to the infestation by the two species of rice stem borers. In the transgenic plant population, 20-80% of the R_2 plants from the five tested R_1 lines were completely resistant

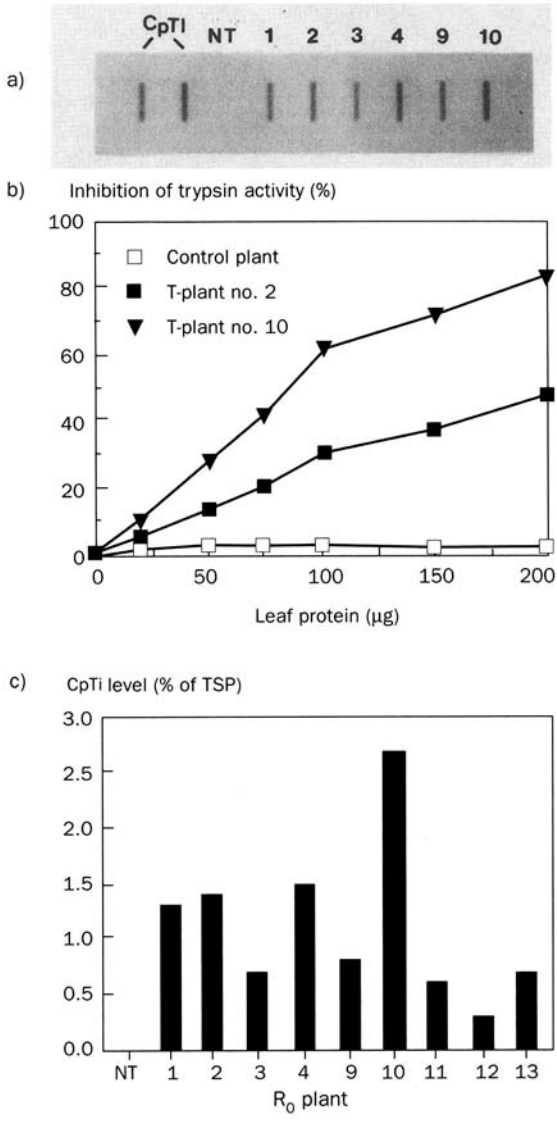

Fig. 1. Analysis of CpTi in transgenic rice plants. a) Detection of CpTi protein in rice leaf protein extracts by slot-blot immunoassay. The identification numbers of transgenic lines are marked on the top. NT, nontransformed control. Purified CpTi (100 ng and 200 ng, respectively) was used as a positive control. For each sample, 10 μg of leaf soluble proteins was applied. b) Inhibition of trypsin activity by leaf protein extracts from two transgenic rice plants, No. 2 and No. 10. c) Estimated levels of CpTi protein in different primary (R_0) transgenic plants. TSP, total soluble proteins.

Table 1. Bioassay of R_2 transgenic rice plants for resistance to rice stem borers.

R_1 transgenic line	R_2 plants tested[a] (no.)	Plants with whitehead[b] (no.)	Plants with whitehead (%)
1	26	15	58
2	34	27	79
3	29	6	21
4	15	6	40
5	24	17	71
Total	128	71	55 (av)
NT control	42	42	100

[a] R_2 plants of the five R_1 lines used in the field test are all derived from the primary transgenic line No. 2. [b] In scoring resistance/sensitivity to insect infestation, if one shoot (tiller) on a plant was Infested and produced dead panicles (whitehead), the whole plant was scored as sensitive. Only those plants with no infested shoots were scored as resistant. On average, each plant had eight shoots (or tillers). NT, nontransformed plants derived from the transformation procedure. Rice variety Taipei 309 was used for the transformation experiments.

to infestation by the two insects. These resistant plants were not affected by the insect infestation and most panicles produced seeds. Results of the scoring of resistance are shown in Table 1.

The field test was carried out according to rules established in December 1993 by the State Science and Technology Commission of China.

In summary, transgenic rice plants expressing the cowpea trypsin inhibitor gene had increased resistance to two species of rice stem borers. Our results suggest that introducing insecticidal proteinase inhibitor genes into rice is a useful strategy for the control of certain rice insect pests.

Production of water stress-tolerant transgenic rice plants

It has been hypothesized that late embryogenesis abundant (LEA) proteins may play a protective role in plant cells under water stress conditions (Dure et al 1989), but clear experimental evidence supporting the exact function of LEA proteins is still lacking. A group 3 LEA protein, HVA1, was previously characterized from barley (Hong et al 1988). We have taken a transgenic approach to investigating the probable function of the HVA1 protein in stress protection.

Production and molecular analysis of transgenic rice plants

Plasmid pBY520, which contains the barley *HVA1* gene joined to the rice *Act1* promoter, was used to transform rice (*Oryza sativa* L. cv Nipponbare) suspension cells. Plasmid pBY520 also contains the bacterial bar gene joined to the CaMV35S promoter as a selectable marker. From three transformation experiments, 63 independent

lines of 120 plants were regenerated and grown in the greenhouse. More than 85% of the transgenic plants were fertile.

Twenty-nine lines of plants were tested for herbicide resistance by painting a leaf with 0.5% Basta®, and 90% of them showed resistance. The same 29 lines were further analyzed by DNA blot hybridization using the *HVA1* cDNA fragment as the probe, and 80% of them showed the predicted hybridization band pattern (data not shown). The general consistency of results of DNA blot hybridization and those of the herbicide resistance test suggests that both the selectable marker gene and the *HVA1* gene on the same plasmid were efficiently cointegrated into the rice genome.

Analysis of accumulation of HVA1 protein in R_0 transgenic rice plants

A single band of 27 kD in SDS-PAGE gel, which corresponds to the HVA1 protein, was detected by immunoblot in leaf protein extract\ of several transgenic lines (Fig. 2a). Accumulation of HVA1 protein was also readily detected in roots of transgenic plants, although the levels were relatively low (Fig. 3b). There were no additional bands of other sizes detected in the protein extracts of the transgenic or the NT plants. The levels of HVAl protein accumulated in the leaf and root tissues of different transgenic lines were estimated to be in the range of 03-2.5% of the total soluble proteins. The HVA1 protein was not detected in the protein extracts of mature transgenic seeds. However, two strong bands with lower molecular mass, 20 kD and 13 kD. respectively, were detected in both transgenic and control seeds (Fig. 2c). These two proteins may represent endogenous rice LEA-like proteins.

Increased tolerance to water stress and salt stress of transgenic rice plants

Evaluation of the growth performance under water and salt stress conditions was carried out using the R_1 plants from several transgenic lines. Seeds of wild-type rice plants or seeds of transformation procedure-derived NT plants were used as controls. Stress experiments were conducted using 3-wk-old plants grown in the soil. Under constant nonstress conditions. no significant difference was observed between transgenic plants and control plants in their growth performance. Upon withholding water from the trays, the rapid decrease of water content in soil created a water deficit condition. Leaves at the same developmental stage of the transgenic plants wilted about 1-2 d later than did leaves of the control plants. After 4-5 d of water stress, leaves of both control and transgenic plants wilted, but wilting of transgenic plants was considerably less severe. During the first 3 d of water stress, transgenic plants maintained a higher growth rate than control plants (Table 2). After the water-stressed plants were rewatered, the transgenic plants showed better recovery and had a faster growth rate than the control plants. Data in Table 2 also show the average plant height and root fresh weight of the stressed plants after four cycles of 5-d water stress followed by 2-d recovery with watering. Transgenic plants showed significant advantages over control plants in their growth performance under water stress conditions, and this is particularly evident in the growth of roots.

Transgenic rice plants also showed increased tolerance for salt stress in our experiments. Analysis of the accumulation of the HVA1 protein in salt-stressed R_1 plants

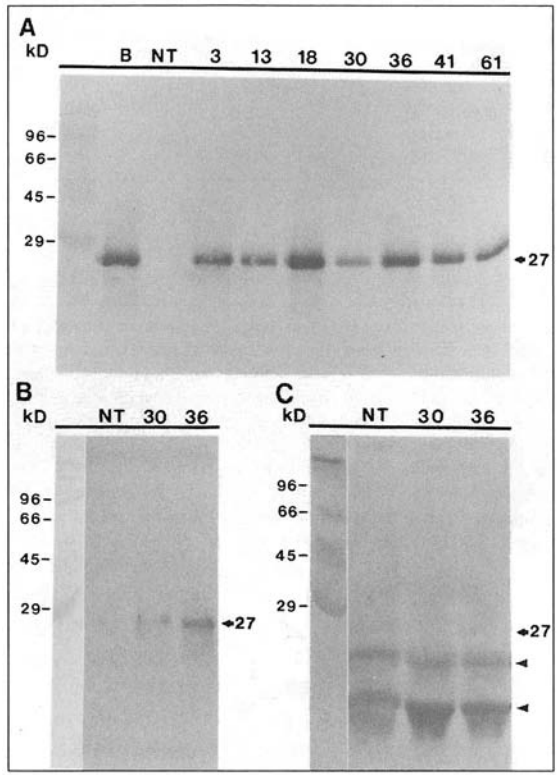

Fig. 2. Immunoblot detection of HVA1 protein accumulation in the first-generation (R_0) transgenic rice plants. Equal amounts of total soluble proteins (20μg) were separated on a 12% SDS-PAGE for immunoblot detection. The identification numbers of transgenic lines are indicated on the top. Molecular mass markers are indicated in kD on the left-hand side. The HVA1 protein, shown as a 27-kD band, is indicated by an arrow at the right-hand side. NT, nontransformed control plant. a) HVA1 protein accumulation in leaves. Lane B contains purified HVA1 protein (250 ng) as the positive control. b) HVA1 protein accumulation in roots. c) HVA1-like protein accumulation in mature rice seeds. The two rice proteins cross-reacting with the HVA1 antibody are marked with filled triangles on the right-hand side.

Table 2. Growth performance of transgenic rice plants in soil under water stress.

Transgenic line	Leaf growth rate[a] (% length increase)	Plant height[b] (cm)	Root fresh weight[b] (g)
NT	69	22±1.4 (100)	0.9±0.1 (100)
30	90	29±1.1 (132)	1.4±0.1 (156)
36	129	37±1.8 (168)	2.1±0.1 (233)
41	113	33±1.8 (150)	2.3±0.3 (256)

[a] The lengths of the two upper leaves were measured before and 3 d after withholding water from the trays. The growth rate was calculated as the percentage length increase of the two leaves during the 3-6 period of water stress, [b] Data were collected 28 d after the beginning of initial water stress (four cycles of 5-d water stress followed by 2-d recovery with watering). The mean length of the two longest leaves on the top of the plants was used as a measure of the plant height. Each value ±SE represents the average of 10 plants, except for root fresh weight which is the average of four plants. The numbers in parentheses are relative values in transgenic plants compared with control plants, which was taken as 100. NT, nontransformed control.

showed that the increased tolerance to stress in transgenic plants was correlated with the expression of the barley HVA1 protein (data not shown).

In summary, our results support the hypothesized role of LEA protein in stress protection. The protective role of LEA proteins suggests the potential usefulness of LEA protein genes as molecular tools for genetic engineering of stress tolerance. As more LEA genes are characterized and their functions in stress tolerance are confirmed, manipulation of endogenous LEA gene expression. in combination with the introduction of additional stress tolerance genes may prove to be an efficient strategy for genetic crop improvement.

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Plant regeneration with high efficiency and production of herbicide (PPT)-resistant transgenic rice plants from protoplasts of wide compatibility rice strain 02428

C. Wu and Y. Fan

Calli of wide compatibility rice strain 02428 were induced from mature embryos on MS4 medium. After transfer to Rm4 medium, the friable calli grew rapidly and were light yellow. The cell suspension lines were established from the calli on Rm4 medium. Protoplasts were isolated from the suspension lines and then embedded in KPR medium with agarose (1.2% low melting point). The first division of the protoplasts was observed 3-4 d after embedding. The KPR liquid medium was added 7 d after culturing of the protoplasts and rotated at 50 rpm. Calli of about 1 mm in diameter were obtained 3-4 wk after culturing of the protoplasts. Plantlets were regenerated via embryogenesis 2 wk after transfer of the calli to N_6 medium. In the field, the plantlets grew, flowered, and set seeds normally. It took less than 3 mo to obtain green plantlets regenerated from the protoplasts. These results were obtained regularly in different experiments using the same experimental conditions. The protoplasts isolated from rice strain 02428 were electroporated with plasmid pFWZ16, containing the CaMV35S promoter/*bar* chimeric gene and the *Act1/Bt* chimeric gene. PPT-resistant transgenic rice plants were then obtained.

Research on rice protoplasts began in the early 1970s. To date, rice plants have been regenerated from the protoplasts of more than 20 genotypes. Some problems in the culturing of protoplasts limit the utility of the technique, for example: 1) the material used (such as Taipei 309, Nonghu 6, etc.) is not conducive to crop improvement; 2) only a few plants are regenerated from protoplasts: and 3) it takes a long time from callus induction to plant regeneration. In this paper, a wide compatibility rice strain, 02428, which plays an important role in breeding for high yield potential for both japonica and indica rices, was used to study factors that affect protoplast culture. The plasmid with the CaMV35S-*bar* gene and the *Act1-Bt* toxic gene was transferred to the protoplasts of the 02428 strain, resulting in the production of herbicide-resistant transgenic rice plants with the *bar* gene and/or *Bt* gene.

Materials and methods

Strain

Rice strain 02428 was developed at the Jiangshu Academy of Agricultural Sciences, China.

Callus induction from mature embryos

Seeds were rotated continuously in 20% NaOCl for 30 min and rinsed with sterile water 5 or 6 times and then transferred to MS + 4 mg 2,4-D L^{-1} + 20 g sucrose L^{-1} + 0.8% agar. The induced calli were transferred to MS4, $N_6$4, and Rm4 media.

Building the cell suspension line

Friable, light yellow calli were transferred to AA2 liquid medium. The ratio of medium to calli was about 5:1. The calli were rotated in the dark at 120 rpm and 26 °C.

Protoplast isolation

Calli in suspension with diameters less than 1 mm were put in an enzyme solution, which was filtered through 30-µm mesh. The protoplasts were collected after centrifugation and washed with CPW7M three times. Protoplasts were suspended in liquid KPR medium.

Protoplast culture

Protoplasts were imbedded in KPR medium with a low agarose melting point at a density of 3-5 × 10^5 ml^{-1}, and cultured in the dark at 28 °C. After 7 d, KPR liquid medium was added and the culture was rotated at 50 rpm.

Callus differentiation and plant regeneration

Calli regenerated from protoplasts (1-2 mm in diameter) were transferred to N_6 medium (N_6 basal component + 50 g sucrose L^{-1} + 0.8% agar). Within 2 wk, roots and shoots had differentiated. Plantlets placed under light turned green within 2-5 d. The regenerated green plantlets were then transferred to solid MS medium (free hormone and sucrose). When the plants reached a height of 10 cm, they were transferred to soil. Agronomic traits of these plants (flowering, fertility, seed shape, etc.) were recorded. Plasmid DNA was purified with CsCl gradient centrifugation.

Protoplast transformation via electroporation, culture selection, and differentiation

The density of protoplasts was 5 × 10^6 ml^{-1}. No DNA was added to the protoplasts in the control. The plasmid pFWZ16 DNA was added to 10 µg/10^6 protoplasts. Transformation was carried out using Beakon 2000 (USA). The electroporation conditions were: electricity pressure A = 10 KV, number of pulses (NP) = 2^6-2^8, time of burst (TB) = 0.2 s, cycle (CY) = 20, electroporation distance (D) = 1 mm, and electroporation solution = 100 µl. After the samples were electroporated, they were put on ice for

10 min and centrifuged for collecting protoplasts. The electroporated protoplasts were cultured under conditions described above. The calli were selected for 6-10 wk using PPT (2-8 mg L^{-1}). The calli, which grew rapidly, were divided into two parts, one being transferred to solid medium containing MS+ 2, 4-D (4 mg L^{-1}) + PPT (8 mg L^{-1}), the other being transferred to a medium containing N_6 + Kinetin (KT, 1 mg L^{-1}) + benzyl adenine (BA. 1 mg L^{-1}) + naphthalene acetic acid (NAA, 0.5 mg L^{-1}). When the plants reached 20-30 cm in height, a solution of PPT (125 mg L^{-1}) + 0.1% Tween 20 was applied on both sides of the leaves.

Identification of transgenic plants using PCR and PCR-Southern blotting

Plant DNA was extracted. The polymerase chain reaction (PCR) products were assayed with electrophoresis on 1% agarose. Used as probes, *bar* or *Bt* gene fragments from pFWZ16 were digested with a restriction enzyme and isolated from low melting point agarose (1.2%). PCR-Southern hybridization was done.

Results and analyses

Callus induction from mature embryos

The sterilized seeds of 02428 were put on MS4 medium. Five days later, light yellow calli were induced from the base of the shoot. The callus induction frequency was 93%.

Callus growth

Calli from 02428 were transferred to $N_6$4, MS4, or Rm4 media. Over a 2-wk period, calli growth varied: slowly on $N_6$4, moderately fast on MS4, and fastest on Rm4. On Rm4, calli were friable and light yellow.

Building the cell suspension line

The friable, light yellow, and fast-growing calli were transferred to AA2 liquid medium. Calli were subcultured every 3 d. After five subcultures, calli from the Rm4 medium grew and formed numerous small cell clusters and a few large cell clusters. The cell clusters were rich in cytoplasm-composed of 10-50 cells. Over the next 5 wk, these clusters continued to grow in the suspension. The same growth progress took 6-8 wk when using MS4 or $N_6$4 media.

Protoplast isolation

Small cell clusters taken from the cell suspension lines were used for protoplast isolation using the enzyme mentioned in materials and methods. The circular protoplasts yielded 0.8-1.2 × 10^7 g^{-1} of fresh weight callus.

Protoplast culture

Protoplasts were cultured in agarose with a low melting point at a density of 3-5 × 10^5 ml^{-1} in a 5-cm-diameter dish. The first cell division was observed 3-4 d after protoplast culture, and the cells divided once every 3-4 d. After 1 wk, 5 ml of liquid KPR me-

dium were added. Protoplasts were cultured and rotated at 50 rpm. The cell clusters in the rotated culture grew more quickly than those in still culture. The KPR medium (5 ml fresh liquid) was changed every 2 wk. Many calli appeared in the dishes 3-4 wk after protoplast culture; the largest were about 1 mm in diameter.

Plants regenerated from callus

Calli about 1 mm in diameter were transferred to 1) N_6 (N6 basal component + 50 g sucrose L^{-1} + 0.8% agar) or 2) N_6 + KT (1 mg L^{-1}) + BA (1 mg L^{-1}) + NAA (0.5 mg L^{-1}). Two weeks later, embryos were formed, and shoots and roots were regenerated through embryogenesis. They turned green after transfer under light. The plant regeneration frequencies from the calli were 13 and 21%, for media 1 and 2, respectively. On medium 2, plants regenerated rapidly, but after transferring 2-3 times, a high percentage of calli turned dark. On both media, most rapid-growing calli did not form embryos, but on N_6 + ABA (1 mg L^{-1}), embryos were formed and plants were regenerated.

Growth of regenerated plants

Upon reaching a height of 2 cm, the plants on N_6+ BA (1 mg L^{-1}) + KT (1 mg L^{-1}) + NAA (0.5 mg L^{-1}) were transferred to MS medium; upon reaching 10 cm, they were transferred into the soil. More than 100 plants were regenerated from protoplasts. These plants averaged 2-5 tillers and flowered normally. One plant would set between 5 and 20 seeds.

Protoplast transformation, selection, and plant regeneration

Two weeks after electroporation, a KPR medium with a PPT concentration of 2-8 mg L^{-1} was used. After 6-10 wk of selection, the calli of the control turned brown and calli growth was completely inhibited. A few light yellow calli were regenerated from protoplasts, which were treated with pFWZ16 DNA. One part of a large callus that grew normally was transferred to MS4 medium with 8 mg PPT L^{-1} (MS4p8). Another part was transferred to a medium of N_6 + 1 mg BA L^{-1} + 1 mg KT L^{-1} + 0.5 mg NAA L^{-1} without PPT (control). The transformed callus grew well on MS4p8 medium whereas the callus in the control died. One month later, plants were regenerated via embryogenesis on a medium composed of N_6 + 1 mg BA L^{-1} + 1 mg KT L^{-1} + 0.5 mg NAA L^{-1}. Upon reaching 7-10 cm in height, the plants were transplanted into soil. At 20-30 cm, the plants were wetted with a PPT solution (125 mg L^{-1}). Results indicated that although plant leaves regenerated from PPT, calli were wrinkled 2-4 d after treatment with the PPT solution (some plants had minor wrinkling, others were severely wrinkled). Plants with minor wrinkling grew normally 7 d after they were treated with PPT. Plants in control were wrinkled, yellow, and died 7 d after treatment of PPT.

Identification of PPT-resistant plantlets and callus

Plantlets from protoplasts without pFWZ16 treatment were used as the negative control, plasmid pFWZ16 DNA was used as the positive control. Results from identifica-

tion of PPT-resistant plantlets and callus with the *bar gene* and *Bt* gene using PCR and PCR-Southern hybridization demonstrated that two kinds of transgenic rice plants were produced: 1) plants with both the *bar* and *Bt* genes, and 2) plants with the *bar* gene only.

Notes

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Molecular approaches to enhance rice productivity through manipulations of starch metabolism during seed development

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In rice seeds, the principal form of photosynthate is starch, which constitutes up to 85% of the dry weight. In view of its dominance as a constituent, the rate and duration of starch synthesis dictate seed weight and, in turn, the overall productivity of this small grain cereal. The rate of starch synthesis is controlled at several enzymatic steps but a key reaction is catalyzed by ADP glucose pyrophosphorylase (AGP). Enzymatic analysis of developing rice seed extracts indicates that the AGP is allosterically activated by 3-phosphoglycerate (3-PGA) and inhibited by Pi. As concentrations of 3-PGA in carbon metabolism may be much lower than in photosynthetic tissues, we suggest that the catalytic activity of AGP is much lower than its maximum potential such that maximum rates of starch synthesis are not achieved during seed development. Here we discuss various strategies to alleviate this potential constraint to carbon flow into starch by the introduction and expression of mutant AGP activities that are less dependent on allosteric regulation for high catalytic activity.

Plant productivity is dictated by source-sink relationships. Much of the effort to manipulate these relationships has been directed at "the source" by increasing net photosynthesis in leaf tissue. Alternatively, a less direct but, nevertheless. equally important approach of increasing yields, is to increase the sink strength, i.e., the plant's ability to convert photosynthate into storage reserves. In rice, a plant considered to be sink-limited during seed development (Chen and Sung 1994), the major storage reserve is starch. Increasing the conversion of photosynthate into starch during seed development should increase the sink strength of this storage organ and may, in turn, increase the yield potential of rice.

Starch synthesis is controlled by the activation and expression of the genes that code for AGP, starch synthase, and branching enzyme. In addition to this molecular control, starch synthesis is controlled biochemically in many plants by the allosteric regulation of AGP (Preiss et al 1989, 1991). Here, we present data that show that the

rice seed AGP is subject to allosteric regulation by the activator 3-PGA and the inhibitor Pi. Because 3-PGA-Pi ratios are likely to be much lower in seed amyloplasts than in leaf chloroplasts, the in situ activity of AGP may be much lower than its maximum potential and, thereby, restricts the flow of carbon into starch in developing rice seeds. To maximize the utilization of photosynthate, we will discuss various strategies to increase starch synthesis by the introduction of various mutant forms of AGP.

Rice seed AGP is allosterically regulated

It is widely accepted that the diurnal oscillation of starch metabolism in leaves is controlled by the allosteric control of AGP by 3-PGA and Pi, which activates and inhibits, respectively, the catalytic activity (Heldt et al 1977, Preiss et al 1991). The role of allosteric regulation of this enzyme in starch metabolism in nonphotosynthetic tissue is less clear with AGPs from some plants showing activation by 3-PGA and inhibition by Pi, while others exhibiting very little or no allosteric regulation by these effector molecules (Sowokinos and Preiss 1982, Plaxton and Preiss 1987, Olive et al 1989, Duffus 1992, Kleczkowski et al 1993). It is unclear whether the lack of or small allosteric response by these enzymes is a native property or whether it is a product of proteolysis of the enzyme during its preparation (Kleczkowski et al 1993).

Although the rice seed AGP has been extensively purified (Nakamura and Kawaguchi 1992), very little information is known about its allosteric properties. To directly address this question, we prepared crude extracts from mid-developing rice seeds and partially purified the AGP enzyme activity by fractionation (30-60%) with ammonium sulfate precipitation, heat treatment at 60 °C for 5 min, followed by chromatography on a MemSep DEAE chromatography column. The enzyme activity (2 units protein mg^{-1}), which eluted as a single peak from the ion-exchange column, was then assayed for its kinetic parameters.

What are the relative levels of these effector molecules in developing seeds? In leaf chloroplasts, these metabolites fluctuate during the diurnal cycle; during photosynthesis, 3-PGA levels increase due to CO_2 fixation while, simultaneously, Pi levels decrease due to the net increase in organic phosphate esters as well as increase in ATP content (Heldt et al 1977). This high 3-PGA-Pi ratio serves to activate the leaf AGP, thereby allowing starch synthesis to occur. In contrast, the amyloplast, the specialized plastid that contains starch, is dependent on the cytoplasm for both carbon and energy. Amyloplasts obtain these metabolic requirements by the uptake of hexose monophosphate and ATP (Okita 1992).

Introduction of variant bacterial AGPs into rice

The enteric bacterium *Escherichia coli* utilizes an identical metabolic pathway as do plants for the production of bacterial glycogen (Preiss and Romeo 1989, 1994). Like the higher plant enzyme, *E. coli* AGP is allosterically regulated although by different effector molecules than those recognized by the plant enzyme. The bacterial enzyme is activated by fructose 1,6-bisphosphate and inhibited by AMP. Several allosteric

mutants of the bacterial enzyme have been obtained including CL1136, SG5, and 618 (Preiss and Romeo 1989, 1994). These AGP mutants differ from the wild-type enzyme in displaying altered allosteric behavior. All of the enzymes require smaller amounts of the activator fructose 1,6-bisphosphate for maximum enzyme activity and are less sensitive to AMP inhibition. For instance, the AGP from strain 618 requires fourfold less activator for 50% activation but 11-fold greater levels of AMP for 50% inhibition than the wild-type enzyme. These mutant enzymes are also unique in that they display high levels of enzyme activity even in the absence of activator. Monsanto scientists have utilized the 618 gene, *glg*C16, to engineer potato plants having an increase in starch content in their tubers (Stark et al 1992). It would be of interest to determine whether starch synthesis and, in turn, the sink strength of developing rice seeds can be increased by the transfer and expression of a bacterial AGP displaying properties similar to those of strain 618 enzyme. The genetic basis for the change in enzymatic properties of the AGPs of CL1136, SG5, and 618 is illustrated by three different point mutations resulting in single amino acid replacements in each of these mutant AGPs. The mutations evident in CL1136, SG5, and 618 have been pyramided to yield the double (G366D, P295D) and triple (G366D, P295D, R67K) mutant enzymes, which display close to 90% of the fully activated enzyme levels in the absence of any activator. Current efforts are directed at constructing a DNA plasmid that would direct expression of the triple mutant *glgC* gene during seed development and the targeting of the AGP to the amyloplasts. Temporal and spatial expression of the glgC mutant will be accomplished by the use of the rice glutelin Gtl 5' flanking sequences, a highly characterized endosperm-specific promoter (Okita et al 1989, Zheng et al 1993), while amyloplast targeting will be conferred by the plastid targeting leader sequence of the maize *Brittle-1* (Sullivan et al 1991).

Engineering of rice by introduction of variant plant AGP genes

Unlike the bacterial enzyme, which is composed of four identical subunits coded by a single gene, the higher plant AGP is composed of a pair of large and a pair of small subunits coded by distinct genes (Hannah et al 1980, Okita et al 1990, Preiss 1992, Preiss and Sivak 1995). Although these subunits share considerable sequence homology, recent evidence indicates that these subunits do not play identical roles in enzyme catalysis. The small subunit appears to play more of a catalytic role as it is capable of forming an active enzyme in the absence of the large subunit (Ballicora et al 1995). The small subunit enzyme, however, requires more than 40-fold greater levels of 3-PGA for activation than the wild-type enzyme. These observations are consistent with the view that the large subunit plays more of a regulatory role and is required to increase the sensitivity of the heterotetrameric enzyme to 3-PGA activation. Because of this specialized function, the possibility of obtaining mutations in the large subunit that confer an allosteric phenotype similar to that displayed by the bacterial mutants CL1136, SG5, and 618 appears feasible. In this effort, the cDNA of the potato large subunit has been mutagenized and coexpressed with wild-type small subunit. One mutant, line 345, was observed to have normal kinetic properties with

regard to substrate binding except that it requires 45-fold greater levels of the activator 3-PGA than the wild type for maximum enzyme activity (Greene et al 1995).

Large AGP subunit mutations have been obtained in the maize Shrunken-2 gene. The mutant *sh2-m1* was isolated more than 20 yr ago and was a product of the insertion of the transposable element *Ds* into *Sh2* (Hannah and Nelson 1976). More recent studies revealed that *Ds* was inserted in the last of 16 exons and located about 60 nucleotides from the translational termination codon (Giroux et a1 1994). While perfectly wild-type genes can be obtained by *Ac*-mediated transposition of *Ds* from the *sh2* locus, other alleles are also synthesized. One of these alleles, *Sh2* Rev6, contains an additional tyrosine and serine residue and conditions a 15-18% increase in seed weight (Giroux et al 1995). Kinetic analysis of the Rev6 AGP reveals a pronounced resistance toward Pi inhibition, indicating that an alteration in allosteric property of the AGP enzyme was responsible for increased starch and, in turn, increased seed weight. Transgenic rice plants bearing *Sh2* Rev6 are currently being evaluated.

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Genetic modification of a rice glutelin cDNA and expression of the engineered glutelin gene in transgenic rice plants

E. Moon and R. Wu

Rice provides up to 80% of the total calories and protein for the people of Asia. To improve the nutritional quality of rice, we modified a rice glutelin gene. Synthetic oligomers and a modified synthetic gene coding for essential amino acids were inserted into a glutelin cDNA. These engineered glutelin genes were introduced into a rice plant using a particle gun, and the transformants were regenerated. Production of the novel proteins in transgenic rice plants will be analyzed when they produce the seeds. For the maximum expression of the engineered gene in rice seeds, we are constructing a series of strong promoters. The strength of each promoter is being tested in immature rice seeds by in situ transient expression assay using the particle gun. We are also modifying the glutelin gene to provide the better target site for the insertion or fusion of useful DNA.

The major cereal grains (rice, wheat, maize, barley, rye, sorghum, etc.) provide 60% of the energy and 50% of the total protein consumed in the world. The essential amino acid composition of the seed storage protein in these cereals is a very important nutritional property. Among the cereals, rice is the main staple diet of two-thirds of the world's population; it provides up to 80% of the total calories and protein in Asia. Therefore, it is especially important to improve the nutritional value of rice proteins. The conventional breeding approach has limitations such as high genetic barriers and a time frame.

The genes encoding storage proteins in most of the main crop plants have been isolated and characterized (for review, Messing 1987, Kreis and Shewry 1989). Extensive sequence comparison revealed that there are several variable regions in the glutelin that show significant divergence in sequence and size variation (Okita et al 1989). These hypervariable regions can serve as targets for modifications through genetic engineering techniques without interfering with the proper processing of these proteins in the seed.

A number of transformation methods—protoplast/polyethylene glycol, electroporation, and particle bombardment—have been successfully applied to transform cereals including rice (Goodman et al 1987, Shimamoto et al 1989). Regeneration of several monocots including rice has also been achieved. Combinations of these powerful techniques make it possible to improve nutritional quality by introducing a modified or new gene using genetic engineering technology (Cocking and Davey 1987, Goodman et al 1987).

The goal of our research is to improve the nutritional value of rice proteins through genetic modification of the glutelin and subsequent gene transfer. Here we describe designing of the synthetic oligomers, insertion of the DNA fragments generated from the oligomers into a glutelin cDNA, and introduction of the engineered glutelin genes into rice plants. We also discuss the modification of the glutelin cDNA to provide an even better targeting site for insertion and construction of strong promoters for the maximum expression of the introduced gene.

Results and discussion

Genetic modification of the rice glutelin cDNA

Inserting a synthetic oligomer coding for methionine, lysine, and tryptophan. To modify the coding sequences of a glutelin gene to increase the percentage of methionine (met), lysine (lys), and tryptophan (trp) residues, we synthesized a set of complementary 36-bp oligonucleotide, encoding 3 met, 5 lys, and 2 trp. After being annealed to form a double-stranded oligonucleotide, this synthetic DNA fragment was inserted into several sites in the variable regios of a glutelin cDNA (Type 11). The modified glutelin cDNA was constructed as a complete gene by adding the 2.3-kb glutelin promoter (Kim and Wu 1991) and the 0.4-kb nos transcription terminator to its 5' and 3' ends, respectively. The promoter and terminator were previously tested to be active using a *GUS* gene as the reporter by the bombardment method into immature rice seeds in a proper expression vector (Moon and Wu, unpubl. data).

Fusing a synthetic protein gene. For a greater effect, a second approach modifies the glutelin gene to make a fusion protein. In this heterologous system, a synthetic gene was designed to code for met and lys. To insert the synthetic gene into the Pstl site in the glutelin cDNA to make a fusion protein, we modified it to have a Pstl site at both ends, and not to have the stop codon at the 3' end. The polymerase chain reaction (PCR) amplification method was used with proper primers and the final modified gene was 294 bp in length containing 16 lys and 12 met codons. The synthetic gene was fused into the Pstl site of rice glutelin cDNA, and for the proper expression, the glutelin promoter and the nos terminator were added to the 5' and 3' ends of the gene, respectively.

Genetic engineering of rice with modified glutelin genes

We have introduced the modified cDNAs into rice plants for expression of the engineered glutelin protein in seeds. The Biolistic PDS-l000/He particle delivery system (BIO-RAD) was used according to the method by Xu and Wu (unpubl. data). Mature

seed-derived embryos were used for initiation of calli, which provided suspension culture cells for transformation. M10 tungsten particles (Sylvania Chemicals, GTE Products Cooperation, or BIO-RAD) were used, with a diameter of 1 mm. Particles were prepared and coated with DNA using the method of Russell (1993). For selection, pDM307 containing the *bar* gene was cotransformed, and the selection period lasted 4-7 wk. Small plantlets regenerated from the resistant calli on the regeneration medium were transferred to hormone-free MS medium for further growth, and then transferred to soil under greenhouse conditions.

Confirmation of the production of the modified glutelin proteins in transgenic rice plants

At the genomic level by PCR and Southern blot analysis. To confirm the integration of the modified genes in transgenic plants using PCR amplification reaction, genomic DNA was isolated using 200 mg of leaf tissue from the regenerants before being transferred to soil. We have designed two sets of PCR primers; each consisting of one of the primers derived from the inserted sequences in an opposite orientation, the other primer in a set is the one derived from the 3' end of the promoter or the one derived from the 5' end of the nos terminator. In these reactions, only the modified glutelin gene will be amplified. The PCR products will be confirmed by hybridization with the glutelin gene.

The larger amount of genomic DNA will be isolated from the transformants growing in pots, and a genomic Southern hybridization using the insert DNA as the probe will be carried out for further confirmation at the genomic DNA level.

Engineered glutelin in rice seeds. When the transgenic plants produce seeds, the seed proteins will be analyzed. The existence of the novel proteins will be confirmed based on the novel peptides produced after specific chemical or enzymatic cleavage of the purified glutelin fraction. In addition, amino acid composition analysis will be carried out to determine the extent of enrichment of 1ys, try. and met in the storage protein population in the transgenic plants.

Construction and assay of strong promoters for maximum expression of engineered genes in rice seeds

To maximize the expression of the genetically engineered gene in the rice seeds, we designed a series of promoters. The basic promoters are the 2.3 kb from the 5' region of a type II (*gt1*) glutelin gene, the 5.1 kb from the 5' region of a type I (*gt2*) glutelin gene, and the promoter from an actin gene. The 350-bp intron from the 5' nontranslated region of the actin gene was also used to construct fusion promoters. To assay the strength of these promoters, a series of test constructs were made using the *GUS* gene with the nos terminator as the reporter. The promoter strength will be assayed by in situ transient expression followed by bombardment into the immature rice seeds.

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Genetic engineering of provitamin A biosynthesis in rice endosperm

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Rice is characterized by a complete absence of provitamin A in its endosperm. Provitamin A, in the form of ß-carotene or structurally related compounds, is the essential precursor for the production of vitamin A in animals and man. Vitamin A deficiency causes 1.3-2.5 million deaths annually among young children worldwide. Furthermore, it is estimated that some 5 million preschool children in Southeast Asia become at least partially blind. Here, we present experiments that aim to initiate the first steps of provitamin A biosynthesis in rice endosperm through genetic engineering. Investigation of the biochemical status of immature rice endosperm has revealed the presence of the isoprenoid precursors necessary for carotenoid biosynthesis but no further hydrocarbon intermediates required to produce ß-carotene. Subsequently, cDNAs encoding the first two of four specific enzymes of this pathway have been transformed into a japonica rice variety and accumulation of the intermediate phytoene could be demonstrated.

According to statistics of the United Nations International Children's Emergency Fund, more than 134 million children worldwide are estimated to be suffering from vitamin A deficiency (Humphrey et al 1992). Improved vitamin A nutrition would be expected to prevent approximately 1-2 million deaths annually among children aged 1-4 yr. An additional 0.25-0.5 million deaths could be avoided if improved vitamin A nutrition could be achieved during late childhood. Improved vitamin A nutrition alone could therefore prevent 1.3-2.5 million out of nearly 8 million late infancy and preschool age children deaths that occur each year in the highest risk countries (West et al 1989).

Rice is the major food staple for most people in Southeast Asia. Its endosperm, however, completely lacks ß-carotene or structurally related compounds that can serve as essential provitamins for the production of vitamin A and retinal and retinoic acid in animals and man. The milled rice kernel consists exclusively of the endosperm

because the embryo and the aleurone layer have been removed during processing. Thus, many children worldwide, particularly in large areas of Southeast Asia, suffer from a variety of mild to severe health problems resulting from vitamin A deficiency.

This project aims to initiate carotenoid biosynthesis in the rice endosperm tissue to increase the daily vitamin A uptake of potentially vitamin A-deficient people who rely predominantly on rice as a food source.

For maize and sorghum, it is known that endosperm cells of cereals can produce and accumulate carotenoids (Buckner 1993). Furthermore, the starch storage tissues of potato and cassava (Penteado and Almeida 1988) accumulate carotenoids in considerable amounts.

To provide the minimum requirements of relevant carotenoids to young infants, and assuming that rice is the sole dietary source, 1-2 mg b-carotene g^{-1} of uncooked rice would be needed in rice endosperm (The Rockefeller Foundation 1993). This is roughly 1/4-1/2 of the amount produced in the endosperm of certain maize varieties, and enough to turn the rice noticeably, though not intensely, yellow.

The carotenoid biosynthesis pathway is a branch of the general isoprenoid pathway. The four enzymes that are necessary for b-carotene biosynthesis in this branch are phytoene synthase, phytoene desaturase, z-carotene desaturase, and lycopene cyclase. The genes for these enzymes are available from higher plants (Ray et al 1987, Bartley et al 1991, Fray and Grierson 1993), purple photosynthetic bacteria (Armstrong et a 1989), cyanobacteria (Linden et al 1994), and nonphotosynthetic bacteria (Misawa et al 1990).

Initially, we analyzed the biochemical status of immature rice endosperm. Incubation assays with radiolabeled precursors of general isoprenoid biosynthesis demonstrated the presence of geranyl geranyl pyrophosphate (GGPP), the substrate for the first specific enzyme of carotenoid biosynthesis, phytoene synthase.

Our strategy is to produce transgenic rice varieties that contain either single hetorologous carotenoid biosynthesis genes or several genes in combination. For this approach, we have chosen sequences encoding the enzymes phytoene synthase and phytoene desaturase from daffodil (*Narcissus pseudonarcissus*), which is a monocot plant like rice. Daffodil phytoene synthase and phytoene desaturase (P. Beyer 1995, University of Freiburg, Germany, unpubl. data) cDNAs have been recently characterized.

These cDNAs have been combined either with a constitutively expressed CaMV35S promoter, or with the endosperm-specific rice Gtl glutelin promoter (Okita et al 1989). As a selectable marker for the identification of transgenic plants, a hygromycin phosphotransferase (*hpt*) gene under the control of a Cah4V35S promoter has been linked to these constructs.

Subsequently, precultured immature embryos of japonica rice variety Taipei 309 were bombarded with a homemade particle inflow device (Finer et al 1992). A total of 203 hygromycin-resistant plants transformed with cDNAs for either phytoene synthase, phytoene desaturase, or cotransformed with both has been recovered from these experiments. Fifty-nine of these plants contained a DNA fragment integrated into the genome of the correct size to represent the expected cDNAs.

Among these transgenic plants, several lines have been identified that accumulated high levels of phytoene in the endosperm of the mature seed. Reverse phase high performance liquid chromatography revealed phytoene levels up to 0.74 µg phytoene per gram dry weight. Accumulation of z-carotene, the product of phytoene desaturase, has not yet been demonstrated. Correct integration and expression of the phytoene synthase transgene was further confirmed by Western blot analysis and RT-PCR. Southern blot analysis of R_1 plants growing in the greenhouse showed stable inheritance of the transgenes from the R_0 to the R_1 generation. Mendelian segregation patterns furthermore suggest a simple integration pattern in a single locus on one chromosome. Biochemical analysis of the R_1 plants will be performed as soon as seeds are available.

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Transfer of *rol* genes to increase panicle-bearing tillers and induce early flowering in indica rice cultivars

P. Nayak, N. Banerjee, S. Mishra, and S.K. Sen

The *rol* genes—*rolA*, *rolB,* and *rolC*—have been used in some plants to produce new genetic variability for agricultural traits. This study analyzed the effects of transferring *rol* genes to increase panicle-bearing tillers and induce early flowering in indica rice. The *rolC* gene was transferred to indica variety Heera to increase the number of panicle-bearing tillers, which are inherently low in this variety. Due to the transfer of this *rol* gene, two plants showed a multiple tillering habit after 10 d of germination, 9-12 tillers per plant compared with only 2-3 tillers in control plants. Increased grain production approached 160%. The transfer of *rolA* gene to indica varieties Kalonunia and Basmati 385 to induce early flowering is also briefly discussed.

Three genes—*rolA, rolB,* and *rolC*—which correspond to open reading frames 10, 11, and 12, respectively, and are present in the TL-DNA of the Ri-plasmid, are responsible for hairy root syndrome in dicotyledonous plants. Although these genes act independently, their combined effects can create a synergistic impact on plant development. It is believed that, although none of these genes are directly involved in phytohormone biosynthesis, their products may influence the mechanism for regulation of plant development and growth that is dependent on the integrated regulatory control of multiple hormones (Klee and Estelle 1991, Dehio et a1 1993). Even though the expression of the *rol* genes in transformed plants is still poorly understood, it has nevertheless been possible to attribute some developmental and morphological changes to the transfer of a single *rol* gene or a combination of them based on direct observation.

The influence of *rol* genes is presumably species- or genotype-dependent. Thus, their effects are not completely predictable. Since influence on plant growth and development can affect productivity, *rol* genes have been used in some plants to produce new genetic variability for agricultural traits (Fladung 1990, Handa 1992, Fladung et a1 1993, Frugis et a1 1993).

We decided to attempt transfer of *rol* genes to indica rice to 1) increase the number of panicle-bearing tillers to enhance yield potential and 2) induce early flowering. Aromatic rice cultivars Heera, Basmati 385, and Kalonunia were the varieties we worked with.

The expression potential of the promoters of the *rolA, rolB,* and *rolC* genes was evaluated through transient expression of the *gusA* reporter gene in protoplasts of rice, tobacco, and mustard. We observed that significant differences do exist between monocot and dicot plant systems involving promoter functions. Thus, we understood that the expression of the *rol* genes may not fall totally in line with the known influences that have been observed in dicot plants.

Increasing panicle-bearing tillers

Heera, a short-duration (60-65 d), photoperiod-insensitive, indica rice variety that has, on average, only two or three panicle-bearing tillers, was selected as a model for increasing the number of panicle-bearing tillers.

The *rolC* gene with the CaMV35S promoter and nos terminator was cloned in pUCl8, which was cotransferred with another plasmid containing the *nptII* gene that confers resistance to kanamycin. Both plasmids were delivered with the help of microprojectile bombardment into embryogenic calli derived from scutellar tissues of mature seeds.

Thirty-three transgenic plants with the *rolC* gene were identified using polymerase chain reaction (PCR) analysis. Only six plants showed increased tillering capacity at the vegetative growth phase after transplanting. Segregation analysis revealed that more than one chromosome contained the transgene. One of the R_0 plants produced nine seeds when selfed. All nine plants showed the presence of the *rolC* gene when analyzed through PCR. Two of these nine plants showed a multiple tillering habit after 10 d of germination. R_2 plants produced 9-12 panicle-bearing tillers per plant compared with only two or three produced in control plants. Although panicle length was shorter in the secondary tillers, grain production per plant increased as much as 160%. Grain quality, weight of each grain, and days to maturity were unchanged. Since Heera has a short vegetative growth phase, the influence of the *rolC* gene on improving tillering capacity was considered to be somewhat constrained.

Following our success with Heera, we transformed two aromatic indica rice varieties, Kalonunia and Basmati 385, using the same procedure. These varieties have a long vegetative phase and further evaluation should show them to be influenced similarly by the *rolC* gene.

Inducing early flowering

Additionally, the *rolA* gene was transferred to Kalonunia and Basmati 385 to induce early flowering. The rationale for using *rolA* gene to induce early flowering is based on observations of transgenic plants of Indian mustard (*Brassica juncea* Czern.) containing the *rolA* gene—early flowering was consistently observed even after the

R_5 generation. Now that transgenic plants have been generated and Southern blot analysis has revealed that they contain the transferred gene, the next step will be to determine if flowering time has been effected.

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Cytosine methylation implicated in silencing of b-glucuronidase genes in transgenic rice

A. Kohli, B. Ghareyazie, H.S. Kim, G.S. Khush, and J. Bennett

Four independent regenerants from the biolistic transformation of the cultivar Anjungbyeo contained multiple copies of the *hpt* and *gus* genes. The *gus* genes became progressively silenced in leaves and roots during the T_0 generation. The silenced state was inherited in subsequent generations. Treatment of T_0 and later generations with 5-azacytidine, an inhibitor of cytosine methylation, reactivated *gus* expression in cells that underwent cell division during the exposure to the inhibitor. These results implicate cytosine methylation in silencing of multiple copies of a transgene in rice.

Numerous examples now exist in plants where the insertion of multiple copies of a transgene leads to loss of expression of some or all copies of the transgene (Finnegan and Miserly 1994, Fluvial 1994). Various transcriptional and post-transcriptional mechanisms have been put forward to explain the phenomenon, including physical interactions between the genes, competition for nondiffusible factors essential for ordered transcription or translation, degradation of sense-antisense hybrids, and selective degradation of overexpressed mRNA. We have observed the silencing of the ß-glucuronidase (*gus*) gene in four out of four independent transgenic lines of rice cultivar Anjungbyeo containing multiple copies of this reporter gene.

Results

All transformants contained multiple copies of the transgene

The *gus*-containing plasmid $pCaI_1Gc$ (Luehrsen and Walbot 1991) was introduced into primary embryogenic calli by particle bombardment, along with plasmid pTRA 132 (Zheng et al 1991) for hygromycin resistance. Four independent transformants, designated HSKI-4, were regenerated after selection on hygromycin B. The polymerase chain reaction was used to show that the young regenerated plants contained both the *hpt* and *gus* genes. Genomic DNA was later extracted from plants after the tillering

stage and digested with the restriction endonuclease *Hin*dIII. which cuts each plasmid once. DNA gel blots revealed multiple bands hybridizing to *hpt* and *gus* probes.

Expression of *gus* gene silenced in all transformants

In $pCaI_1Gc$ the *gus* gene is fused with the first intron of the maize *Adh1* gene and controlled by the CaMV35S promoter. The gene was expressed in hygromycin-resistant calli and also in early leaf tissue of the T_0 generation but became progressively silenced in leaves during T_0 growth, until GUS staining (Kosugi et al 1990) of later leaves was limited to spots and streaks (Fig. 1a). Silencing was particularly severe in the roots. Strong expression was observed in pollen grains of HSKl and HSK4, but severe silencing was reestablished in the leaves and roots of T_1 and later generations.

Reactivation of silenced transgene. One of the epigenetic mechanisms of transgene silencing is methylation of cytosine (Matzke et al 1989, Kilby et al 1992, Ingelbrecht et al 1994). We used 5-azacytidine, an inhibitor of C-methylation (Santi et al 1983), to test for the involvement of methylation in silencing *gus* in HSK1-4. Reactivation of *gus* expression was readily demonstrated in root tips of T_0 plants treated for 5 d with daily supplements of 30 µM 5-azacytidine. A detailed study of line HSK1 showed that *gus* expression could also be reactivated in many tissues of later generations, but only in cells that had undergone cell division in the presence of the inhibitor. Figures 1b and 1c show the roots of line HSK1 stained for GUS activity after growth for 5 d in the absence and presence, respectively, of 30 µM 5-azacytidine.

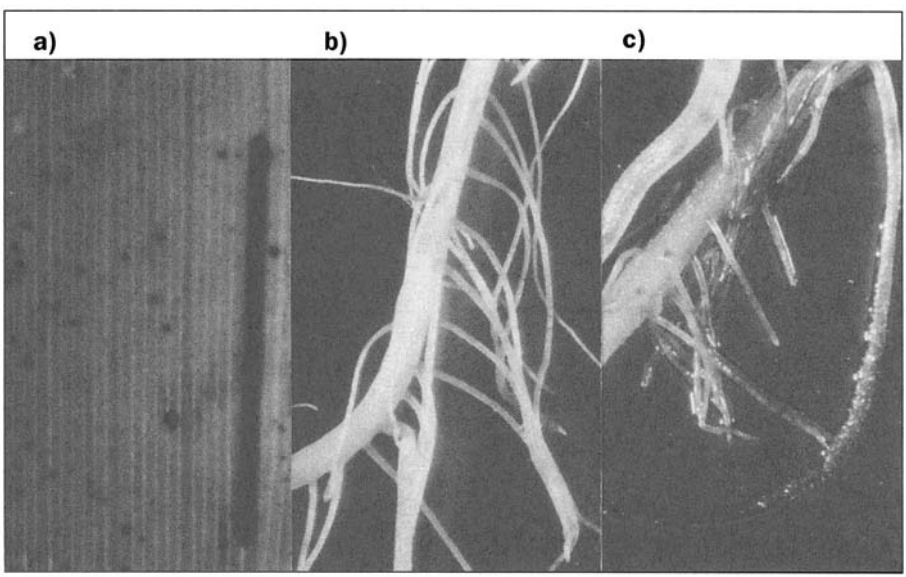

Fig. 1. Silencing and reactivation of the *gus* reporter gene in transgenic rice. (a) Leaf of line HSKl shows spots and streaks of GUS activity following severe silencing of the gene. Roots of line HSKl stained for GUS activity after growth for 5 d in the absence (b) and presence (c) of 30 µM 5-azacytidine.

Discussion

Methylation was previously implicated in the silencing of the CaMV35S promoter in petunia (Linn et al 1990), but the crucial methylation site(s) were not identified. Plants contain at least two classes of DNA methyltransferases capable of methylating C: those methylating CG and those methylating CNG (Pradhan and Adams 1995). There is also evidence for methylation of cytosines at other locations (Ingelbrecht et al 1994). The silencing of *gus* in rice leaves and roots may be due to C-methylation of the *as1* cis-element of the CaMV35S promoter. This element contains two copies of the motif TGACG, known to be important for expression from this promoter in leaves, stems, and roots of transgenic tobacco (Lam et al 1989).

Several groups have reported transformation of rice with a CaMV35S/*gus* constructs without observing silencing (e.g., Battraw and Hall 1990, Kyozuka et a1 1990, Terada and Shimamoto 1990). In one case, the construct was very similar to $pCaI_1Gc$ in also containing the maize *Adhl* intron (Kyozuka et al 1990). Although we cannot eliminate the possibility that cultivar Anjungbyeo may have an unusually marked tendency for de novo methylation, we favor the hypothesis that the methylation-induced silencing of *gus* observed in our experiments is due to the high copy number of this gene in our transgenic lines. Methods for achieving a lower copy number for transgenes in biolistic transformations are under investigation.

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Integrated sequence array and genome structure of integration sites in transgenic rice

K. Wakasa and M. Takano

Twenty-seven transformed rice plants appeared to have a simple integration pattern except five plants that showed a multiple integration pattern. In four of these plants, inheritance of a transgene indicated DNA integration into one chromosome. To know the precise feature of the transgene, we conducted an extensive genomic sequencing and sequence motif analysis over the integration site of two of the transgenic plants, #478 and #559. The sequence analysis revealed that the plants carried transgenes with an inverted structure, one of which included a large duplication of the rice genome of more than 2 kb. DNA integration occurred at the repetitive sequence regions and/or at AT-rich regions, suggesting that they are the preferred structures for DNA integration.

The transgenic plant system is becoming more and more important in basic and applied plant science. However, little is known about the mechanism of DNA integration into the plant genome. It is important to understand the role of DNA sequencing and the topological genome structure to understand this mechanism and to establish an efficient method for integrating genes that does not unnecessarily change the plant genome. We report here on the transgene array and sequence motif surrounding integration sites.

Transformation

Protoplasts were isolated from calli induced from mature seeds of rice variety Norin 8 on MS agar medium. These were maintained in R2 liquid medium (Fujimura et al 1985) with 2 mg 2,4-D L^{-1} according to the modified protocol of Wakasa et al 1984. Maps of two plasmids, pUKC and pMY402, are shown in Figure 1. A purified plasmid DNA sample resuspended in 0.1 mM EDTA was used without carrier DNA.

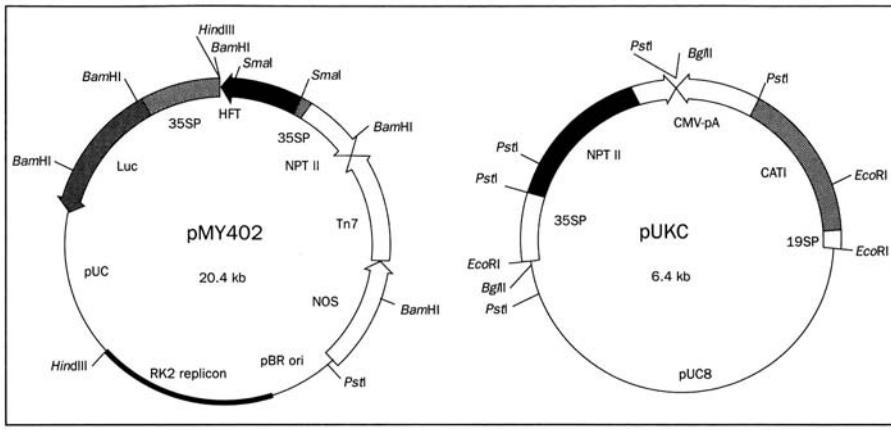

Fig. 1. Restriction enzyme maps of pUKC and pMY402. Neomycin phosphotransferase II (NPT II); hygromycin phosphotransferase (HPT); luciferase (Luc); 19SP, CaMV19S promoter; 35SP, CaMV35S promoter. Other genes are not discussed in this paper.

The DNA-calcium-phosphate mixture solution was prepared using the standard method. Thirty to 40 µg of plasmid DNA dissolved in 1 ml of DNA solution and 3 to 4×10^7 protoplasts were gently mixed. One milliliter of 40% polyethylene glycol (PEG 6000) was added after 2 h. One to 2×10^6 protoplasts were cultured in l-ml-conditioned R2 medium (pH 5.8). Selection was performed twice on N6 medium containing 20 µg hygromycin B ml^{-1} or 35 to 50 µg G418 ml^{-1}**.** Resistant calli were transferred to the N6 medium without hormones for plant regeneration.

No segregation of multiple bands in progeny

Among 27 primary transformed rice plants, five plants presented multiple bands, suggesting multiple integration or a complex array. Based on Southern blot hybridization, four of these plants presented concomitant inheritance of bands. As summarized in Table 1, all bands in each plant always transmitted together to the progeny without any segregation. This means that all transgenes are located on the same chromosome of each individual. Plant #478, which was one of them, and plant #559, which contained a rearranged size of the *hpt* gene, were subjected to an analysis of the structures of the transgenes and the target genome.

DNA analyses

Total DNA samples were isolated from leaves of the untransformed rice (Norin 8) and plants #478 and #559, according to the CTAB (cetyltrimethyl-ammonium bromide) method. Prior to agarose gel electrophoresis, 3 µg of each DNA sample were digested. A genomic Southern blot hybridization was performed.

Table 1. Concomitant inheritance of transgenes in progeny plants.

Lines (Plasmids)[a]	Number of progeny plants		
	Total	With transgenes[b]	Without transgenes
98 (pU)	9	6	3
113 (pU)	16	13	3
237 (pU)	9	8	1
478 (pM)	19	12	7

[a]pU, pUKC; pM, pMY402. [b]Each plant had the same bands as the parents.

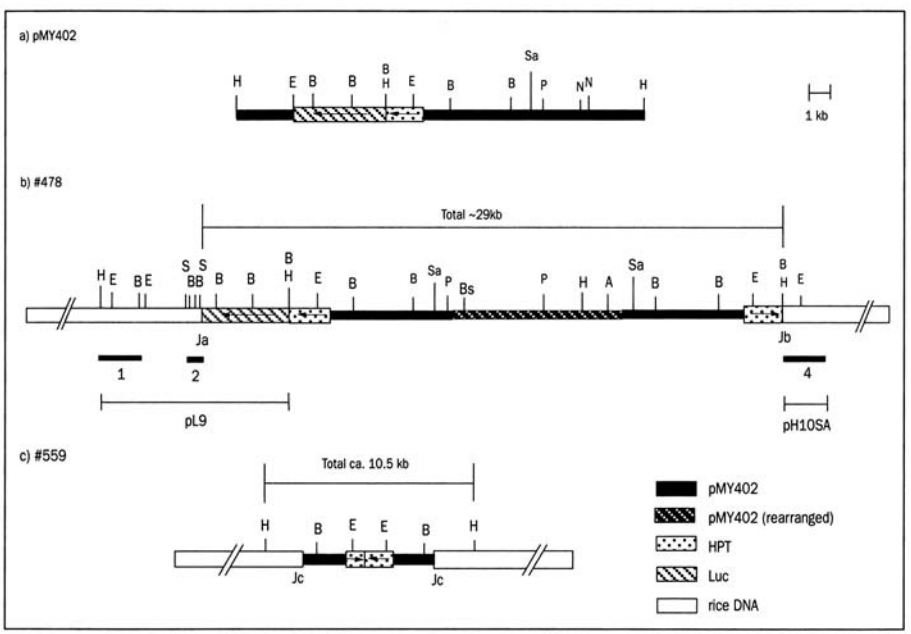

Fig. 2. Transgene arrays of plants #478 and #559, based on sequence and Southern blot analyses. a) Diagram showing linearized plasmid, pMY402. b) and c) Diagrams of transgenes in plants #478 and #559. The probes to isolate corresponding clones from untransformed rice genome were indicated (1, 2, and 4 in plant #478). A, *Acc*I; B, *Bam*HI; Bs, *Bss*HII; E, *Eco*RI; H, *Hin*dIII; N, *Nae*I; P, *Pst*I; S, *Sac*I; Sa, *Sal*I; S3, *Sau*3AI.

The clones were isolated from the genomic libraries of the transgenic rice and the untransformed rice, which were constructed by cloning the DNA samples digested with *Hin*dIII, *Bam*HI or the partially *Sau*3A I into the 1DASH2 vector. Whole inserts of phage clones were subcloned in Bluescript 2 for restriction enzyme mapping and DNA sequencing.

Transgene arrays

Gel blot and sequence analyses yielded the transgene arrays in Figure 2. In plant #478, the integration complex was composed of two plasmid fragments, totaling 29 kb in length and was conjugated invertedly (Fig. 2b). In plant #559, the symmetrical feature was presented. The sequence analysis revealed an inverted duplication of the transgene, including a rice genome sequence of more than 2 kb.

Structure of junction regions

The summarized results of the junction structures obtained from the sequence analyses are shown in Table 2. At all three junctions, the AT-rich and/or repetitive sequences were the target of DNA integration. pL9, which contained the Ja junction, displayed two levels of AT content. The first 350 nucleotides from the recombination site were composed of 72% AT nucleotides. The rest of the genomic sequence had a low AT content (32%). Analysis of target DNA from the untransformed rice genome showed that this AT-rich fragment was a part of a large AT-rich sequence consisting of about 650 bp, which contained several characters showing a scaffold attachmentlike structure (Gasser et al 1989). In junction Jb of pH10SA, integration occurred in the AT-rich sequence of retrotransposon. In both cases (Ja and Jb junctions), the importance of AT richness was supported by the fact that even in a repetitive sequence, integration occurred at the AT-rich region. Telomere repeats were also the target of DNA integration in plant #559. In this case, these repeats were found to be followed by three AT-rich regions with more than 550 bp containing *Drosophila* satellite DNA repeats.

Conclusion

Transgenes in plants #478 and #559 presented inverted arrays. A concomitant inheritance and rearranged feature of the transgene resulted from this complex array. Summarized results in Table 2 suggest the presence of a preferred genome structure for DNA integration.

Table 2. Characteristics of the three junctions and surrounding structures.

Transgenic plant	Junction	Integration region	Surrounding sequence
# 478	Ja	AT-rich (SAR)[a]	Repetitive sequence
# 478	Jb	AT-rich	Retro-transposon
# 559	Jc	Telomere repeats	AT-rich (satellite DNA)

[a]SAR = scaffold attachment region.

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Isolation and characterization of lipoxygenases from rice induced by incompatible infection of rice blast fungus, *Magnaporthe grisea*

Y.-L. Peng, Y. Qin, Y. Yang, and J. Huang

It has been previously reported that incompatible infection of rice blast fungus (*Magnaporthe grisea*) induces increased activity of lipoxygenases (LOXs) in rice. LOXs from incompatible and compatible *M. grisea*-infected rice leaves were compared by DEAE-Toyopearl column chromatography. It was found that leaf-LOX 3 was markedly induced in response to incompatible infection of the pathogen. This leaf-LOX 3 was further separated into two isoforms (CM-LOX 1 and CM-LOX 2) through CM-Toyopearl column chromatography. The two LOXs were then purified to homogeneity by gel filtration and Mono-Q chromatography. Sodium dodecylsulfate polyacrylamide gel electrophoresis showed a single polypeptide with a molecular weight of 98 kDa for CM-LOX 1 and 102 kDa for CM-LOX 2. Optimal pH was 5.5 for CM-LOX 1 and 6.5 for CM-LOX 2. The Km's of CM-LOX 1 and CM-LOX 2 were 115 and 54 mM for linoleic acid, and 73 and 57 mM for linolenic acid, respectively. The study also indicated that the two LOXs are immunologically related.

Lipoxygenases (LOXs) are ubiquitously distributed enzymes among plants, which catalyze hydroperoxidation of polyunsaturated fatty acids containing a *cis,cis*-1,4-pentadiene-conjugated double-bond structure. In plants, linoleic and linolenic acids are their major substrates. Although the precise physiological functions of LOXs have not been determined, these enzymes have been implicated in growth and development, senescence, wounding, and pest resistance (Siedow 1991). In particular, the importance of LOXs in plant defense against diseases has recently attracted attention, and induced increases in LOX activity have been described in at least 15 plant-pathogen systems (Qin 1995). Melan et al (1993) reported cDNA cloning of an *Arabidopsis thaliana* LOX and showed induction of the gene by *Pseudomonas syringae.* Some other studies also analyzed protein and mRNA accumulation levels of LOX in pathogen-infected plants using heterologous antisera or DNA probes (Koch et al 1992,

Slusarenko et a1 1993). Plants in which LOXs have been studied up to date usually have a number of LOX genes and isozymes, and these LOXs in different organs and subcellular locations may play distinct physiological functions. Ohta et al (1991) identified three LOX isozymes in rice leaves and demonstrated leaf-LOX 3 is markedly induced in response to incompatible infection of rice blast fungus (*Magnaporthe grisea*). Peng et al (1994) isolated a novel LOX cDNA from the pathogen-infected rice leaves and showed evidence indicating specific mRNA and protein accumulation of the enzyme as an early defense response. Here, we report the identification, purification, and characterization of two rice LOXs induced by *M. grisea* infection.

Materials and methods

Plant and pathogen growth and inoculation

Rice (*Oryza sativa* cv Aichiasahi) at the five-leaf stage was inoculated with either the compatible race 007 or the incompatible race 131 of *M. grisea.* Rice seedlings and the pathogen conidia were prepared as described by Peng and Shishiyama (1988). At 48 h after inoculation, the top leaves were harvested and quickly frozen in liquid nitrogen (-80 °C) until needed.

Extraction, assay, isozyme identification, and purification of induced LOXs

Rice leaves (6 g) were first ground in liquid nitrogen to a fine powder and the enzymes were extracted in 30 ml of a 50-mM sodium phosphate buffer, pH 6.5, containing 1% Tween 20 and 0.5 mM PMSF by stirring for 4 h. The slurry was centrifuged at 10,000 g for 30 min and the supernatant was dialyzed overnight against Buffer A (25 mM Tris-HCl, pH 7.5, containing 10% glycerol, 1% Tween, 0.1 mM EDTA, and 0.1 mM PMSF). The enzyme extracts were then loaded to a DEAE-Toyopearl column (1.6 × 20 cm) equilibrated with buffer A' (same as buffer A except for 0.1% Tween 20). After washing with 40 ml of buffer A', LOX was eluted with an 80-ml linear gradient of 0.00 to 0.30 M NaCl in buffer A'. The induced fraction of LOX was pooled, concentrated to 10 ml with the Amicon centriplus concentrator, and desalted through a Sephadex G-25 column equilibrated with buffer Bl (50 mM sodium acetate, pH 5.0, containing 10% glycerol, 0.1% Tween 20, 0.1 mM EDTA, and 0.1 mM PMSF). The fraction was then charged to a CM-Toyopearl column equilibrated with B2 (same as B1 except for pH 5.3) and washed with 50 ml of buffer B2 and 40 ml of buffer B3 (same as B1 except for pH 6.3). Active fractions from this column were respectively concentrated and passed through a column for gel permeation of Sephacryl S-100, which was equilibrated and washed with buffer A'. The LOXs were finally purified by FPLC on the Mono Q column equilibrated with Buffer A' and eluted with a 45-ml linear gradient of 0.00-0.30 M NaCl in buffer A'.

LOX activity was measured as previously described (Ohta et a1 1991). All the extraction and purification procedures were carried out at 4 °C. Proteins were measured by the dye binding assay (Bradford 1976).

Characterization of induced LOXs

Optimal pH was determined by assaying LOX activity in 0.1 M sodium acetate (pH 3.5-4.5), 0.1 M sodium phosphate (pH 6.0-7.0) and 0.1 M Tris-HC1 (pH 7.5-9.0), which contained 7.5 mM linoleic acid and 0.1 % (w/v) Tween 20.

Substrate specificity was determined by measuring the enzyme activity in 0.1 M sodium phosphate (pH 6.5) containing 1.5 mM substrate, 0.1% Tween 20. Linoleic and linolenic acids as well as their methyl esters were used as substrates. Apparent Km was calculated by Hanes-Woolf plot for the reaction of LOXs with linoleic and linolenic acids (Christensen and Palmer 1974).

The regiospecificity of the enzymes was determined as follows. Approximately one unit of the enzymes was incubated at 4 °C for 1 .5 h with 4 ml of a 2 mM linolenate solution containing 0.07% Tween 20 in 0.1 M sodium phosphate, pH 6.5. The reaction products were extracted with an equal volume of chloroform/methanol (v/v=2/1). After concentrated to dryness by nitrogen gas, the product was dissolved in *n*-hexane and fractionated by Shimadzu LC-10A HPLC on a micro-pack Si-5 column (4 mm × 30 cm) eluted as previously reported (Ohta et al 1991).

LOXs used for characterization were collected from CM-Toyopearl chromatography.

Results and discussion

Identification of LOX isozymes induced by incompatible blast infection

LOXs from rice leaves were separated into three fractions (leaf-LOXs 1, 2, and 3) by DEAE-Toyopearl chromatography (Fig. 1). Comparison of elution profiles of LOX isozymes clearly indicates that leaf-LOX 3 was markedly induced in the incompatible race-infected leaves relative to the compatible and noninfected one (Fig. 1). This confirms a previous report by Ohta et al (1991). However, this leaf-LOX 3 was separated into two isozymes by CM-Toyopearl chromatography, designated as CM-LOXs 1 and 2 (Fig. 2), indicating that at least two LOXs were induced in rice after infection of blast fungus. Activity increase in LOXs has been reported in a number of plant-pathogen interactions, and several recent studies followed mRNA and protein levels of one LOX in plants under infection. However, to address the biological function of induction of different LOXs in plant disease resistance, we strongly suggest that biochemical studies be carried out to determine the number of induced LOX isozymes. Since the isozymes are similar in their molecular weight, it may be difficult to distinguish induction of different isozymes only by Northern and Western blots.

Purification and characteristics of induced LOXs

The induced CM-LOXs 1 and 2 were purified from rice leaves by a procedure involving DEAE-Toyopearl, CM-Toyopearl, gel permeation of Sephacryl S-100, and Mono Q chromatography (Table 1). Specific activity of the purified enzymes was 9.25 unit mg^{-1} for CM-LOX 1 and 11.89 unit mg^{-1} for CM-LOX 2 (Table 1). This is much lower than rice LOX 2 from embryo (Ohta 1988). However, sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) revealed a single polypeptide with

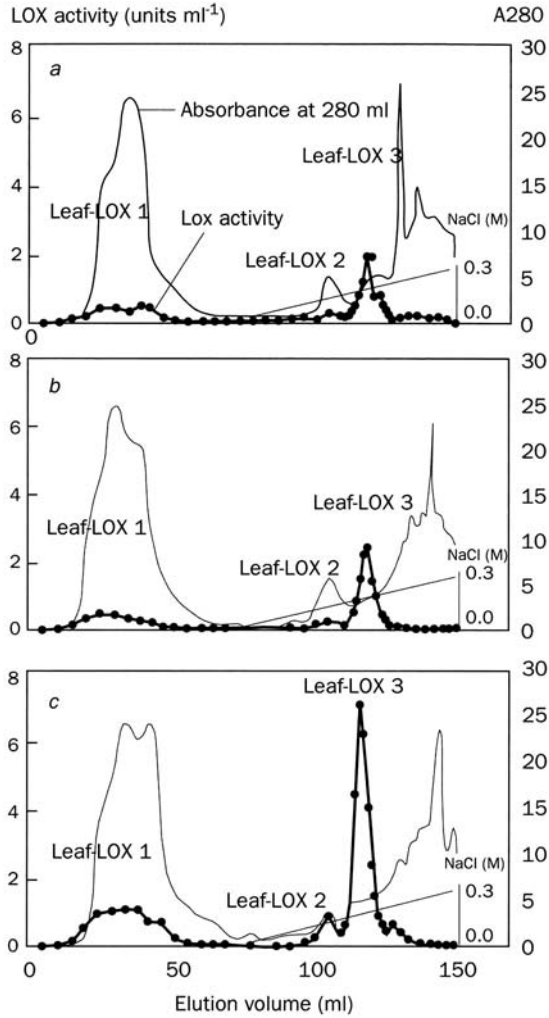

Fig. 1. Elution profiles of lipoxygenases in *M. grisea*-infected and noninfected rice leaves on a DEAE-Toyopearl column: a) noninfected leaves; b) race 007-infected leaves; c) race 131-infected leaves.

molecular weight of 102 kDa for CM-LOX 2 and 98 kDa for CM-LOX 1 (Fig. 3), indicating their electrophoretical purity.

Various isozymes of LOX are distinguished on the basis of their pH optima, product regiospecificity, and immunological relationship. To compare the two and the previously reported LOXs, we investigated the effect of pH on the activity, substrate specificity, and product regiospecificity of CM-LOXs 1 and 2. The optimal pH is 5.5 for CM-LOX 1 and 6.5 for CM-LOX 2, and both LOXs showed much activity reduc-

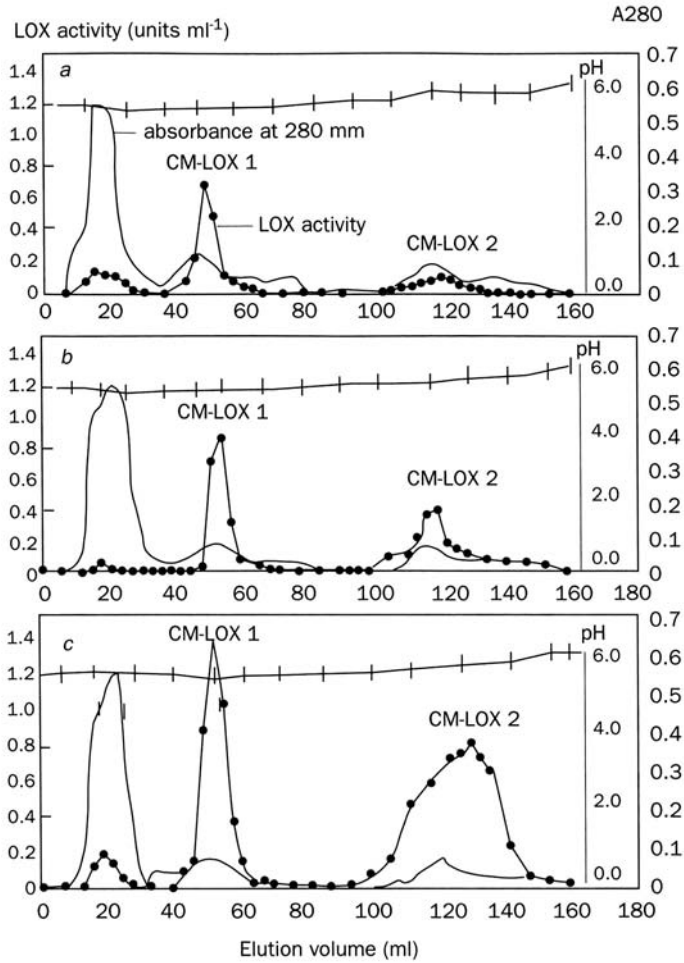

Fig. 2. Elution profiles of leaf-LOX 3 on a CM-Toyopearl column: a) noninfected leaves; b) race 007-infected leaves; c) race 131-infected leaves.

tion as pH of reaction buffers became lower than 4.5 or higher than 7.0. Both LOXs prefer linolenic acid as their substrate, although they are also active against linoleic acid and methyl ester of these fatty acids (Table 2). Respective Km's of CM-LOXs 1 and 2 are 115 and 54 mM for linoleic acid, and 73 and 57 mM for linolenic acid, respectively. The lower Km of the two LOXs further confirmed their preference for linolenic acid. Collectively, these data indicate that CM-LOXs 1 and 2 are two distinct isozymes. However, both LOXs insert exclusively molecular oxygen into the C13 position of linoleic and linolenic acids and the ratio of 9-hydroperoxy to 13-hydroperoxy fatty acid reaction products was about 14% for linoleic acid and less

Table 1. Purification of two induced lipoxygenases from rice infected with an incompatible race of blast fungus. [a]

Purification step	Protein (mg)	Activity (Units)	Specific activity (units mg^{-1})	Yield (%)	Purification (fold)
Crude extracts	582.40	228.80	0.393	100	
DEAE-Toyopearl	115.10	76.71	0.666	31.55	1.692
Ultrafiltration	24.04	64.85	2.698	26.68	6.859
Sephadex G-25	20.82	60.06	2.855	24.71	7.335
CM-Toyopearl (LOX 1)	2.35	9.61	4.089	3.72	10.937
CM-Toyopearl (LOX 2)	6.35	25.35	3.990	10.43	10.144
Sephacryl S100 (LOX 1)	0.387	3.43	9.074	1.41	23.069
Sephacryl S100 (LOX 2)	2.432	10.00	4.112	4.11	10.454
Mono Q (LOX 1)	0.091	0.84	9.254	0.35	23.529
Mono Q (LOX 2)	0.365	4.35	11.894	1.79	30.238

[a] Twenty grams of Infected rice leaves were used.

Table 2. Substrate specificity of CM-LOX 1 and CM-LOX 2.

Substrate	Relative activity (%)	
	CM-LOX 1	CM-LOX 2
Linolenic acid	100	100
Linoleic acid	53.9	86.0
Methyl linoleanate	38.5	18.0
Methyl linoleate	28.5	14.0

Fig. 3. SDS-PAGE of purified LOXs from *M. grisea*-infected rice leaves: Line a, purified CM-LOX 1; Line b, purified CM-LOX 2.

than 1 % for linolenic acid. Dot blot experiments showed that polyclonal antiserum to CM-LOX 1 cross-reacted with CM-LOX 2, indicating the two LOXs are immunologically related. The two LOXs have molecular weight, substrate, and product specificity similar to that of LOX2:Os:1, a new LOX recently isolated from the same source (Peng et al 1994). For comparison, determination of the partial amino sequence of CM-LOXs 1 and 2 is under way in our laboratory.

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Preliminary studies on cloning of blast resistance genes

Z.H. He, Y.L. Wu, J.Q. Lin, S.J. Chen, and D.B. Li

Cloning of rice genes that confer resistance to the blast fungus caused by *Magnaporthe grisea* was conducted using a pair of near-isogenic lines, H7R (resistant) and H7S (susceptible), by mRNA differential display and random amplified polymorphic DNA assays. Preliminary results showed that six primers produced special polymerase chain reaction products in the resistant line. Some special mRNAs were found in H7R 8-24 h after inoculation with fungus race ZB15. Eight resistance-related (RR) cDNA fragments derived from those mRNAs were found in H7R. Some pathogenesis-induced (PI) cDNA fragments were observed in both lines inoculated with the races. Of these RR and PI cDNA fragments, three RR cDNA (240, 250, 400 bp) and two PI cDNA (200 and 500 bp) fragments were selected for further study. Northern hybridization and cloning of the fragments are in progress.

Rice blast caused by *Magnaporthe grisea* is one of the crop's most destructive diseases. It often leads to huge yield losses in China. Breeding for resistance is the most effective control method. However, lines developed through traditional breeding often break down due to the diversity of fungus races. Genetic engineering in crop resistance breeding promises to be a powerful tool in overcoming the disease. Some blast resistance genes have been mapped on chromosomes by restriction fragment length polymorphism (Yu et al 1991, Zhu et al 1993, McCouch et al 1994, Wang et al 1994). Two polymerase chain reaction (PCR)-based markers, which can distinguish blast resistance genes *Pi2(t)* and *Pi11(t),* have been identified (Lu et al 1994, Hittalmani et al 1995). Also, advanced work has been done on molecular biology of the fungus (Leong et al 1994, Valent and Chumley 1994). However, we still lack molecular information on characteristics of blast resistance genes and "gene-for-gene'' interaction between the host and pathogen.

This paper presents some preliminary results of studies on resistance and response genes to blast by mRNA differential display (DD) and random amplified polymorphic DNA (RAPD) assays.

Materials and methods

A pair of near-isogenic lines (NILs)—H7R (resistant) and H7S (susceptible)—was used. They differ by the presence or absence of resistance gene *Pi-rl(t)* to race ZB15 (He and Zhen 1990). Genomic DNAs were extracted from the seedlings of these lines. RAPD assays were done using the DNAs and 120 10-mer primers (synthesized at the University of British Columbia, Canada) in 25 µl reaction size: 50 ng DNA, 6 pmol random primer, 10 mM Tris-HC1, pH 9.0, 50 mM KCl, 0.1% Triton-l00, 2 mM $MgCl_2$, 200 µm each of dNTPs, and 1 unit Taq (Promega's product). The seedlings were inoculated with race ZB15, and total RNA was isolated using TRIzol reagent (GIBCO BRL's product) 8, 12, 16, 24, 36 h, and 2-7 d after inoculation. DD was done as described by Peng and Pardee (1992) and GenHunter Corporation's mRNA differential display system except that ^{32}P and silver staining were used. Twenty combinations of two anchored primers T11CA and T11GC and 10 arbitrary primers of 10-mer were investigated.

Results

RAPD fragments existing only in resistant line

After PCR, most of the 120 primers produced 5-15 DNA bands, of which primers 603, 611, 613, 619, 623, and 679 were found to produce special fragments of about 1.5, 0.7, 0.6, 1.5, 0.55, and 0.60 kb in the resistant line H7R, respectively (Fig. 1). Other primers produced highly identical bands in both lines, implying that two lines

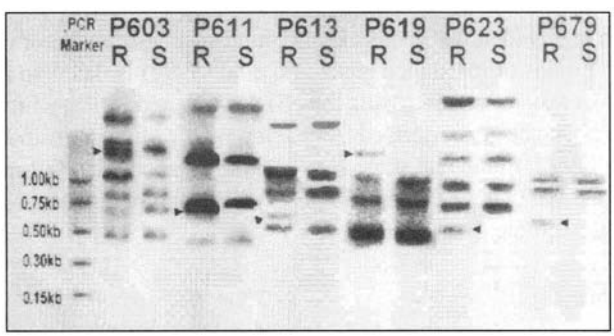

Fig. 1. RAPD patterns of H7R and H7S. PCR was made at 94 °C, 30 sec; 36 °C, 2 min for 40 cycles, then 72 °C, 10 min. PCR products were analyzed on 2% agarose gel. The special DNA products (with arrow) are shown, appearing only in H7R, derived from six primers. The PCR DNA marker is shown at the left.

of the NIL pair were highly uniform in genetic background. One of these fragments might be linked to resistance gene *Pi-rl(t).* Further identification and cloning of RAPD markers are underway with the F_2 population of H7R/H7S.

Pathogenesis-induced cDNA fragments in both lines

Most of the DD assays showed 100-150 cDNA bands on 6% denatured or nature polyacrylamide gel. Ten pathogenesis-induced (PI) cDNA fragments were found to appear in both lines 8-24 h after inoculation, with 20 pairs of the anchored primers and arbitrary primers. Among cDNA fragments, PI-3 and PI-8 were observed to appear stably in the resistant and susceptible lines 16-24 and 8-12 h after inoculation. The fragments were strongly induced by challenging of the pathogen and may have a role in defense response. PI-3 and PI-8 were about 500 and 200 bp. Northern blot analysis and characterization of the fragments are being conducted.

We could not find any differential cDNA after 2 d of inoculation. Whether or not gene induction happens at an early period of the interaction between rice and *M. grisea* is an interesting question.

cDNA fragments relative to resistance response

Eight cDNA fragments were obtained from the 20 combinations of DD assays, which appeared only in H7R infected for 8-24 h. It may be the resistance-related (RR) cDNA induced by the pathogen. The primer pair of T11GC and CTTGATTGCC resulted in six RR cDNAs from 180 to 400 bp. Among them, RR3, RR4, RR5, and RR7 appeared in H7R 8, 16, and 24 h after infection. RR6 and RR8 appeared only in H7R 24 h after infection. It was possible that these cDNAs were derived from or regulated by the gene *Pi-rl(t).* Three RR cDNA fragments—RR3, RR4, and RR5—were reamplified. Additional Northern blot and cloning of fragments are under way.

Discussion

The NILs with highly identical genetic background are considered promising materials for molecular studies of resistance genes. Lu et al (1994) isolated an RAPD marker linked with blast resistance gene using the NILs K80R and K79S, which we selected. H7R(S) with resistance genes derived from Tetep will facilitate our studies. In another study, He et al (1992) found that some special proteins appear in H7R inoculated with the blast fungus. We believe that mRNA DD will reveal potential resistance-related genes and then facilitate their cloning.

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Genetic and molecular analysis of a cultivar specificity locus from the rice blast fungus *Magnaporthe grisea*

S.A. Leong, M.L. Farman, and N. Nitta

A *Magnaporthe grisea* cultivar specificity gene toward rice cultivar CO 39 was previously mapped to one arm of chromosome 1 between restriction fragment length polymorphism (RFLP) markers CH5-120H and 5-10-F. These RFLP markers map 11.6 and 17.2 cM, respectively, on either side of *AVRCO39.* Using Achilles' cleavage methods, CH5-120H and 5-10-F were shown to be separated by 600 kb. A chromosome walk to *AVRCO39* was initiated from these flanking RFLP markers. More than 500 kb have been covered in 20 walking steps. The DNA conferring avirulence has been delimited to a 7.2-kb region. Preliminary hybridization studies on DNA of the virulent *M. grisea* strain Guy11, using portions of the 7.2-kb region, suggest that an alternate is not present, or if present, has undergone extensive mutation.

Flor (1955) proposed the gene-for-gene hypothesis to describe the relationship of races of the flax rust and cultivars of its flax host. In its simplest form, this hypothesis states that for every resistance gene in the host plant, there exists a complementary avirulence (cultivar specificity) gene in the pathogen that allows the host to recognize the pathogen and resist development of the diseased state. Since its first proposal, the gene-for-gene hypothesis has been found to be applicable to many host-pathogen interactions including that of the rice blast fungus *Magnaporthe grisea* and its host *Oryza sativa* (Valent et al 1991, Silue et al 1992, Lau et a1 1993, Smith and Leong 1994) as well as subspecific groups of *M. grisea* and their respective hosts (Kato 1983, Valent et al 1986, Valent and Chumley 1994). This fundamental relationship is of great practical interest as *M. grisea* is rapidly able to overcome new disease resistances in rice soon after their deployment (Bonman et al 1992). Moreover, *M. grisea* exists as a complex genus with numerous subspecific groups that are interfertile but differ in their host range (Valent and Chumley 1991). How these different subspecific groups interrelate evolutionarily is of great concern as some of these alternate hosts are frequently found growing in close proximity to or in rotation with

rice, and *M. grisea* isolates infecting these alternate hosts can sometimes also infect rice (Kato 1983, Mackill and Bonman 1986, Iwamoto et al 1992).

The molecular basis of host/cultivar specificity and pathogenic variability in *M. grisea* is only beginning to be understood with the recent cloning of the avirulence genes *AVR2-YAMO* (cultivar specificity) and *PWL2* (host specificity) from rice pathogenic isolates of *M. grisea* (Valent and Chumley 1994). Both genes function as classical avirulence genes (Flor 1955) by preventing infection of a specific cultivar or host. *AVR2-YAMO* encodes a 223-amino acid protein with homology to proteases while *PWL2* encodes a 145-amino acid polypeptide that is glycine-rich. Based on the predicted amino acid sequence of the proteins, both gene products could be secreted. Homologs of both genes appear to be widely distributed in rice as well as in grass-infecting isolates of *M. grisea* (Valent and Chumley 1994, Zeigler et al 1995) and confirm the prediction obtained through genetic analysis that *M. grisea* isolates infecting monocots other than rice contain cultivar specificity genes for rice (Yaegashi and Asaga 1981, Valent et al 1991, Valent and Chumley 1994) as well as host specificity genes that preclude infection of other hosts (Yaegashi 1978, Valent et al 1986), while rice pathogenic isolates of *M. grisea* contain cultivar specificity genes for rice and other grass hosts (Iwamoto et al 1992) as well as host specificity genes that prevent infection of other monocot hosts (Valent and Chumley 1994).

In some cases, homologs of *AVR2-YAMO* and *PWL2* have been shown to be functional and to exhibit the same host or cultivar specificity as *AVR2-YAMO* or *PLW2* (Valent and Chumely 1994). Whether other alleles confer new host/cultivar specificities will be important to learn. By analogy, homologs of the corresponding disease resistance genes may specifiy novel resistances that are recognized by these cultivar and host specificity gene alleles. The availability of cloned cultivar and host specificity genes from *M. grisea* and the corresponding disease resistance genes provides an experimental avenue to test this hypothesis. Toward this end, we describe here our efforts to clone the *M. grisea AVRCO39* locus and to identify its corresponding resistance locus in rice cultivar CO 39.

Cloning of a locus controlling cultivar specificity on CO 39

High-density genetic map of *M. grisea*

The genetic map of *M. grisea* 2539/Guy11 was improved by the addition of 87 new cosmid-derived restriction fragment length polymorphism markers to the map of Farman and Leong (1995). The current map consists of 203 markers representing 132 independently segregating loci and spanning approximately 860 cM with one telomere unlinked (N. Nitta, M. Farman, and S.A. Leong, unpubl. data). This value is in good agreement with the map sizes reported by Romao and Hamer (1992) and Sweigard et al (1993). Thirty of the new markers were single-copy DNA isolated from cosmids containing the retrotransposon MAGGY. These were randomly distributed in the genome. Thirty-three of the new markers were derived from the map of Sweigard et al (1993), which has permitted integration of the two maps (N. Nitta, M. Farman, S.A. Leong, unpubl. data). The integrated maps of chromosomes 1 and 2 are shown in Figure 1.

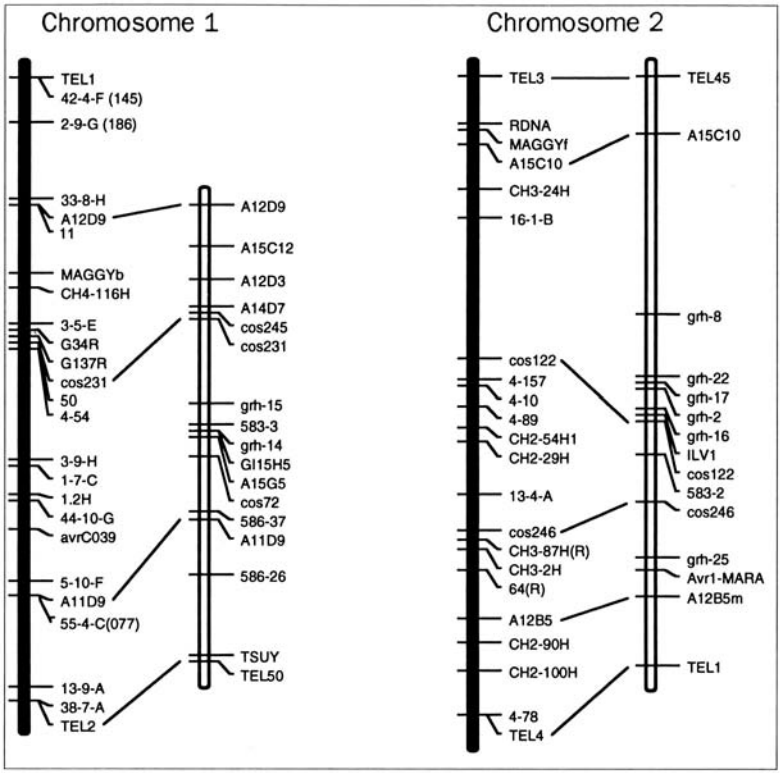


Fig. 1. Integrated maps of chromosomes 1 and 2 of *M. grisea.* The black chromosomes are from the maps in the authors' USDA laboratory and the white chromosomes are from the maps of Sweigard et al (1993). To simplify the representation, only the 6043 genome of the translocation chromosomes from the map of Sweigard et al is depicted. 1 cm = 10 cM.

Identification and genetic mapping of *AVRCO39*

Guy11, 2539, and 101 progeny from a cross of these strains were inoculated onto rice cultivars CO 39 and 51583. Both parents and progeny were pathogenic on 51583 (Smith and Leong 1994). On CO 39, Guy11 was virulent and 2539 was avirulent; avirulence was found to segregate 1:1 among the progeny tested. Further inoculation studies involving progeny of test backcrosses and sibcrosses indicated that a single gene conferring avirulence was segregating. The gene specifying avirulence on CO 39 was designated *AVRCO39* and shown to map on one arm of chromosome 1 (Fig. 1).

Chromosome walk to *AVRCO39*

A chromosome walk was initiated from markers 1.2H and 5-10-F. Achilles' cleavage (Koob et al 1993) was used to assess the physical extent of the walk. Cleavage of the

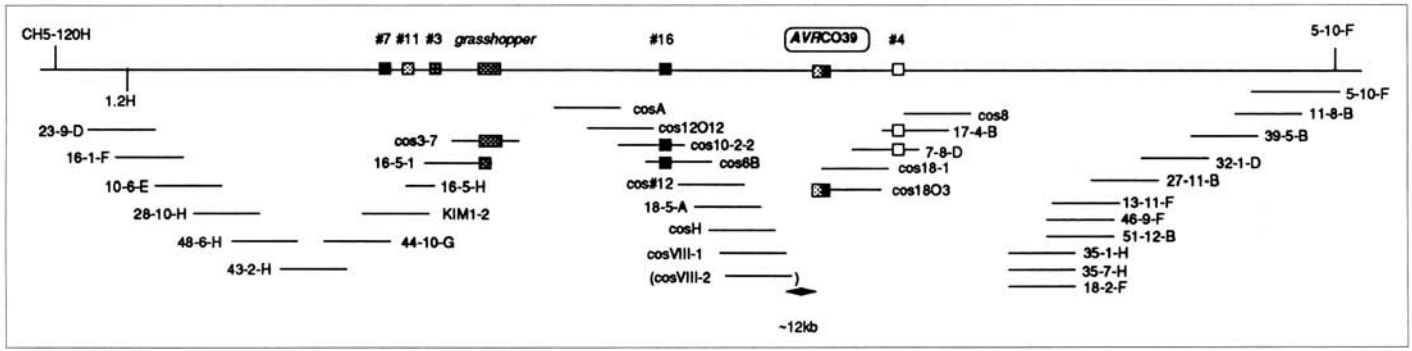


Fig. 2. Progress of the chromosome walk to *AVRCO39*. The locations of five subclones (#3, 4, 7, 11, and 16) of the 280-kb fragment released by Achilles' cleavage at cosmids 43-2-H and 18-2-F are shown in the small boxes. The position of the grasshopper element is also shown. *AVRCO39* is located on cosmid 1803 between subclones #4 and #16.

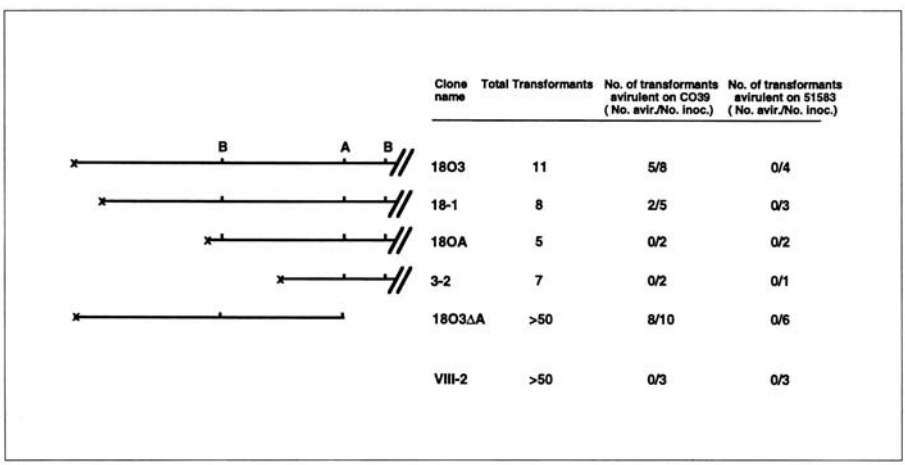
genome at these markers released an intervening fragment of around 600 kb. The strategy and progress of the chromosome walk to *AVRCO39* are represented in Figure 2.

Identification of the corresponding virulence locus in Guy11

A cosmid library of Guy11 was constructed in the vector pMLF2, a new version of cosmid pMLF1 (Leong et al 1994) that allows use of the half site fill-in method to clone genomic DNA. A partial library was screened with an endclone from cos1803. This probe did not hybridize with a reference lane of Guy11 genomic DNA. This observation suggested that Guy11 has deleted part of the *AVRCO39* locus, that the sequence has diverged rapidly, or that a novel foreign DNA has inserted into the region in Guy11. Hybridization of the endclone of 1803 with DNA of progeny used to map *AVRCO39* confirmed that the deletion cosegregated fully with the virulent phenotype (data not shown).

Transformation of candidate cosmids into Guy11

The results of inoculation of transformants carrying the putative cloned *AVRCO39* locus onto CO 39 are shown in Figure 3. Only cosmid 1803, its derivative 18O3ΔA, and 18-1 were able to convert Guy11 from virulence to avirulence to rice cultivar CO 39. The virulence of these transformants toward rice cultivar 51583 was unaffected. Not all the 18O3ΔA transformants were avirulent. This is not surprising as the incoming DNA must integrate into the chromosome to be stably maintained and expressed in the transformants.



Clone name	Total Transformants	No. of transformants avirulent on CO39 (No. avir./No. inoc.)	No. of transformants avirulent on 51583 (No. avir./No. inoc.)
18O3	11	5/8	0/4
18-1	8	2/5	0/3
18OA	5	0/2	0/2
3-2	7	0/2	0/1
18O3ΔA	>50	8/10	0/6
VIII-2	>50	0/3	0/3

Fig. 3. Summary of results of inoculation of *M. grisea* transformants on CO39. Guy11 was transformed with five cosmid clones and one deletion derivative of cosmid 1803. The total number of transformants generated with each clone is shown in the first column. The second and third columns show the results of the inoculation experiments performed to date.

Genetic analysis of resistance of rice cultivar CO 39 to strain 2539 *(AVRCO39)*

To determine whether the interaction of *M. grisea (AVRCO39)* with rice represents a gene-for-gene system, we studied the inheritance of resistance to *M. grisea* in a cross of rice cultivars CO 39 (R) and 51583 (S). The F_2 seeds were screened for resistance to strain 6082 *(AVRCO39).* From our genetic studies of the pathogen (Smith and Leong 1994), we anticipated that the F_2 will show a 3:1 (R:S) segregation resistance to 6082 assuming that resistance is dominant and the parents are homozygous for R or S. This expectation has been met.

Prospective

With *AVRCO39* in hand, we have the opportunity to better understand the molecular cues that illicit resistance to blast disease. Interestingly, indica cultivar CO 39 is considered to be highly susceptible to blast disease and is used as a susceptible host for genetic analysis of both major and minor gene resistance to blast in the Philippines (Wong et al 1994). CO 39 has shown resistance to only a limited number of lineages of *M. grisea* isolates attacking rice (Smith and Leong 1994, Zeigler et al 1995). Thus *AVRCO39* may have been mutated to nonfunction or to the corresponding *VIRCO39* in the majority of rice-pathogenic isolates of *M. grisea* in the Philippines. It will be important to learn what the molecular nature of the corresponding locus is like in the virulent strain Guy11 as well as determine the structure of the corresponding locus in the avirulent and virulent isolates of *M. grisea* representing the different lineages of rice pathogens in the Philippines (Zeigler et al 1995). It will be interesting to determine whether genetic resistance to *AVRCO39* is widespread in rice varieties employed today and historically in rice culture in the Philippines as well as in alternate hosts of *M. grisea.* Such an analysis will provide a better understanding of the evolution of this gene in nature. Finally, the availability of a cloned copy of *AVRCO39* avails us the opportunity to explore the development of transgenic plants having broad spectrum resistance to rice blast disease.

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Rice tungro spherical virus: expression of the 3' nonstructural proteins

V. Thole and R. Hull

This study characterized the genomic information of the rice tungro spherical virus (RTSV) and investigated the variations of the different virus isolates. The 3' terminal half of the genomic RNA of a Philippine isolate and the 3' ends of a Thai and an Indian isolate of the RTSV genome were analyzed. Polyclonal antisera were separately raised against the putative protease, the N-terminal part of the RNA-dependent RNA polymerase, and against two peptides of two small open reading frames with unknown functions for identification of the nonstructural proteins in infected rice plants. Polyprotein processing was investigated with in vitro transcription/translation assays.

Rice tungro disease is caused by a coinfection of rice tungro spherical virus (RTSV) and rice tungro bacilliform pararetrovirus (RTBV). In this complex, RTSV enables the leafhopper transmission of both viruses and RTBV is responsible for severe disease symptoms in infected rice plants.

The plant picornavirus of RTSV comprises a single-stranded RNA genome of approximately 12 kb. The entire sequence of a Philippine isolate of RTSV was published by Shen et al (1993). The genome encodes one long open reading frame (ORF) encoding a large polyprotein of about 390 kDa, which is probably cleaved into smaller polypeptides by viral encoded protease(s). The large ORF contains three coat proteins (CPs), motifs for a neomycin phosphotransferase-binding protein, a protease, an RNA-dependent RNA polymerase, and possibly proteins of unknown function (Fig. 1). Downstream of the large polyprotein, two small ORFs (sORF1 and sORF2) are located, which are thought to be expressed from subgenomic RNAs. The expression of sORFl would involve a +1 frameshift and result in a product of 11 kDa; sORF2 could encode a peptide of 9 kDa (Shen et al 1993). However, it is still unknown whether these proteins are expressed in vivo.

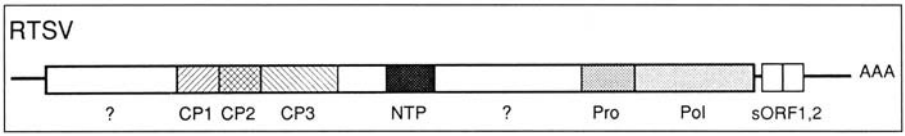

Fig. 1. The genome organization of RTSV. The genome encodes, as part of a large polyprotein, three coat proteins (CP1, CP2, and CP3), motifs for an NPT-binding protein (NPT), a protease (Pro), a polymerase (Pol), and proteins with unknown function. At the 3' end of the genome, two small open reading frames (sORF1 and sORF2) are expressed (Shen et al 1993) and a poly (A) tail is located.

Materials and methods

To investigate the genomic information of RTSV and to study the variation between different rice tungro isolates, the 3' half of the genomic RNA of a Philippine isolate and the 3' ends of a Thai and an Indian isolate of the RTSV genome were cloned and sequenced; polyclonal antisera were raised against nonstructural proteins as fusion proteins with glutathione-S-transferase, and polyprotein processing was studied with in vitro transcription/translation assays.

Results and discussion

Molecular cloning and sequencing of the 3' terminal half of the RTSV genome of the Philippine isolate revealed an overall nucleotide sequence similarity of 93% with the published complete sequence of the Philippine isolate of Shen et al (1993). The 3' ends of the putative protease and the predicted polymerase show a greater nucleotide sequence diversity (11-15%) but these clustered nucleotide variations only result in a few amino acid changes at both C termini (99% homology). Therefore, the sequence analysis displays a significant nucleotide sequence variation of virus isolates within one geographical area. However, the amino acid sequence diversity is very low and this enabled us to find the appropriate target sequences for conferring resistance against different virus isolates.

Almost the entire putative protease and a part of the 5' end of the predicted polymerase were expressed as an N-terminal fusion with glutathione-S-transferase in the pGEX system in *Escherichia coli.* Polyclonal antisera were separately raised against the fusion proteins in rabbits and each antiserum reacts specifically with the relevant fusion protein. These antisera were used to detect these four proteins in infected rice plants, leafhoppers, and in virus preparations by enhanced chemiluminescent and chromogenic detection assays. Plant materials were analyzed as crude extracts and were fractionated or concentrated by ammonium sulfate, acetone, and/or immunoprecipitation. Rice plants were examined from different stages of infection and samples were checked for infectivity by RTSV CP antisera (Druka et al, unpubl. data). The antiserum raised against the protease detected a protein of about 35 kDa in crude extracts of infected rice plants (V. Thole et al, unpubl. data). The antiserum

raised against an N-terminal part of the polymerase detected only very faintly a protein of about 70 kDa in infected leaves (data not shown).

Polyprotein processing was studied with in vitro transcription/translation assays using rabbit reticulocyte lysate and wheat germ extracts. It was shown by several constructs containing regions of the C terminus of the polyprotein that processing occurs and a consistent product of 35 kDa was identified, which comigrated with the protease found in infected rice plants. Based on sequence similarity, this protein resembles the picornaviral 3C protease, the comoviral 24 K protease, and the potyviral NIa (nuclear inclusion body a) protease and has several characteristics in common with cellular serine proteases. This was confirmed by immunoprecipitation, complementation assays, and mutational analysis (V. Thole et al, unpubl. data).

The nucleotide sequence comparison of the two Philippine isolates downstream of the polyprotein showed major differences. A direct repeat of 255 nucleotides in the 3' noncoding region of the published sequence was not found in the 3' untranslated region of our Philippine isolate. Sequencing sORF1 from several independent cDNA clones from the Philippine isolate revealed a stop codon in the middle of sORF1. The presence of this additional stop codon was confirmed by a direct RNA sequencing of the Philippine isolate and by analyzing the sequence of this region from a Thai and an Indian isolate. The latter two each contained another additional stop codon in the sORF1, one of these was found in a clone of the Philippine isolate as well.

Antisera were raised against fusion products containing sequences of the two sORFs and used for identification of these peptides in the infected rice plants. In these assays, no specific proteins were detected in the extract of the infected plants. This might be a result of the low concentration of the proteins in the infected plant or probably, the proteins were expressed only during a short period of time. Another probable reason is that the proteins were expressed in a compartment of the cell which complicated the extraction of the proteins. However, the additional stop codons found in the region of sORFl would only allow the expression of a 5-kDa peptide provided a frameshift at the 5' end of sORFl occurs in vivo. Furthermore, the nucleotide sequence of the 3' end of RTSV contains several other sORFs of similar size (5-6 kDa) with the exception of sORF2 which could encode a 9-kDa peptide.

These results suggest that sORF1 does not express an 11-kDa peptide in the infected rice plants. Picornalike viruses have a 3' nontranslated region varying in size from 35 to 350 nucleotides. Therefore, if neither sORF was expressed, the 3' noncoding region of RTSV of about 1,240 nucleotides would be unusually long and to our knowledge, several viruses contain a very long noncoding region (larger than 1,000 nucleotides) only at their 5' ends. The 3' terminal region of RTSV shows a strong secondary structure, the formation of stem-loop structures could play a role in the viral RNA replication. Further investigations are necessary to shed light on the genome organization of the 3' end of RTSV.

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Investigation of the leader sequence and upstream genes of rice tungro spherical virus

A. Druka, S. Zhang, Y. Yan, T.M. Burns, and R. Hull

Rice tungro disease, the most severe viral disease of rice in South and Southeast Asia, is caused by a complex of two viruses, rice tungro spherical virus (RTSV), which provides the leafhopper transmission characters, and rice tungro bacilliform virus, which contributes most of the symptoms. RTSV has been classified as a probable plant picornavirus and has a polyadenylated RNA genome of approximately 12 kb. The sequence of RTSV suggests that most of the coding information is for a polyprotein of 393 kDa starting at nucleotide 515. Its leader sequence upstream has been investigated to ascertain the first nucleotide assuming a covalently linked protein. There is a possibility of an extra U residue. The leader sequence itself has features that resemble those that effect translational control and replication of picornaviruses. The positions of the three coat protein (CP) species in the polyprotein have been mapped, showing a possible protein (Pl) about 70 kDa upstream of CP1. An antiserum against P1 recognizes a protein of about 40 kDa in membrane-rich fractions from infected plants. Individual antisera, raised against the three CP species, have been used to investigate the properties of these proteins in both sap extracts and purified virus preparations.

Rice tungro disease, the most serious viral disease of rice in South and Southeast Asia, is caused by a complex of two viruses. Rice tungro spherical virus (RTSV) causes few symptoms on its own and is transmitted by rice green leafhoppers, primarily *Nephotettix virescens.* Rice tungro bacilliform virus (RTBV) causes severe symptoms but is only leafhopper-transmitted in the presence of RTSV. Thus, in the disease, RTBV causes most of the symptoms and provides the leafhopper transmission characteristics.

RTSV has a single-stranded polyadenylated RNA genome (Jones et al 1991) encapsulated in three coat protein (CP) species (Shen et al 1993, Zhang et al 1993).

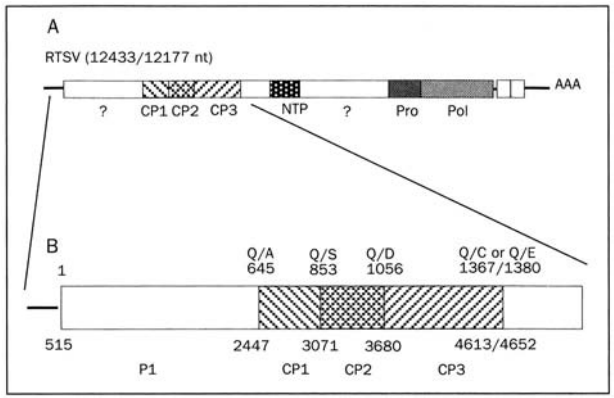

Fig. 1. Genome organization of RTSV (based on Shen et al 1993). A) The overall genome organization with the RNA indicated as a thick line and the coding regions as boxes. Features of the polyprotein are indicated: CP1, CP2, and CP3 are the three coat proteins, NTP is the NTP binding motif, pro and pol are the cysteine protease and RNA-dependent RNA polymerase motif region, respectively. B) Details of the 5' part of RTSV genome. The numbers above and below the polyprotein are the amino acid number and nucleotide number, respectively; the cleavage sites for the coat proteins are indicated.

The genome organization of RTSV (Fig. 1) from the published sequence (Shen et al 1993) shows that it encodes a large polyprotein of 393 kDa and has two short open reading frames at the 3' end. Various motifs have been found in the large polyprotein, which suggest functions for its products. Among these are a putative cysteine protease and an RNA-dependent RNA polymerase. These properties of RTSV show that it has many features in common with picornaviruses (Shen et al 1993, Zhang et al 1993).

Leader sequence

The 5' end

Like the picornaviruses and plant viruses that encode a large polyprotein and have a 3' poly(A) sequence, RTSV might also have a covalently linked protein (VPg) at the 5' end. To determine if the putative VPg hid any nucleotides 5' to the published 5' nucleotide, clones were made using the 5-RACE procedure from RTSV RNA which had been treated with proteinase K and compared with clones from untreated RNA. cDNA was synthesized, tailed with dCTP or dATP, double-stranded DNA generated, and the resultant clones sequenced. Of the ten clones selected for sequencing whose RNA template had been treated with proteinase K, only one had an additional nucleotide (U) to that published for the 5' end (Shen et al 1993).

Nontranslated region

Picornaviruses are characterized by long 5' nontranslated regions (NTR) that have at least two unusual features. First, there are several AUG translation start codons upstream of the start codon of the polyprotein which, if RNA were translated by normal scanning mechanism, would interfere with the translation of the polyprotein. Cap-independent translation of picornaviral RNA initiates at elements termed "internal ribosome entry sites" (IRES) (Wimmer et al 1993). These comprise several stem loops, including one that is branched and a pyrimidine-rich tract separated from an AUG codon by a spacer of 15-20 nucleotides giving a motif of YnXmAUG (Fig. 2). In type 2 IRES elements (e.g., EMCV), the AUG is the initiation codon; in type 1 elements (e.g., poliovirus). this AUG is cryptic and initiation occurs 19-154 nucleotides downstream at the next AUG codon. RTSV has a leader sequence of at least 514 nucleotides up to the first AUG codon for the polyprotein. This leader sequence contains seven other AUG

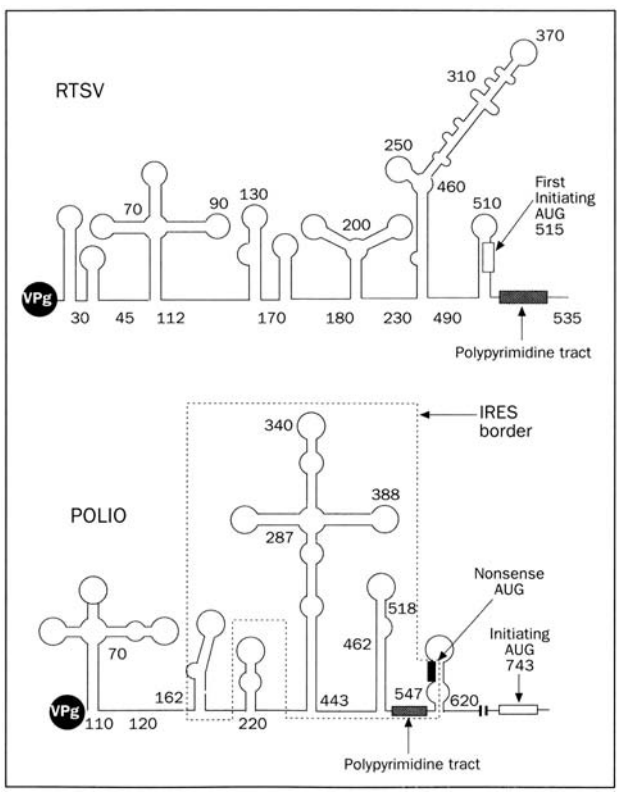

Fig. 2. Predicted foldings of the 5' nontranslated regions of RTSV and poliovirus genomes. Folding was done using the MFOLD and SQUIGGLES program of the UWGCG package. The VPg is indicated at the 5' end; that of RTSV has not been verified. The poliovirus IRES is indicated by the dotted box and other features mentioned in the text are shown.

codons, each followed closely by a stop codon. Thus, it resembles picornaviral leader sequences and the resemblance goes even further with the predicted folding that shows several stem-loop structures (Fig. 2). RTSV has a marked polypyrimidine tract (523-CUUUCUUCUUUC) (Fig. 2) but, unlike the picornaviruses, this is just after the polyprotein AUG codon. In spite of this, the structure of this part of the leader sequence is suggestive of an IRES function.

The second feature is that the 5'-terminal 100 or so nucleotides of the picornaviral NTR are involved in viral replication (Adino et al 1993) and there is strong evidence that the viral polymerase of poliovirus interacts with the cloverleaflike structure at the 5' end of the NTR (Harris et al 1994) (Fig. 2). It is interesting to note that the predicted structure of the RTSV NTR resembles that of poliovirus in having a cloverleaflike structure at the 5' end.

Coding region

P1

The N terminus of coat protein 1 (CP1) is located at nucleotide 2447, which is 1,932 residues downstream of the first AUG codon for the polyprotein (Fig. 1). This means that there is potentially a protein of 644 amino acids (about 70 kDa) upstream of CP1. To investigate this putative protein (termed P1), the cDNA of the sequence from nucleotide 515 to 2427 was cloned in the pMAL vector to create a fusion protein with the maltose-binding protein. The fusion protein was expressed in *Escherichia coli,* purified, and an antiserum raised against it. This serum recognized a protein of about 40 kDa in infected plants. Fractionation studies of extracts from infected plants indicated that the protein is found in a high-speed (30,000x g, 30 min) pellet fraction which suggests that it might be associated with membranes. This protein is much smaller than expected and could result from processing of P1 or translation initiation at a downstream AUG. The next AUGs are at nucleotides 1088 and 1106, neither of which is in a good context for initiation of translation (Lutcke et al 1987), whereas that at 515 is in a reasonably good context.

Coat proteins

RTSV has three CP species processed from the polyprotein (Shen et al 1993, Zhang et al 1993). N terminal analyses showed that they were cleaved at Q/A, Q/S, and Q/D for the N termini of CP1, CP2, and CP3, respectively; the C terminus of CP3 has been presumed from its size (33 kDa) and a Q/C or Q/E site close to the predicted site. The coding regions for CP1 , CP2, and CP3 were each cloned in pMAL, the fusion proteins expressed in *E. coli,* and antisera were raised against them. These sera were used to probe blots of proteins from purified virus preparations and sap extracts from plants infected with a Philippine isolate of RTSV. The sera to CP1 and CP2 identified proteins of the expected sizes (about 23 and 22 kDa, respectively) in both purified virus and sap. CP3 antiserum revealed a band of about 33 kDa in the purified virus while bands of about 45, 47, and 49 kDa were identified in the sap extracts. Since these larger bands were not recognized by CP2 antiserum, they could be due to either CP3 not

being processed at the predicted C terminus or CP3 being modified. An antiserum raised against the protein downstream of the presumed C terminus of CP3 (from nucleotide 4598 to 4963) did not recognize the larger bands, suggesting that the protein found in the sap was modified. Cellulase (celluclast) is used in virus preparation and treatment of extracts that contained the 45-49 kDa CP3 species reduced the size to 33 kDa. Considerable attempts have been made to identify the nature of the modification as this might have some significance in the use of RTSV CP in transgenic protection. The proteins show up well in Western blotting but are not stained easily with Coomassie blue or silver stain. They do not react to Schiff's reagent or with lectins such as concanavalin A. They are not recognized by an antiserum to ubiquitin and do not appear to be complexed with fatty acids or nucleic acid. Work is continuing to elucidate the cause of this phenomenon.

On comparing the reactions in Western blots of the three CP antisera with one raised against the whole virus, it was shown that CP3 is the most immune. This was confirmed by electron microscopy of purified virus preparations immunogold-labeled with the three CP antisera.

Conclusion

We have noted many similarities between the 5' part of the RTSV genome and the picornaviruses. These similarities would help us identify research approaches that can be taken to understand the expression and replication of RTSV and that will provide information for developing nonconventional approaches to resistance. The similarities raise various questions concerning the evolution of these viruses. Since RTSV does not infect its insect vector and is thought to only interact with the exoskeleton during its transmission. there is no obvious link between the animal-infecting picornaviruses and the plant-infecting RTSV.

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Negative regulation of an elicitor-responsive *cis*-element by a labile repressor

Q. Zhu, T. Dabi, and C. Lamb

A rice gene encoding phenylalanine ammonia lyase (PAL), designated ZB8, is induced in suspension-cultured cells by elicitors from the cell wall of *Magnaporthe grisea.* Using in vitro transcription initiation and in vivo functional analysis, we have dissected the architecture of the ZB8 promoter. A silencer and a general enhancer are located between -435 and -302 and between -302 and -174, respectively, relative to the transcription initiation site at +1. The minimal promoter for ZB8 gene is determined. Accurate in vivo and in vitro basal level transcriptions of ZB8 gene are dependent on both the TATA box and initiator element. It has been confirmed that the elicitor response of ZB8 gene is transcriptionally regulated. The ZB8 gene is also induced by protein synthesis inhibitors such as cycloheximide. This cycloheximide-mediated induction is not a result of increased RNA stability or reduced rate of RNA turnover but a reflection of transcriptional activation. The elicitor- and cycloheximide-responsive element(s) are colocalized in the same region (-54 to -35) proximal to the TATA box. The ZB8 gene is superinduced by the treatment of fungal elicitors together with cycloheximide. Gel retardation assays reveal a DNA-protein complex which disappears upon addition of cycloheximide. These data strongly suggest that there is a labile repressor(s) involved in the regulation of the elicitor responsive element of the PAL ZB8 gene.

Phenylalanine ammonia lyase (PAL) is the key enzyme in controlling metabolic flux into phenylpropanoid pathway which leads to the synthesis of certain important molecules such as phytoalexins and lignin monomers (Dixon et al 1983). PAL genes are induced during plant hypersensitive response to pathogen attacks and in suspension cells treated with elicitors from the cell walls of the fungus ***Magnaporthe grisea.*** In all cases so far studied, the activation of PAL genes are at the level of transcription. PAL promoters directed expression of reporter genes and reflected the expression

patterns of their endogenous PAL genes, suggesting that the *cis*-elements responsible for stress induction are located in PAL promoters. The signal transduction pathways from elicitor to the transcriptional activation of PAL genes are largely unknown (Dixon et al 1994, Lamb et al 1989).

We are interested in the signal transduction between elicitor and transcriptional activation of the rice PAL gene. In this paper, we describe the isolation, characterization, the minimal promoter definition, and the regulation of the ZB8 gene. We studied the interaction of transfactors with elicitor responsive *cis*-elements and demonstrated that negative regulation is involved in the elicitor activation of ZB8 gene.

Isolation and characterization of a gene encoding phenylalanine ammonia lyase

PAL genomic sequences were isolated from a rice (*Oryza sativa* L.) genomic library using a polymerase chain reaction-amplified rice PAL DNA fragment as a probe. There is a small family of PAL genes in the rice genome. The nucleotide sequence of one PAL gene, ZB8, was determined. The ZB8 gene is 4660 bp in length and consists of two exons and one intron. It encodes a polypeptide of 710 amino acids. The transcription start site was 137 bp upstream from the translation initiation site. Rice PAL transcripts accumulated to a high level in stems, with lower levels in roots and leaves. Wounding of leaf tissues induced ZB8 PAL transcripts to a high level. In rice suspension-cultured cells treated with fungal cell wall elicitors, the ZB8 PAL transcript increased within 30 min and reached maximum levels in 1-2 h. The transcription of the ZB8 gene was investigated by fusing its promoter to the reporter gene b-glucuronidase (GUS) (Jefferson et al 1987) and transforming the construct into rice and tobacco plants, as well as rice suspension-cultured cells (Li et al 1993). High levels of GUS activity were observed in stems, moderate levels in roots, and low levels in leaves of transgenic rice and tobacco plants. Histochemical analysis indicated that in transgenic rice, the promoter was active in root apical tips, lateral root initiation sites, and vascular and epidermal tissues of stems and roots. In rice flowers, high GUS activity was observed in floral shoots, receptacles, anthers and filaments; GUS activity was also detected occasionally in lemma and awn tissues. In tobacco flowers, high GUS activity was detected in the pink part of petals. Consistent with the activity of endogenous PAL transcripts, wounding of rice and tobacco leaf tissues induced GUS activity from low basal levels. Tobacco mosaic virus infection of tobacco leaves induced GUS activity to a high level. Fungal cell wall elicitors strongly induced GUS activity and GUS transcripts to high levels in transgenic rice suspension-cultured cells. We demonstrated that the promoter of ZB8 gene is both developmentally regulated and stress-inducible (Zhu et al 1995a).

Minimal promoter definition of the ZB8 PAL gene

The functional architecture of the proximal region of the ZB8 PAL promoter was analyzed by transcription of PAL promoter/GUS fusion templates both in vitro and in

vivo. The in vitro transcription system consists of incubating template and whole cell extract to generate initiation complexes, and addition of nucleotide triphosphates to support elongation, followed by primer extension assay to detect authentic transcripts (Zhu et al 1995b). Accurate in vitro transcription of PAL promoter/GUS fusion has been examined in rice whole cell extracts, and the optimal ranges for several reaction components, including DNA template, whole cell extract, mono- and divalent cations were determined with specific initiation from the in vivo start site. Three to four rounds of RNA polymerase II-mediated transcription were initiated in standard assays. The in vivo transcription system was developed to use transgenic rice suspension-cultured cells. The expression of PAL promoter/GUS fusion in transgenic suspension-cultured cells treated with elicitors mimicked the pattern of the endogenous ZB8 gene (Zhu et al 1995a).

We determined the minimal 5' flanking sequence required for in vivo transcription of the ZB8 gene by using the p81, p54, p35, and p20 constructs (5' ended at –81, –54. –35, and –20, respectively) to generate transgenic suspension-cultured cells. The basal transcription of the rice ZB8 PAL gene in unelicited cells is low and therefore poly (A)+ RNA was isolated for primer extension analysis. The levels of ZB8/GUS expression in transgenic cells containing the p81 and p54 gene fusions were higher than in p35 transgenic cells. In p20 transgenic cells, no GUS transcripts were detected, indicating that the ZB8 promoter 5' truncated at –20 was inactive in vivo.

We then examined initiation of transcription by these 5' truncated ZB8 promoters in vitro using the p81 m, p54m, p35m, and p20m (5' ended at –81, –54, –35, and –20, respectively) plasmids as templates for transcription by rice whole cell extracts. Consistent with ZB8 promoter activity in vivo, the promoter 5' truncations to –81, –54, and –35 were accurately transcribed, but the promoter truncated to –20 was inactive. While, as in vivo, the promoters extending to –81 and –54 were more active than the 5' truncation to –35, p35m nonetheless gave accurate initiation of transcription in vitro. These data indicate that 5' sequences to –35 are the minimal requirement for transcription initiation both in vivo and in vitro, and suggest that the region between -35 and –21, which contains the putative ZB8 TATA sequence, is important for transcription initiation from this minimal promoter.

We next studied the functional properties of the TATA box and initiator sequence of the ZB8 PAL gene. Substitution of the $_{-34}$TATTTAA$_{-28}$ sequence with GCGGGTT or 2-bp substitutions to give TCGTTAA and TATGGAA inactivated the minimal promoter. Moreover, the function of the TATTTAA sequence was dependent on its position relative to the initiation site. Hence, this element is an authentic TATA box essential for RNA polymerase II-dependent transcription. Substitutions in the-$_{3}$TCCAAG$_{+3}$ initiator element at the –1 (C to A or G) and +1 (A to C or T) residues caused inaccurate initiation, whereas mutations at the other residues of this conserved element or sequence substitutions between the TATA box and initiator had little effect. TATA box and initiator functions were confirmed by analysis of the effects of promoter mutations on expression in transgenic rice cell suspensions. We conclude that the proximal region of the PAL promoter has a simple functional architecture involving a TATA box appropriately positioned upstream of the initiator (Zhu et al 1995c).

Negative regulation of elicitor-responsive elements by a labile repressor

We have demonstrated that the rice ZB8 PAL gene is transcriptionally activated in suspension-cultured cells treated with elicitors from fungal cell walls. The transcription activity of the ZB8/GUS gene fusion reflected the expression pattern of the endogenous ZB8 gene in transgenic rice suspension-cultured cells (Zhu et al 1995a). The elicitor-induced accumulation of GUS transcripts was very rapid; it started to accumulate at 30 min and with maximum level at about 1-2 h after treatment. These data suggested that the induction may occur via preexisting molecules in cell. We then used protein synthesis inhibitors (Galling 1982) to monitor whether de novo protein synthesis is required for the signal transduction between the elicitor and the ZB8 gene expression. Pretreatment of rice suspension-cultured cells with cycloheximide, a eukaryotic cytoplasmic protein synthesis inhibitor, did not inhibit the accumulation of the GUS and the endogenous ZB8 transcripts induced by elicitors, rather, it stimulated the expression of the ZB8/GUS gene fusion and the endogenous ZB8 gene by itself. The cycloheximide-mediated induction was, irrespective of the coding regions, under the control of the ZB8 promoter. This indicates that it operates at the level of transcription.

To address the function of cycloheximide on the induced expression of the ZB8/*GUS* gene fusion, we did several parallel experiments. Studies of effectiveness of cycloheximide on protein synthesis inhibition showed that the cycloheximide effectively inhibits the protein synthesis in rice suspension cells. At a final concentration of 10 $\mu g\ ml^{-1}$, about 95% of protein synthesis was inhibited, indicating that the inhibition of protein synthesis may be mediating the induced expression of the ZB8/GUS gene fusion. The effects of anisomycin, another eukaryotic protein synthesis inhibitor, was nearly as effective as cycloheximide on the induction of ZB8/GUS gene fusion. By using transcription inhibitor actinomycin D to pretreat the suspension cells, we further confirmed that the cycloheximide-induced expression of ZB8/GUS was at the transcription level. RNA stability experiments also showed that the cycloheximide did not affect the decay rate of GUS mRNA in suspension cells. This is in agreement with the observation that cycloheximide did not affect the GUS mRNA stability in transgenic tobacco plants (Lam et al 1989). Superinduction was observed in transgenic suspension cells treated with elicitor and cycloheximide together.

To dissect the elicitor response elements, we used transgenic rice suspension cells with various 5' and internal deletions in ZB8 promoter/*GUS* gene fusions. The regions from –302 to –174 and from –54 to –35 were found to contain elicitor response elements. The cycloheximide response elements were colocated in the same regions. In vitro DNA-protein interaction assays showed that a DNA (–54 to –35)-protein complex tended to disappear in the reactions using nuclear proteins extracted from cycloheximide-treated cells, suggesting that there was a labile repressor(s) involved in the elicitor response of ZB8 promoter. Our results opened up new perspectives in understanding the regulation of plant defense genes in response to elicitor treatment.

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Isolation of a 35-kDa chitinase from suspension-cultured rice cells and its potential in the development of sheath blight-resistant transgenic rice plant

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A glycopeptide elicitor isolated from *Rhizoctonia solani* Kuhn, the rice sheath blight pathogen, was used to search for defense genes in suspension-cultured rice cells. H_2O_2 and superoxide anion were produced in cultured cells within 10 min after elicitor treatment. Lipid peroxidation was observed within 1 h after elicitor application. Lipoxygenase activity increased 6 h after treatment. Phenylalanine ammonia-lyase and peroxidase activity increased within 1 h while 4 coumarate-ligase activity increased within 6 h after treatment. Significant increase in phenolic synthesis occurred 12 h after elicitor treatment. Six pathogenesis-related proteins appeared 24-36 h after elicitor treatment Both ß-1-3-glucanase and chitinase activities increased at 12-48 h after treatment. Western blotting of extracts from elicitor-treated rice cultured cells with bean chitinase antiserum showed three bands with molecular mass of 35, 42, and 56 kDa. Of these, the 35-kDa band was prominent. The chitinase was purified and its isoelectric point was 8.3. The purified chitinase inhibited growth of the pathogen in vitro. A suppressor, which is also a glycoprotein with active carbohydrate moiety, was isolated from the fungal culture. It suppressed the induction of all defense mechanisms induced by the elicitor. Only the elicitor-induced 35-kDa basic chitinase was not suppressed.

Sheath blight, caused by *Rhizoctonia solani* Kuhn, is a serious disease of rice. So far, no resistance gene has been identified (Bonman et al 1992). However, defense genes are common in all plants. Selection of useful defense genes and inducing them to overexpress in plants may help to develop an effective disease-resistant cultivar. Suspension-cultured plant cells and fungal elicitors have been used to detect defense genes in plants (Vera-Estrella et al 1993). Pathogens are known to produce suppressors that suppress the activation of defense genes (Vidhyasekaran et al 1992). The present studies were undertaken to detect elicitor-inducible defense genes in rice-cultured

cells which are least suppressed by the suppressor. Both elicitor and suppressor were isolated from *R. solani.*

Materials and methods

The elicitor was isolated from the cell wall of *R. solani* (Anderson-Prouty and Albersheim 1975). Suspension-cultured rice cells were obtained by the method described by Vidhyasekaran et al (1990). The suspension-cultured cells were treated with the elicitor preparation (50 µg glucose equivalents) and phenolic content of the cells was estimated using standard procedure.

The suppressor was isolated from the culture filtrate obtained by growing the fungus in Richard's medium for 15 d. The culture filtrates were concentrated in vacuo at 50 °C to 10% of their original volume. Methanol was added and stored overnight at 5 °C. Precipitates were removed by filtration. The filtrates were dried, dissolved in water, and treated with activated charcoal at 3% (w/v). The unadsorbed solution was partitioned with three volumes of ethyl acetate; the water fraction was evaporated to dryness. Then, it was dissolved in 2 ml of water, placed on a Sephadex G-75 column, and eluted with distilled water. Five-ml fractions were collected and their ability to induce electrolyte leakage from cultured cells was assessed. The fractions were added to rice suspension-cultured cells at 10 µg ml^{-1} and incubated for 6 h in a shaker. The solutions were washed off from treated cells and then the elicitor (10 µg ml^{-1}) was added. The phenolic content of the cells was assessed 24 h after elicitor treatment. The fraction that suppressed the elicitor-induced phenolic synthesis was used as the suppressor.

To assess the defense gene activation, the elicitor solution (10 µg ml^{-1}) was added to rice cell cultures and various analyses were made at intervals following standard procedures. Chitinases were purified by chitin affinity chromatography and chitinase isozymes were identified by Western blotting. Antifungal activity of purified chitinases was assessed by the method described by Benhamou et al (1993). Suppression of defense mechanisms was assessed by treating the rice-cultured cells with the suppressor 6 h before treatment with the elicitor. Defense-related enzyme activities were assessed at 18 h after elicitor treatment.

Results

An elicitor was isolated from the cell wall of *R. solani.* It was found to be a glycoprotein and the carbohydrate moiety had the elicitor activity. H_2O_2 and superoxide anion were produced in suspension-cultured rice cells within 10 min after elicitor treatment. Lipid peroxidation was observed within 1 h after elicitor application. Lipoxygenase activity increased 6 h after treatment. Elicitor treatment induced an increase in phenylalanine ammonia-lyase (PAL) activity within 1 h after treatment. Significant increase in 4 coumarate-ligase (4CL) activity was detected in cultured cells 6 h after elicitor treatment while significant increase in phenolic synthesis was observed 12 h after treatment (Table 1).

Table 1. Induction of defense-related enzymes and chemicals in suspension-cultured rice cells due to elicitor treatment.

	% increase over untreated control at different time intervals						
	10 min	20 min	30 min	1 h	6 h	12 h	24 h
H_2O_2 production	700	570	484	0	0	0	0
O_2 production	6	25	8	0	0	0	0
Lipid peroxidation	0	0	0	4	25	36	41
Lipoxygenase	0	0	0	3	13	17	21
PAL	0	0	0	8	22	80	67
4CL	0	0	0	0	40	61	52
Phenolics	0	0	0	0	12	56	149

Table 2. Suppression of elicitor-induced defense-related enzymes and chemicals by the suppressor in rice suspension-cultured cells.[a]

Pretreatment (6 h)	Treatment (18 h)	Enzyme activity (n mol^{-1} min^{-1} g^{-1}cells)				Phenolics (µg g^{-1} cells)
		PAL	4CL	b-1,3-glucanase	Chitinase	
Water	Water	1083a	1282a	7.8a	2.4a	1098a
Water	Elicitor	1910b	1940b	11.1b	3.1b	1670b
Suppressor	Water	1031a	1181a	7.8a	2.5a	1045a
Suppressor	Elicitor	1081a	1203a	7.9a	2.5a	1168a

[a]Values followed by the same letter do not differ significantly from each other according to Duncan's multiple range test.

Peroxidase activity increased 1 h after elicitor treatment. New proteins (PR-proteins) accumulated 24-36 h after elicitor treatment. Proteins with molecular weights of 16, 23, 24, 35, 42, and 56 kDa accumulated in the elicitor-treated cells. b-1,3-glucanase activity increased 12-24 h after elicitor treatment while significant accumulation of chitinase was observed 24-48 h after elicitor treatment. However, an increase in chitinase was observed even 6 h after elicitor treatment.

Western blotting of extracts from elicitor-treated cells with bean chitinase antiserum showed three bands with molecular mass of 35, 42, and 56 kDa. Of these, the 35-kDa band was prominent. The elicitor-inducible 35-kDa chitinase was purified from elicitor-treated cells. The purified chitinase had an isoelectric point of 8.3.

The 35-kDa chitinase inhibited growth of the rice sheath blight pathogen in vitro. Morphological changes appeared within 1 h following exposure of mycelium to the purified chitinase. The hyphal tip showed marked swelling and subsequently lysis of hyphal tip was also observed.

A suppressor was isolated from the culture filtrate of *R. solani.* It was also a glycoprotein with active carbohydrate moiety. It suppressed the elicitor-induced accumulation of PAL, 4CL, phenolics, b-1,3-glucanase, and chitinase (Table 2). However, the 35-kDa chitinase band alone appeared in suppressor + elicitor-treated cells.

Discussion

An elicitor and a suppressor have been isolated from *R. solani.* This is the first report of the occurrence of these molecules although a similar occurrence of elicitors and suppressors has been reported in several pathogens (Vidhyasekaran 1993). Early outburst of H_2O_2 and O_2 indicates that they may be involved in signal transduction system (Apostol et al 1989). Lipid peroxidation products are known to be inhibitory to fungal pathogens (Ohta et al 1991) and an increase in lipoxygenase activity has been observed in rice cultured cells due to elicitor treatment. Coordinated induction of PAL and 4 CL, the first and last enzymes of phenylpropanoid main pathway to regulate synthesis of phenolics, is reported for the first time in monocots although a similar induction has been widely reported in dicots (Robbins et al 1985).

Appearance of PR-proteins and induction of b-1,3-glucanase and chitinase in cultured cells due to elicitor treatment have been reported in many hosts (Vidhyasekaran 1993). The suppressor has suppressed all these defense mechanisms. This probably explains the very wide host range of *R. solani* and the absence of resistant varieties in rice. However it is interesting to note the presence of an elicitor-inducible, basic, antifungal chitinase in suppressor plus elicitor-treated cells. Overproduction of this chitinase probably might induce resistance.

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Post-transcriptional regulation of the rice *waxy* gene

Z.Y. Wang, F.Q. Zheng, G.Z. Shen, J.P. Gao, D.P. Snustad, M.G. Li, J.L. Zhang, and M.M. Hong

The waxy (*Wx*) gene of rice encodes a granule-bound starch synthase required for synthesis of amylose in endosperm. As in other cereals, the amylose content of rice endosperm is thought to play important roles in grain yield, palatability, and processing quality. Therefore, we studied the genetic control of starch composition, a vital step in designing breeding and/or genetic engineering strategies aimed at enhancing both yield and eating quality. We used RNA gel blots to compare relative levels of steady-rate *Wx* transcripts in seeds of cultivars with different amylose content. We isolated and characterized a cDNA clone, designated cWx6, equivalent to the 3.3-kb Wx transcript, and the 5' and 3' termini of the cDNA insert on clone cWx6 were sequenced. Results confirm that the extra 1.1-kb segment present at the 5' terminus of the insert of clone cWx6 contains the 5' and 3' terminal sequences of intron 1 of rice *Wx* gene. Based on these, we hypothesize that the amylose content of rice endosperm is regulated at the level of *Wx* transcript processing, and, more specifically, at the stage of intron 1 excision from *Wx* pre-mRNA.

The waxy (*Wx*) gene of rice encodes a granule-bound starch synthase required for the synthesis of amylose in endosperm (Sano 1984, 1985; Villareal and Juliano 1989). We previously cloned and sequenced *Wx* genes from three rice cultivars: *Oryza sativa* subsp. *japonica* Hanfeng cultivar (Wang et al 1990), *O. sativa* subsp. indica 232 cultivar, and *O. sativa* L. f. *spontanea* Dongxiang (wild rice) (Wang et al 1994). The coding region of the *Wx* gene from the Hanfeng cultivar consists of 13 exons interrupted by 12 introns (Wang et al 1990). Subsequently, a large (1,126 bp) intron was identified in the leader region of the rice *Wx* gene.

As in other cereals, the amylose content of rice endosperm is thought to play important roles in grain yield, palatability, and processing quality. Therefore, an understanding of the genetic control of starch composition in rice seeds will be important in designing breeding and/or genetic engineering strategies aimed at enhancing both yield and eating quality of this important food source.

Three distinct patterns of Wx transcripts in different cultivars

Given the role of the *Wx* gene in controlling amylose synthesis in rice endosperm (Okagaki and Wessler 1988, Okagaki 1992) and the variation in amylose content in rice cultivars (Sano et al 1986), we thought that the *Wx* gene might be differentially expressed in various rice cultivars. To investigate this possibility, we used RNA gel blots to compare the relative levels of steady-state *Wx* transcripts in seeds of cultivars with different amylose content. *O. sativa* subsp. *indica* 232 cultivar (amylose content 20%), *O. sativa* subsp. *japonica* Hanfeng cultivar (amylose content 16%), and *O. sativa* subsp. *japonica* Fengnuo cultivar (amylose content 0%; glutinous rice) were used in the initial experiments.

Total RNAs were isolated from developing seeds harvested at 12 d after pollination (DAP). RNA gel blots were prepared, and the RNAs on the blots were hybridized with a ^{32}P-labeled restriction fragment that contains both 5' noncoding and coding sequences of the rice *Wx* gene. The RNA gel blots from the three cultivars exhibited distinct patterns of probe hybridization. The RNA sample from the 232 cultivar contained a single RNA species that hybridized to the *Wx* probe. This RNA was approximately 2.3 kb in size, the predicted size of mature *Wx* mRNA. In the case of the Hanfeng cultivar, the mature *Wx* mRNA and a larger RNA species (3.3 kb in length) were detected. In the Fengnuo cultivar (no amylose). only the 3.3-kb RNA species was present. In addition to the two major hybridizing RNAs described above, a minor hybridizing RNA was present in all three cultivars. The significance of this minor hybridizing RNA is unknown.

The results of the RNA gel blot experiments showed that although the levels of *Wx* transcripts were found to vary during seed development in a cultivar-specific manner, the pattern of transcripts present in a given cultivar did not change during seed development in any of the cultivars examined.

Characterization of the 3.3-kb Wx RNA

The 3.3-kb *Wx* RNA species present in Hanfeng and Fengnuo cultivars is about equal in size to the mature *Wx* mRNA (2.3 kb) plus intron 1 (1.1 kb). To test for the presence of the intron 1 sequences in the 3.3-kb RNA, RNA gel blots of total RNA were probed with *Wx* DNA restriction fragments SBg, BgE, and EBg derived from the left, internal, and right portions of intron 1, respectively. These three intron 1 probes hybridized with the large 3.3-kb RNA species, but not with the mature *Wx* mRNA. These results demonstrate that the 3.3-kb *Wx* RNA species contains major portions of intron 1 and strongly suggest that the 3.3 kb RNA represents a *Wx* transcript from which intron 1 has not been excised.

We isolated and characterized a cDNA clone, designated cWx6, equivalent to the 3.3-kb *Wx* transcript, and the 5' and 3' termini of the cDNA insert in clone cWx6 were sequenced. The results confirm that the extra 1.1 -kb segment present at the 5' terminus of the insert of clone cWx6 contains the 5' and 3' terminal sequences of intron 1 of rice *Wx* gene.

Splicing of intron 1 from Wx pre-mRNA and amylose content in endosperm

To study the effects of splicing of intron 1 from *Wx* pre-mRNA on the amylose content in rice endosperm, an analysis of *Wx* transcripts. *Wx* protein, and amylose content of 31 rice cultivars was carried out. The results revealed that endosperm amylose and *Wx* protein contents are correlated with the ability of the cultivar to excise intron 1 from the leader sequence of the *Wx* transcript. Cultivars with high endosperm amylose content (group I) contain high levels of amylose, *Wx* protein, and the mature 2.3-kb *Wx* mRNA. Cultivars with intermediate amylose content (group II) produce substantial amounts of a large 3.3-kb *Wx* pre-mRNA, with intron 1 still present, in addition to the mature *Wx* mRNA, and intermediate levels of *Wx* protein. Glutinous rice (group III cultivars) contains no amylose, no *Wx* protein, and no mature *Wx* mRNA. Only the incompletely spliced 3.3-kb *Wx* pre-mRNA is present in group III cultivars (Table 1). Based on these results. we hypothesize that the amylose content of rice endosperm is regulated at the level of *Wx* transcript processing, and, more specifically, at the stage of intron 1 excision from the *Wx* pre-mRNA.

Table 1. Levels of *Wx* RNA, Wx protein, and amylose content in 31 rice cultivars.

Cultivar	pre-RNA[a]	mRNA[a]	Wx protein[b]	AC (%)[c]
Group I (indica rice)				
232	0	476	430	20.0
9332	0	458	816	27.8
9225	0	352	697	24.8
92103	0	637	832	27.3
92105	0	287	832	27.7
9248	0	310	832	26.7
9267	0	410	832	26.0
IR36	0	605	816	24.5
Group II (indica rice)				
9223	46	31	33	6.7
9335	14	41	179	12.3
92106	18	16	33	6.8
9344	44	106	35	10.2
9345	55	40	60	10.0
92125	13	27	23	9.2
IR661	8	16	69	11.2
Group II (japonica rice)				
Hanfeng	38	120	66	16.0
02428	32	18	28	11.1
CG 3	43	116	24	14.3
HD708	16	24	62	12.5
HD361	33	63	51	15.3
JC8785	19	38	71	9.3
QG	23	21	11	9.2
22321	48	42	14	—[d]

Continued on next page

Table 1 continued.

Cultivar	pre-RNA[a]	mRNA[a]	Wx protein[b]	AC (%)[c]
Group III (glutinous rice)				
Fengnuo	18	0	0	0
Xin	16	0	0	0
ZH194	16	0	0	0
ZH196	35	0	0	0
ZR 1	22	0	0	0
ZR9	24	0	0	0
XH25	35	0	0	0
XH47	10	0	0	0

[a]Relative radioactivity [Relative amount of total RNAs was loaded in each lane for each cultivar, and probing of these blots with a ^{32}P-labeled $p(dT)_{15}$ (Boehringer, cat. No. 814270) probe confirmed that all lanes contained similar levels of poly $(A)^+$ RNA (data not shown).] [b]Relative amount. [c]Amylose content. [d]Not detected.

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Glutelin genes in rice species

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The structure of the 5' flanking region of glutelin genes, amplified from the various wild rice species, was analyzed by cloning and sequencing. The results showed that beyond the essential boxes (legumin, CAAT, AACA, and TATA), the 5' region of rice glutelin genes has numerous putative enhancers (long-direct and short-direct repeats) and putative regulatory segments (RY repeats, -300 bp elements, nuclear protein binding sites) although portions of a few elements have been deleted in some wild species. The sequence length and structure of glutelin 5' regions varied among rice species. On the basis of the length, the degree of homology, and the corresponding base substitutions and deletions in the 5' regions of glutelin genes, the authors suggest that glutelin genes in the subfamily Glua can be classified into three kinds of members, each with its 5' region of 0.5, 0.9, or 1.2 kb. The distribution of these genes among the genomic DNAs of rice species is polymorphic.

A rice glutelin cDNA was first isolated from cultivated rice by Takaiwa et al (1986). More glutelin cDNAs were isolated and classified into two types, I and II, which can be distinguished, respectively, by stop coden TAA or TAG and by one or two polyadenylation signals (Takaiwa et al 1987a). Since these early reports. three genomic clones (*Gt1, Gt2,* and *Gt3*) for rice glutelin were isolated and studied in the laboratory of T.W. Okita. Comparison of DNA sequences from relevant regions of these clones showed that two of them, *Gt1* and *Gt2,* are closely related. *Gt3* shows little or no homology to *Gt1* and *Gt2.* All three clones had 5' flanking regions of less than 0.9 kb (Okita et al 1989). Two new glutelin genes, *Glua3* and *Glua4,* were later added to subfamily A (Takaiwa and Oono 1991) that already contains *Glua1* (Type I) and *Glua-*2 (Type II). Takaiwa et al (1 991) proposed a new subfamily of glutelin genes, subfamily B, in which three member genes have been sequenced. Furthermore, direct repeat, enhancer core, and legumin box (Takaiwa et al 1987b); -300 bp element, RY repeats,

and inverted repeats (Okita et al 1989); and nuclear protein binding sites (Kim and Wu 1990, Takaiwa and Oono 1990) have been reported to be related to glutelin gene expression in cultivated rice. This paper reports the structure of 5' regions of glutelin genes and their distribution with special emphasis on that of glutelin subfamily A genes from wild rice species.

Materials and methods

Polymerase chain reaction (PCR) was used to amplify the 5' region of the glutelin genes from various rice species (both cultivated and wild). The two ends of the 5' region of a known glutelin gene (Takaiwa et al 1986) were used to synthesize two primers,

a 2-1 (5' CAAGCTTTTGGAAAGGTGCCG 3') and
a 2-2 (5' GCTCTAGAGTTGTTGTAGGACTAATGAA 3').

Each has a *Xba*I or a *Hin*dIII linker. The amplified 5' regions were cloned into the plasmid M13 *mp* 19 to produce recombinant DNA molecules.

Deletion and sizing

Double-stranded recombinant DNA of M13 was extracted and digested with exonucleaseIII to produce successively shortened insert DNA using the Erase—a Base System (Promega). Transformation of *Escherichia coli* JM101 was carried out with the deleted recombinant DNA using an *E. coli* Pluser Apparatus. Sizing was performed by electrophoresis of the single-stranded shortened recombinant DNA from M13.

Sequencing and analysis

The single-stranded recombinant DNAs were used as a template and annealed to the fluorescent primers supplied in the auto sequencing kit (Pharmacia). Sequencing reactions were carried out according to the procedures suggested by the supplier; the products were loaded in an automated laser fluorescent DNA sequencer (Pharmacia ALF). Data thus obtained were processed by GCG (Genetics Computer Group) sequence analysis software package.

Southern blot analysis was used to figure out the distribution of glutelin genes in the genomic DNAs of the rice species.

Results and discussion

The amplified DNAs were analyzed by gel electrophoresis. Each lane gave two major bands and several minor ones. Major bands were identitied as true glutelin 5' regions of the species by Southern blot analysis using the 5' region from a glutelin gene isolated from Tainung 67 (cultivated rice of genome AA) as a probe. There were two major bands, 1.2 kb and 0.9 kb in length, amplified from each rice species except that from *Oryza eichingeri* and *O. officinalis* in which the 0.9-kb band was substituted by a 0.5-kb band. Only the 0.9-kb band has been shown to appear in glutelin genes of cultivated rice (Takaiwa et al 1987b, 1991), the other two bands are first described in this paper.

Table 1. 5' region sequence length of glutelin genes from various species of rice.

Species[a]		Genome	Length (kb) estimated from gel electrophoresis	Actual length (bp)
Oryza perennis	(W0107)	AA	1.2	1119
O. eichingeri	(W1519)	CC	1.2	1116
O. punctata	(W1564)	BBCC	1.2	1111
O. punctata	(W1564)	BBCC	0.9	911
O. grandiglumis	(W1194)	CCDD	0.9	913
O. australiensis	(W0008)	EE	0.9	912
O. sativa	(Gt1)	AA	—	779
O. sativa	(Gt2)	AA	—	878
O. eichingeri	(W1519)	CC	0.5	481
O. sativa	(Gt3)	AA	—	842

[a]Only some species were chosen to be cloned and sequenced. The length of the 5' region of *Gt1, Gt2,* and *Gt3* was calculated based on the published sequences (Okita 1989). The sequence of the 5' region of glutelin gene of each wild rice species has been deposited in the Data Bank of Japan (DDBJ), Tsukuba, Japan. The accession number of each sequence is as follows:

clone W0107-1.2:D26363
clone W1564-1.2:D26365
clone W1519-1.2:D26367
clone W0008-0.9:D26369
clone W1564-0.9:D26364
clone W1519-0.5:D26366
clone W1194-0.9:D26368

Sequencing of the seven cloned 5' regions of glutelin genes from the wild species of five genomes revealed their lengths (Table 1), which correspond to their molecular weights estimated from the gel electrophoresis of the PCR products. These sequences can be grouped into three categories according to the length of the PCR-amplified DNAs, 1.2, 0.9, and 0.5 kb, respectively.

All seven 5' region sequences were arranged to give maximum alignment. Each sequence has at its upstream 5' end an AGCTT or GCTT that is part of the cutting site of *Hin*dIII. This and the recovered primer sequence at both ends of the seven sequences assured that the amplified sequences are the true 5' regions of the glutelin genes. Sequences of the *Gt1*, *Gt2*, and *Gt3* reported by Okita et al (1989) were also included in the alignment. In comparison with each other, it is clear that the 0.9-kb 5' region has a short deletion of 20 bp, with respect to the 1.2-kb 5' region, from -834 to -815 bp (from the translation initiation codon ATG), and another long deletion of 204 bp from -470 to -267 bp. As for the 0.5-kb 5' region, it has two long deletions: one is 463 bp in length (from -1055 to -593 bp) and another is 211 bp (from -470 to -260 bp) that coincide with the long deletion in the 0.9-kb 5' region. Such long deletions combined with minor deletions of bases account for the actual length (in bp) of each 5' region sequence listed in Table 1.

There are 209 base substitutions or deletions that have occurred in corresponding positions in the sequence of the 1.2-kb and the 0.9-kb 5' regions. The same amount of such corresponding substitutions and deletions can also be found between the *Gt1* and *Gt2* sequences. The substituted or deleted bases in the 5' region sequence of *Gt1*

Table 2. Base substitution, deletion, and homology in the boxes, repeats, and elements of glutelin genes flanking the 5' region.

		AA CC BBCC	BBCC CCDD EE	*Gt1*	*Gt2*	*CC*	*Gt3*[a]
		(1.2 kb)	(0.9 kb)			(0.5 kb)	
Direct repeat	4	.GACATAATGCAAAA**G**	T, T	H0.9kb[b]	H1.2kb	Del	VLH[c]
	3	ATGTCAT**G**TTGCGAAA AGAGGAGAG	A	H0.9kb	H1.2kb	H0.9kb	VLH
	2	TAG**A**ACAG**A**GC	G, G	H0.9kb	Del	H0.9kb	VLH
	1	ATGTCATATTGCAAAA GAA**AG**AAAG		H1.2kb	H1.2kb	H0.9kb	VLH
Box	VI	**G**TCA	C	H0.9kb	H1.2kb	Del	VLH
	V	TAAGTCA**TAAC**TGATG A	CGTT	H0.9kb	H1.2kb	Del	VLH
	IV	AT**C**ATCCATGTCATAT TG	T	H0.9kb	H1.2kb	H1.2kb	VLH
	Ill	AC**A**AATG**A**TG**T**GTCAA TTA	., C, C	H0.9kb	H1.2kb	H1.2kb	VLH
	II	CTTCCGTGTACCACA	Conserved	———Conserved———		HH	
	I	ATATCAT..GAGTCAC	Conserved	.:AA	—Conserved—	HH	
		TTCA					
-300 bp element	6	TGCAAAGTT	Conserved	—Conserved—	Del	LH	
	5	TGCAAAA**G**	T	H0.9kb	H1.2kb	Del	NH
	4	TGC**G**AAAG	A	H0.9kb	H1.2kb	H0.9kb	LH
	3	TGCAAAAG	Conserved	—— Conserved —		LH	
	2	C**A**AA TG**T**GTCA	., C	H0.9kb	H1.2kb	H0.9kb	LH
	1	**A**T..GAGTCA	C	AA	H1.2kb	H1.2kb	LH
RY repeat	7	**T**ATG	C	H0.9kb	H1.2kb	Del	NF
	6	CATGCAAG	Del	Del	H1.2kb	Del	H
	5	CATGCATC	Conserved	—Conserved—	Del	H	
	4	CATG	Conserved	—Conserved—	Del	H	
	3	CATG	Conserved	—Conserved—	Del	H	
	2	CATG	Del	Del	H1.2kb	Del	H
	1	CATGCATG	Del	Del	H1.2kb	Del	H

Enhanscer core		.TGGTGTT	G	H0.9kb	H1.2kb	Del	Del
13bp AACA box		AACAAACTCTATC	Conserved	——————Conserved ——————			
Legumin box		CTTAACATT**C**A**A**ATGC A**T**G	T, G, A	H0.9kb	H1.2kb	H0.9kb	H0.9kb
Immature seed nuc fac BS		CTTCCGTGTA	Conserved	—— Conserved ———— HH			
CAAT Box		TGGACATTAACAAAC TCTATCTTAACA	Conserved	——Conserved ————			
Inverted repeat		AATC (-271)	Del	Del	H1.2kb	Del	AATC (-324)
		AATC (-459)	Del	Del	H1.2kb	Del	NF
		AATC (-766)	C	H0.9 kb	H1.2kb	Del	NF
		CTAA (-408)	Del	Del	H1.2kb	Del	CTAA (-317)
		CTAA (-684)	Conserved	H1.2kb	H1.2kb	Del	CTAA (-374)
		CTAA (-966)	Conserved	H1.2kb	H1.2kb	Del	CTAA (-504)
SV40 enhancer			—[d]	—	—	—	TGAAAAA
7bp direct	6	TGCAAAG	——— Conserved ———			Del	LH
	5	TGCAAAA	——— Conserved ———			Del	VLH
	4	AGCAAAA	——— Conserved ———				LH
	3	TGC**G**AAA	A	H0.9kb	H1.2kb	H0.9kb	LH
	2	AGCAAAA	Del	Del	H1.2kb	Del	LH
	1	TGCAAAA	————Conserved————		LH		

[a] The *Gt3* 5' region has its own direct repeat 4, CCAAAACCAAAAGCA; direct repeat 3, CCAAAAGCA; direct repeat 2, AAAAATAAAAGCA, and direct repeat 1, TCCCACAAAAAC and six RY repeats, CATG, and four inverted repeats. Positions of these repeats do not coincide with those found in other 5' region sequences. [b] Sequence homology of *Gt1* 5' region to that of the 0.9-kb 5' region. [c] NH = nonhomology; LH = low homology; VLH =very low homology; HH = high homology of *Gt3* 5' region sequence to the corresponding sequence of 1.2-kb 5' region of AA, CC, and BBCC genomes; H = homology; NF = not found; Del = deleted. [d] Not found.

Bases In boldface In the lane of the 1.2-kb 5' region are shown to have been respectively substituted by the base or deleted as shown in the lane of 0.9 kb. for example, -T. **G**-T, m **AG-..** etc.

are like those in the sequence of the 0.9-kb 5' region. The same can be found between the sequences of *Gt2* and the 1.2-kb region.

The structural diversity of the examined 5' region is summarized in Table 2. It includes base substitutions, deletions, and the degree of homology among the 5' region sequences (Table 2). In view of the long direct repeats, for example, four and three had been identified in the *O. sativa* cultivar Mangetsumochi (Takaiwa et al 1987b) and *O. sativa* cultivar M201 (Okita et al 1989), respectively. The alignment shows the sequences and positions of the four direct repeats. All four direct repeats are conserved in the sequences examined except that the 0.5-kb sequence lacks direct repeat 4 and *Gt2* lacks direct repeat 2. The *Gt3* sequence has its own four direct repeats, and their positions do not coincide with those identified in the other sequences. Its first direct repeat is located from -15 to -4 bp relative to ATG.

The distribution of glutelin genes of subfamily A was figured out by hybridizing the *Eco*RI cut genomic DNA from various rice species to a probe prepared from a glutelin gene coding sequence of the cultivated rice. Each positive band is likely to accommodate a glutelin gene. However, the number and size of the positive band are different from one species to another. Wild rice species, especially the tetraploid species (BBCC, CCDD), generally have more glutelin genes than the cultivated ones. Due to the high homology of the coding sequences among the rice species, short sequences, which are respectively specific to the 5' region on the three member genes (*Glua1, Glua2,* and *Glua3*) of subfamily A were cloned and used as probes to differentiate them. The *Glua1* genes are usually located in the bands of larger molecular weight (from 7.6 kb to 23.7 kb) while the *Glua2* genes are in the bands of smaller molecular weight (from 4.3 kb to 6.3 kb). More extensive distribution (from 2.8 kb to 19.4 kb) can be found for the *Glua3* genes. Each member gene in subfamily A shows its polymorphic distribution among the genomic DNAs of the rice species. Our results first show the distribution of *Glua* genes in rice species based on the structure of the 5' region of the glutelin genes.

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Molecular analysis of genes that control flowering in rice

G. An

We have isolated and characterized cDNA clones from rice, which encodes a MADS domain-containing protein. One of the clones, OsMADS1, encodes a protein that shows 56.2% identity to AGL2 and 44.4% identity to AP1. The MADS box region was the most homologous to other MADS domain-containing proteins. Northern blot analysis indicated that the rice MADS box gene was preferentially expressed in floral organs. In situ localization studies showed that the transcript was uniformly present in young flower primordia and later became localized in palea, lemma, and ovary. Ectopic expression of *OsMADS1* with the CaMV35S promoter in transgenic tobacco plants dramatically alters development, resulting in early-flowering plants with reduced apical dominance. These results suggest that the *OsMADS1* gene is involved in flower induction and that it may be used for genetic manipulation of certain plant species.

Molecular characterization of a group of homeotic genes that control flower development revealed that the gene products contain a conserved MADS box (a DNA binding domain of certain proteins involved in the regulation of eukaryotic gene expression) region (Sommer et al 1990, Yanofsky et al 1990, Huijser et al 1992, Jack et al 1992, Mandel et al 1992b, Trobner et al 1992, Bradley et al 1993). MADS box genes have also been isolated from several plant species including tomato (Pnueli et al 1991), tobacco (Kempin et al 1993), petunia (Angenent et al 1992), *Brassica napus* (Mandel et al 1992a), and maize (Schmidt et al 1993).

Transgenic approaches were undertaken to study the functional roles of the MADS box genes. Expression of one of the MADS box genes, AG, from *Arabidopsis thaliana, B. napus*, petunia, tobacco, and tomato resulted in homeotic conversion of sepal to carpel and petal to stamen. suggesting that the MADS box gene plays an important role in controlling floral organ development. Antisense expression of the tomato AG resulted in morphological changes of the three inner whorls of transgenic plants.

Results

We have isolated a cDNA clone, OsMADS1, from l ZapII cDNA library prepared from immature rice flower mRNA. DNA sequence analysis showed that the rice clone encodes a putative protein of 257 amino acid residues that contain the conserved MADS box domain and K box domain. Among characterized MADS box proteins, the OsMADS1 amino acid sequence is most homologous to AP1 and SQUA. In addition, OsMADS1 shows extensive similarity to the functionally anonymous *Arabidopsis* MADS box genes, *AGL2* and *AGL4.* DNA blot experiments show that the rice genome contains a high number of genes that encode MADS box proteins.

The OsMADS1 transcripts were present in palea, lemma, and carpels, but not in the anthers or vegetative organs. The gene was active during the young inflorescence stage and the expression continued into the early and late vacuolated pollen stages. In situ experiments revealed that the OsMADS1 transcript was uniformly present in young flower primordia during early flower development and later became localized in certain floral organs.

To characterize the functional role of OsMADS1, we have used tobacco plants as a heterologous expression system. The cDNA clone encoding the entire OsMADS1 coding region was placed under the control of CaMV35S promoter. The chimeric molecule was transferred to tobacco plants. Results showed that most of the primary transgenic plants flowered much earlier compared with the control plants which were transformed with the Ti plasmid vector alone. These plants were significantly shorter and contained several lateral branches. These phenotypes were inherited to the next generation as a dominant Mendelian trait. Northern blot analysis was conducted on transgenic plants that displayed the early flowering phenotype. The results showed that all of the plants accumulated the OsMADS1 transcripts in both vegetative and reproductive organs. Although there were significant differences in gene expression among transgenic plants, the relative expression level was similar between the leaf and flower. Transgenic plant no. 7, which displayed the most severe symptoms, accumulated the highest level of the transcript. Plants no. 4, 5, and 6, with less severely altered phenotypes, expressed the gene at reduced levels, indicating that the level of OsMADS1 RNA correlated with phenotype. However, progeny from the same parent displayed phenotypic variation. The basis of this variation was investigated with T_1 offspring of the transgenic plant no. 2 in which the transgene segregates as a single locus. OsMADS1 homozygotes were much shorter (34.2 ± 0.8 cm) compared with heterozygotes (51.6 ± 1.4 cm), while the wild-type tobacco plants were 119.8 ± 2.2 cm. The homozygotes flowered 2 d earlier than the heterozygotes and 8 d earlier than the wild type. This indicates that the variation was due to gene dosage. Table 1 summarizes characteristics of four independently transformed plants from the T_1 generation. Transgenic plants flowered 7-10 d earlier than the wild type and their height and internode length appeared to be significantly reduced.

Table 1. Comparison of phenotypes of transgenic plants with nontransformed control. For each transgenic line, 10-20 plants were analyzed.[a]

Transgenic line (no.)	Days to flowering	Height (cm)	Internode length (cm)
1	53.0 (2.0)	61.2 (5.8)	5.7 (0.5)
2	54.2 (0.3)	47.6 (1.9)	4.6 (0.2)
3	53.0 (0.4)	64.3 (3.5)	5.8 (0.3)
7	50.6 (0.9)	40.2 (4.4)	3.5 (0.3)
C	61.0 (0.2)	119.8 (2.2)	9.0 (0.3)

[a]Standard errors are shown in parentheses. Progenies carrying the transgenes were Identified by visually scoring T_2 seedlings for kanamycin resistance. The kanamycin-sensitive segregants were used as controls (C). Days to flowering includes the time from seed germination to first anthesis. Height and internode length were measured when fruits were fully developed (90 d postgermination).

Discussion

We have studied the role of the rice MADS box gene by expressing it in tobacco plants. Ectopic expression of the rice *OsMADS1* gene resulted in early flowering and dwarf phenotypes. Although the exact mechanism by which the gene exerts its effects is not known, we have demonstrated that *OsMADS1* is potentially useful for shortening flowering time and for reducing apical dominance in certain plant species. These interesting phenotypes were not apparent in their juvenile state. In the transgenic *OsMADS1* plants, the dwarf phenotype may be the result of altered hormonal status due to early flowering.

Early flowering and dwarf phenotypes are important agronomic traits since a balance between vegetative and reproductive growth is a crucial factor that controls crop yields. Enhancement of harvest index in grain crops has been accomplished by the use of dwarfing genes. However, isolation of these genes has been difficult. Moderated expression of the *OsMADS1* gene by means of tissue-specific promoters may make it useful as an alternative source of early flowering and dwarfing gene to increase crop productivity.

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Isolation and characterization of genes that control inflorescence

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An analysis of the development of rice inflorescence is being carried out using various molecular genetic techniques. Scanning electron microscopy (SEM) is being used to study various stages in panicle development. We report a SEM characterization of a wild-type flower and two mutant spikelets: multiple pistil 1 (*mp1*) and multiple pistil 2 (*mp2*). In both mutants, an increased number of stamen and carpel primordia was observed due to a primary defect in the size of the floral meristem. We have cloned from rice cDNAs fragments of two genes that contain the conserved MADS domain found in floral organ identity genes of other species. We have also cloned a fragment of a rice cDNA that bears homology to the conserved domain of the *Arabidopsis* gene, *LEAFY,* which is required for establishment of the floral meristem. The presence of a homolog for the *LFY* gene product in rice also has been demonstrated through immunohistochemistry. Antibodies raised against the *Arabidopsis* LEAFY protein detect the presence of a nuclear-localized homolog in sections of developing rice panicles.

Genes that control floral production, shape, number, and sterility need to be characterized for a basic understanding of floral development. Molecular genetic analysis of flowering in two distantly related dicot plant species, *Arabidopsis* and *Antirrhinum* (snapdragon), suggests a conserved mechanism for floral organ specification in the developing meristem. Many of the floral organ specification genes and the floral meristem identity genes in these two model species encode transcription regulators (Coen and Meyerowitz 1991, Weigel and Meyerowitz 1994). The availability of the *Arabidopsis* and *Antirrhinum* organ specification genes has resulted in the cloning of sequence homologs that form a diverse range of plant species (reviewed in Weigel and Meyerowitz 1994.)

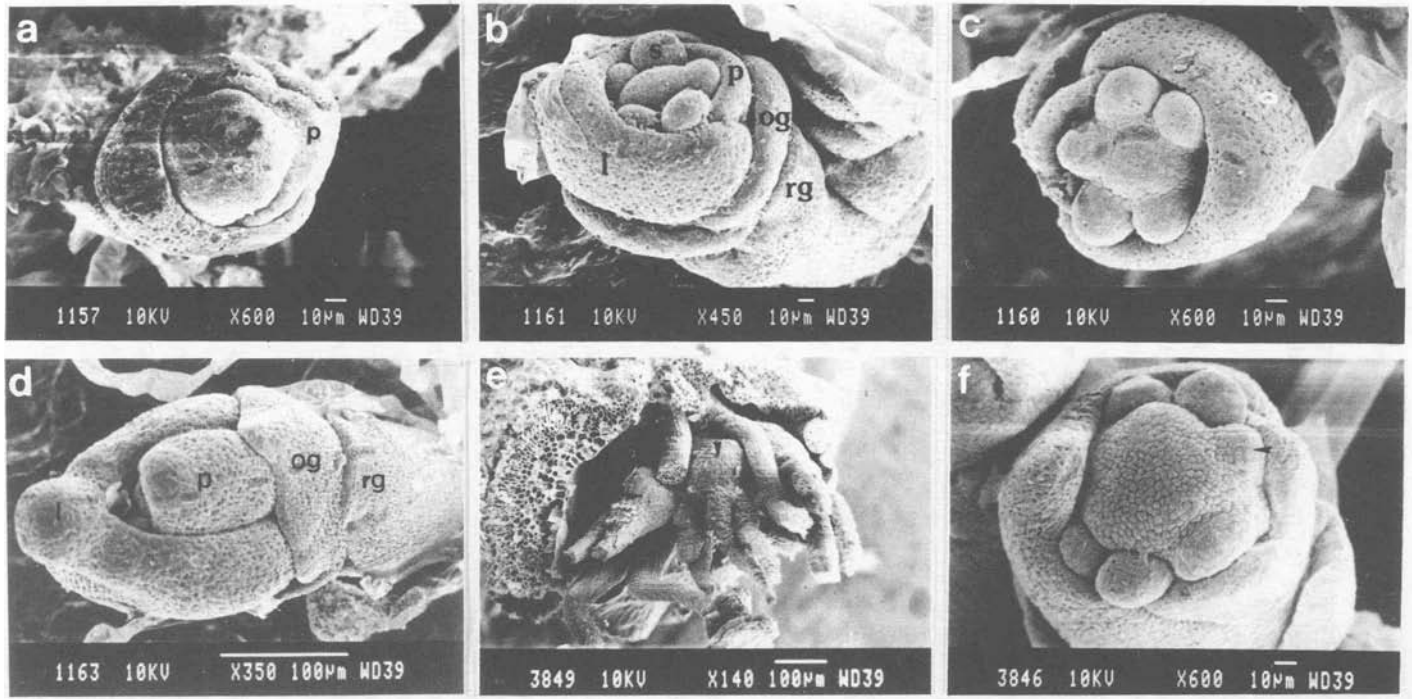

Fig. 1. a) SEM of developing wild-type flower. The determination of the rudimentary glumes and the outer glumes is complete. The lemma (l) and palea (p) have also been specified. The central mass of cells form the primordia for lodicule, stamen, and carpel development. b) Wild-type flower where the rudimentary glumes (rg), outer glumes (og), lemma (l), and palea (p) have been specified. c) Rice flower at the same stage as in (b), but with a view looking down at the developing stamen primordia present in two alternating whorls. d) Rice flower where the lemma (l) and palea (p) development is nearly complete and they enclose the developing inner organs. e) SEM of developing mutant spikelet multiple pistil 1 *(mp1).* The lemma, palea, and most of the stamens have been dissected. Marked with arrows are the developing three carpels instead of the single carpel seen in a wild-type spikelet. f) A young developing spikelet *(mp1)* where the development of an extra stamen primordia is marked with an arrow. In addition, the size of the central dome of cells that forms the primordium for carpel development is at least twice as large as that seen in a wild-type spikelet.

Morphogenetic characterization of panicle development

SEM analysis of wild-type and mutant panicles

Scanning electron microscopy (SEM) is being used to study the stages in wild-type panicle development, beginning from spikelet primordia, and finally, the formation of organs (Fig. 1a,b,c,d). We have also characterized two previously known mutants *mp1* and *mp2* (kindly provided by G. Khush of IRRI) that lead to a primary defect in the number of carpels being increased from one to three (Fig. 1e,f). We observed an increased number of stamen primordia and the formation of mosaic structures with stamenoid features, together with stigmatic papillae. These mutants, therefore, affect the number of organs that develop in a single flower.

Isolation of new mutants that affect floral development

We also have carried out ethyl methane sulfonate mutagenesis of rice seeds to isolate mutants that affect panicle or floral characteristics. Initial screening of 142 M_2 families is being done. Of the several mutants observed, those with interesting changes in floral characteristics were two mutant lines with awned lemmas and one line with a transformation of the outer glumes and lodicules to large lemmalike glumes and with fewer stamens. Further genetic characterization including determination of the inheritance pattern, etc., will be taken up for analysis.

Isolation of rice genes homologous to the MADS domain of *Arabidopsis* genes

We are attempting to clone genes from rice that encode homologs of the floral organ and meristem identity genes of *Arabidopsis* and *Antirrhinum.* We have isolated RNA from panicles in early stages of development and used the RNA in reverse-transcription polymerase chain reaction (RT-PCR) experiments to clone MADS box (a DNA-binding domain of certain proteins involved in the regulation of eukaryotic gene expression) genes. Using MADS domain-specific primers, we have obtained two different clones that contain this conserved motif. One of these is the *OsMADS1* gene that has been recently characterized (Chung et al 1994). The other clone has a MADS domain that is closely related to the domain present in *AGAMOUS* of *Arabidopsis* (Yanofsky et al 1990) and AG homologs from other species. Further characterization of this clone is in progress.

Isolation of floral meristem identity genes

Another *Arabidopisis* gene that is conserved through evolution but does not contain a MADS box is *LEAFY* (Weigel et al 1992). Using a conserved domain for the *LEAFY* gene as a probe, we have detected the presence of a single or low-copy gene in rice. Using primers for the conserved domain in an RT-PCR experiment, on RNA from flowers at early stages of development, we have amplified the putative LEAFY homolog

of rice (Fig. 2). This clone is being used to obtain longer cDNAs. The open reading frame (ORF) in this short cDNA has been cloned inframe in the *Escherichia coli* expression vector pMALp. Here the ORF is produced as a fusion with the maltose-binding protein (MBP) of *E. coli.* The fusion protein can be purified using amylose-agarose affinity chromatography. Dot blot Western analysis was done with the affinity-purified fusion protein (MBP-LFY) and as a control of the purified MBP. Antibodies raised against the *Arabidopsis* LFY protein were used to determine the cross reactivity of the cloned fusion protein. We found that the fusion of MBP and the conserved domain of LFY from rice cross-reacted with high specificity with these antibodies. These results confirm the DNA sequence conservation between *Arabidopsis* and rice cDNAs in this short domain.

We also are attempting to clone the homolog of the *Antirrhinum* floral and meristem regulatory gene *FIMBRIATA* (Simon et al 1994). Using primers specific for a domain of the *FIM* gene, we have detected the presence of a cross-hybridizing fragment in an RT-PCR experiment. Attempts to clone this gene are in progress.

Conserved immunoreactive antigens in rice panicles

We have used antibodies raised against the *Arabidopsis* LEAFY protein on sections of developing rice inflorescence and have detected the presence of the putative rice LEAFY homolog. This cross-reacting antigen appeared specifically in the early stages of panicle development. The first detectable signal was in the primary rachis branch primordia, which was followed by a signal in the secondary rachis branch primordia and followed by a signal in the glumes (Fig. 3). No signal was detected in the adult flower. In addition, this cross-reacting antigen was specifically nuclear-localized. These results suggest the expression of this nuclear protein in specific early stages of floral development.

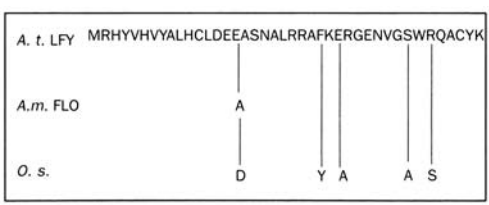

Fig. 2. Alignment between a 41 amino acid-conserved domain of the *LEAFY* gene product from *Arabidopsis* (A.t. LFY), the *FLORICULA* gene product from Antirrhinum (A.M. FLO), and the putative LEAFY homolog of rice (O.s.).

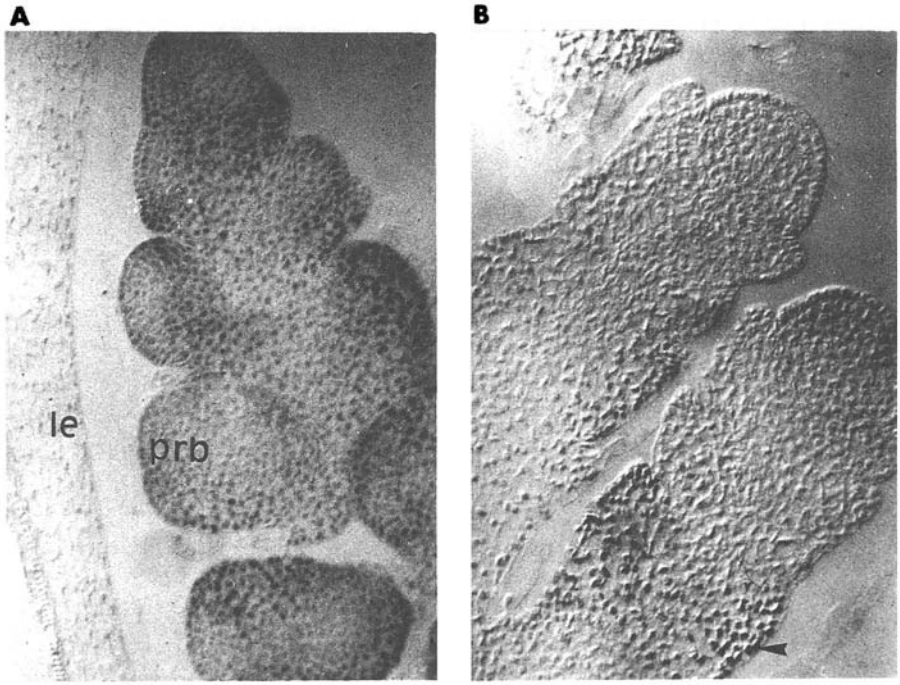

Fig. 3. Immunoreactivity of LEAFY specific antibody on section of rice panicles. a) Cross-section of a developing panicle at a very early stage enclosed in a leaf sheath (le). The primary rachis branch primordia (prb) are developing at this stage. Some staining is seen as darker groups of cells. b) Cross-section through a developing spikelet primordia where the rudimentary glumes are stained and are marked with an arrow.

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High-molecular weight proteins associated with response of rice to various abiotic stresses

A. Grover, S.L. Singla, and A. Pareek

So far, little has been achieved in characterizing gene products that may impart resistance against various abiotic stresses. We have analyzed various proteins in rice that are specifically modulated to respond to salinity (NaCl), high and low temperatures, and drought, by using one- and two-dimensional protein gel electrophoresis coupled to highly resolutive silver staining and radiolabeling. By subjecting proteins to various stresses and to abscisic acid, we have been able to distinguish polypeptides that accumulate in response to a specific stress stimulus from those that accumulate in response to a number of different stress stimuli. Data on the expression pattern of three high-molecular weight (110, 104, and 90 kDa) stress proteins of rice are presented in this paper. Further, we compare 104- and 90-kDa stress proteins in salt-sensitive (Basmati 370) and salt-tolerant (CSR10 and CSR19) rice cultivars.

Intensive efforts are currently being made to genetically engineer rice plants for better growth, performance, and yield. Abiotic stresses (primarily drought, submergence, salinity, and high and low temperatures) limit rice productivity to a significant extent. Further, increasing soil salinity due to extension of irrigation networks and an increase in ambient temperature due to the greenhouse effect may amplify the problems attributed to abiotic stresses in the years to come (Toenniessen 1991). Our group is interested in identifying, isolating, and characterizing genes that have the potential to confer rice with resistance to abiotic stresses. Here. we report our efforts to identify proteins/genes that are associated with the response of rice plants toward salinity (NaCl), high and low temperatures, and drought.

Materials and methods

Detailed protocols for growing and subjecting rice seedlings to various stresses are published elsewhere (Singla and Grover 1993, 1994; Pareek et al 1996). Analysis of stress proteins was carried out before and after imposing the stress to identify specific alterations that are caused by the stress treatments. The analysis of stress proteins was done by one- and two-dimensional polyacrylamide protein gel electrophoresis following silver staining and radiolabeling (Singla and Grover 1993, 1994; Pareek et al 1996). We have purified two specific stress proteins, SAP 104 and SAP 90 (see later part of the text for more details), and raised polyclonal antisera against these proteins following methods published earlier (Pareek et al 1996).

Results and discussion

In the present work, we are attempting to identify stress proteins in rice seedlings that are associated with sublethal stress conditions. Our hypothesis is that, often, priming or pretreating plants to sublethal stress levels results in the induction of stress tolerance, or so-called "acquired or induced stress tolerance" (Grover et al 1993).

Divergent stress proteins of rice

The level of specific stress proteins either accumulate or decline in response to a given stress type or more than one stress type. The accumulation pattern of selective stress proteins in rice shoot tissues is depicted as a Venn diagram (Fig. 1; A. Pareek, S.L. Singla, and A. Grover, University of Delhi South Campus, unpubl. data). We have also identified a few proteins (such as those with molecular weights of 110, 104, and 90 kDa) that accumulated in response to all the stresses tested in this study (i.e., salinity, high and low temperatures, drought, and abscisic acid). Accordingly, we refer to these proteins as stress-associated proteins (SAPs, see Pareek et al 1996). The main outcome of this analysis is 1) that multiple protein alterations occur in response to abiotic stresses, confirming that plant response to these environmental cues is multigenic in nature; and 2) some of the reactions pertaining to the response of rice plants to different abiotic stresses may be common.

Rice SAP 104

We noted that when 5-d-old rice seedlings were subjected to high temperatures, the shoots and roots synthesized and accumulated a 104-kDa polypeptide within 1-2 h of imposing the stress. The higher levels of this polypeptide persisted for nearly 16 h when the stress was continued for 24 h or up to 8 h when seedlings were recovered at 28 °C after 4 h of high temperature. Subsequently, we purified and raised highly specific antisera against this polypeptide. In Western blotting experiments, we found that this protein is accumulated, to different extents, in rice seedlings subjected to salinity, drought, and low temperature and exogenous application of abscisic acid. Seedlings of *Triticum aestivum, Sorghum bicolor, Pisum sativum, Zea mays, and Brassica juncea* and mycelia of *Neurospora crassa* showed accumulation of the immunological homo-

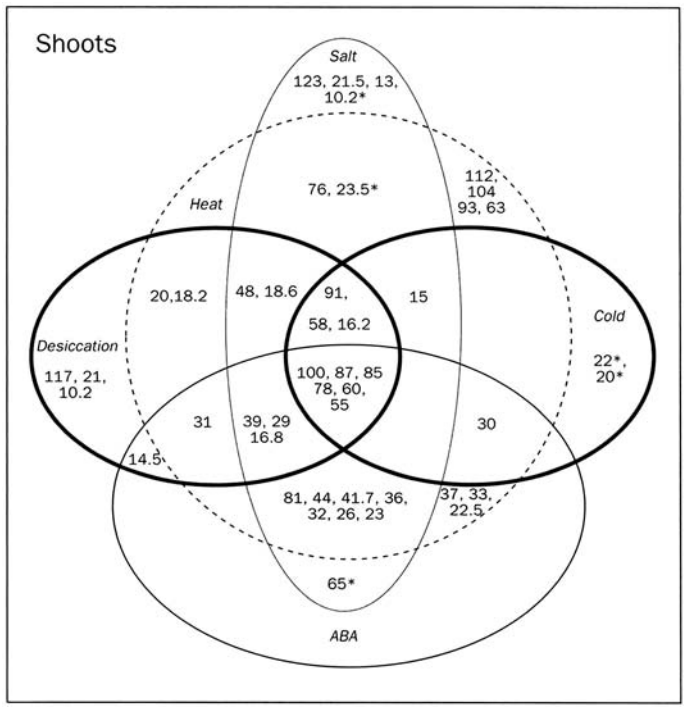

Fig. 1. Venn diagram showing rice shoot proteins that are altered in response to high temperature (45 °C, 8 h), cold stress (5 ± 2 °C, 96 h), desiccation stress (16 h air-drying), salinity stress (200 mM NaCl, 96 h), and exogenous abscisic acid (ABA) application (10^{-4}M, 24 h). The numbers indicate the molecular weight of the stress proteins (kDa), which either accumulate (without an asterisk) or decline (marked with an asterisk) in response to a given stress treatment. The proteins shown in the overlapping region of the circles represent polypeptides that are altered in response to the specified stresses; those polypeptides shown in the nonoverlapping areas are altered exclusively in response to the specified stress condition.

logues of this protein. Details about SAP 104 are discussed by Singla and Grover (1994) and Pareek et al (1996), however, its identity is still unclear. We recently microsequenced a few tryptic peptides of this protein and are presently analyzing its possible homology with other stress proteins identified in the literature.

Rice SAP 110

Antibodies raised against yeast HSP 104 recognize a heat-inducible polypeptide with a molecular mass of 110 kDa in the shoot tissues of young rice seedlings (Singla and Grover 1993). Our data show that the rice SAP 110 protein accumulates in shoots of rice seedlings but not in the topmost leaf of 90-d-old plants (just prior to flowering) in

response to heat shock (Singla and Grover 1993). It is important to note that while, in relative terms, rice seedlings are considered heat tolerant (Howarth and Ougham 1993), a marginal increase in ambient temperature at the time of anthesis can cause a high degree of pollen sterility, which results in empty spikelets (Yoshida 1981). At a comparable growth stage, we found that leaves of *O. australiensis* accumulate this protein marginally in response to heat shock, while levels of this protein are either markedly declined or remain unaltered in 14 other wild rices tested (S.L. Singla, A. Pareek, and A. Grover, University of Delhi South Campus, unpubl. data). Importantly, we note that this protein also accumulates in shoots of rice seedlings in response to salinity, desiccation, and low temperature.

Rice SAP 90

HSP 90 proteins are shown to be important in inducing heat tolerance in several microbial and animal cells. We note that heat-shocked rice seedlings accumulate two polypeptides of 87 and 85 kDa, collectively referred to as SAP 90 proteins. We have purified these polypeptides and raised highly specific polyclonal antisera against them. In Western blotting experiments, we found that these proteins accumulate in various amounts in rice seedlings subjected to salinity (NaCl), drought, and low temperature. These proteins also accumulate when rice seedlings are grown in pots under natural conditions but subjected to drought by withholding watering. Seedlings of *Triticum aestivum, Sorghum bicolor, Pisum sativum, Zea* may, and *Brassica juncea* and mycelia of *Neurospora crassa* showed accumulations of the immunological homologues of this protein. Pareek et al (1996) presents additional details about SAP 90. Our recent experiments suggest that 87-kDa rice protein is a member of the eukaryotic stress-90 family. This is shown by microsequencing of the rice HSP 87 tryptic peptides (A. Pareek, S.L. Singla, and A. Grover, University of Delhi South Campus, unpubl. data).

Comparison of salt-susceptible and salt-tolerant cultivars

We used three cultivars of rice (Basmati 370, CSR10, and CSR19) to examine the relative levels of various stress proteins discussed above. Basmati 370 is a salt-susceptible cultivar; CSR 10 and CSR 19 are considered salt-tolerant types. All the salt-responsive proteins found in Pusa 169, as mentioned above, were found in these three cultivars as well. Silver-stained gels showed no appreciable variation in their levels in these cultivars. Therefore, one-dimensional protein gel electrophoresis, even with highly sensitive silver staining, is not suficiently resolutive to detect variations in the levels of stress proteins in the contrasting types. Further, levels of two specific stress proteins (SAP 104 and SAP 90) were tested by the Western blotting technique. Basmati 370 seedlings showed appreciable accumulation of SAP 104 and SAP 90 proteins with the application of 50 and 200 mM NaCl, within a 3- to 6-d interval. CSR19 seedlings showed no change in the levels of these proteins in this duration of the stress. On the other hand, CSR 10 cultivar accumulated both the SAP 104 and SAP 90 proteins at about the same rate as noted for Basmati 370. We are extending these observations on SAP 104 and SAP 90, and further aim to test other stress proteins discussed in this study for their role as markers for stress tolerance.

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Rice proteasome: multicatalytic proteinase complex involved in cell proliferation

M. Umeda, Y. Manabe, and H. Uchimiya

Proteasomes are known to function mainly in the ATP-dependent degradation of proteins conjugated with ubiquitin. We isolated a rice cDNA clone that showed high homology with a proteasome subunit named C2. Southern blot analysis revealed that the gene is encoded at a single locus in the rice genome. The anti-C2 subunit antibody cross-reacted with a major protein of 34 kDa in the crude lysate of rice cells. After glycerol density gradient centrifugation, the 34-kDa protein was recovered in a fraction that showed a maximum peptidase activity in the presence of sodium dodecyl sulfate. This suggests that the C2 subunit is incorporated into the 20S complex of the rice proteasome. We found that the mRNA and protein levels were decreased in roots of rice seedlings after 4-6 d of salt stress. The reduced expression of proteasome may be associated with retarded root elongation under saline conditions. Using carrot cultured cells, we showed that the protein level of the C2 subunit in the nucleus was induced at the early stage of somatic embryogenesis. These results suggest that proteasomes are involved in cell proliferation in plant cells.

Proteasome is a multicatalytic proteinase complex that has been identified in archaebacteria and found in life forms from yeasts to humans. There are two types of eukaryotic proteasomes, with sedimentation coefficients of 20S and 26S, respectively. Energy derived from ATP is required for formation of the 26S complex (molecular mass of more than 1500 kDa) that consists of the 20S complex (around 700 kDa), which is itself composed of 13-15 different subunits and a characteristic set of other protein components (35-110 kDa). The 26S complex seems to be an active form and catalyzes ATP-dependent degradation of various cytoplasmic proteins conjugated with ubiquitin.

An ATP- and ubiquitin-dependent proteolytic system appears to be essential for selective removal of unnecessary proteins, such as abnormal proteins and those with a rapid turnover in cells. This system should be important in maintaining homeostasis in plant cells. Proteasome particles have been found in several plants, such as tobacco, potato, mungbean, pea, and wheat. However, the molecular and biochemical characteristics of each subunit have not been analyzed as well as proteasomes in yeast and animals.

In this paper, we describe the molecular and biochemical characterization of the proteasome and discuss its role in cell proliferation.

Molecular characterization of the C2 subunit of rice proteasome

Through large-scale sequencing of randomly selected cDNA clones of rice (Uchimiya et al 1992, Umeda et al 1994), we obtained a cDNA clone that was homologous to a proteasome subunit of *Drosophila melanogaster.* The cDNA contained a putative open reading frame (ORF) of 813 nt (30 kDa), which was highly homologous to that of the proteasome C2 subunit. The C2 subunit is assumed to be the largest component of the 20S complex and is classified into an alpha-type subunit.

Southern blot hybridization was performed to analyze the copy number of proteasome C2 subunit in the rice genome. Under high stringency conditions during probe hybridization, we detected only one major band with several restriction enzymes. This suggests that the C2 subunit gene exists at a single locus in the rice genome. Several minor hybridization signals were also found, indicating that sequences homologous to the C2 subunit are present in the genome. Therefore, the rice genome may contain a proteasome gene family, as found in animals and yeast.

Evidence for incorporation of the C2 subunit into the 20S proteasome complex

Anti-C2 subunit polyclonal antibody was produced with the GST-C2 subunit fusion protein as antigen. By Western blotting using the antibody, we could detect a major 34-kDa band in the crude lysate of the rice cells.

To characterize the C2 subunit in the rice 20S complex, crude lysate was fractionated by glycerol density gradient centrifugation and each fraction was analyzed by Western blotting. The maximum level of the 34-kDa protein was detected in fraction 13. We then measured peptide degradation activities of individual fractions. Although almost no activity was detected in the absence of sodium dodecyl sulfate (SDS), addition of 0.04% SDS, a potent artificial activator of latent 20S proteasome, markedly activated the peptide degradation activities in fractions 12-14. The correspondence of the 34-kDa protein with peptidase activity indicates that the antibody cross-reacted with the rice C2 subunit, which was incorporated into the 20S complex.

Expression of the C2 subunit under salt stress

Expression of the C2 subunit under stressed conditions was then analyzed. Among the abiotic stresses examined, salt stress had a distinct effect on expression. The root portion was submerged in 0.5% NaCl, which is known to inhibit the growth of rice seedlings. Northern hybridization showed that the mRNA level was elevated in shoots, especially after 4-6 d under salt stress. On the other hand, the transcripts in roots decreased after 2-4 d of salt treatment. To investigate the expression at the protein level, we then analyzed crude lysate of rice seedlings by Western blotting. Although the amount of the C2 subunit did not change much in shoots, it was reduced in roots after 4 d of salt stress and could not be detected after 6 d. The consistency of the results at the mRNA and protein levels suggests that expression of the C2 subunit in roots was repressed at the mRNA level by salt stress. In rice plants, root elongation and aerenchyma development in roots were highly retarded under saline conditions. Therefore, the reduced expression of proteasome in roots may be correlated with the decrease of nuclear activities of rice cells under salt stress.

Behavior of the proteasome subunit in nucleus and cytoplasm

We analyzed the behavior of the C2 subunit in nucleus and cytoplasm. In the crude nuclear extract of rice cultured cells, we could detect a faint band of the 34-kDa protein. A similar faint band was also observed in the nucleus of carrot cells. However, in the process of somatic embryogenesis of carrot cells, the protein level of the C2 subunit changed in the nucleus; in proembryogenic masses, we could detect a faint band of the C2 subunit as in callus, whereas the major band was clearly observed at the globular stage. Through the heart to torpedo stages, the protein level was reduced again. It is known that cells are rapidly divided at the early stage of somatic embryogenesis. Therefore, the behavior of the C2 subunit observed here may also reflect the nuclear activity of cell proliferation.

Proteasomes in animals and yeast are assumed to be involved in nuclear activities of cells during rapid growth, possibly by regulating proteinous fractions in the cell cycle. Therefore, proteasomes in higher plants also may be essential to the regulation of the cell cycle and differentiation. In animal cells, high levels of proteasomes have been found predominantly in the nucleus of immature proliferating cells and that proteasomes move rapidly between the cytoplasm and the nucleus during oogenesis and embryogenesis in lower organisms. Therefore, ubiquitin-dependent proteolysis in the nucleus may play an important role in the differentiation and cell division of rice cells.

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Isolation and characterization of Tnr1, a mobile genetic element in rice

H. Ohtsubo, T. Tenzen, and E. Ohtsubo

We have identified and characterized several kinds of mobile genetic elements in rice. These include transposable DNA elements, retroposons, and retrotransposons, which are scattered all over the chromosomes. Tnr1, 235 bp in length, is a novel rice transposable element with a long terminal inverted repeat of 75 bp. Its sequence is different from those of any other transposable elements belonging to Ac/Ds, En/Spm, and Mu families. We have isolated six members of Tnr1 located at different loci and determined their nucleotide sequences. The Tnr1 members were similar in size and highly homologous (about 80%) to the Tnr1 sequence originally identified in the waxy gene in *Oryza glaberrima* and *O. sativa.* Almost all the chromosomal sequences that flank the Tnr1 members were 5'-PuTA-3' and 5'-TAPy-3', indicating that Tnr1 transposes to the 5'-PuTAPy-3' sites, duplicating the TA sequence. Considering its small size, Tnr1 is assumed to be a defective element. To isolate an autonomous Tnr1 element, we carried out polymerase chain reaction using primers that hybridize to the terminal inverted repeat sequences. The consensus sequence derived from nucleotide sequences of clones obtained is 1.76 kb in length, similar in size to those of the transposons of IS*630*-Tc1 family, which are known to generate duplications of the TA sequence upon transposition.

We have been studying roles of mobile genetic elements on genomic rearrangements in rice. We have identified transposable elements, such as Tnr1 (235 bp; Umeda et al 1991, Tenzen et al 1994), Tnr2 (147 bp; Mochizuki et al 1992), Tnr3 (1.5 kb; Motohashi et al 1996), retroposon p-SINE1 (Mochizuki et al 1992), and retrotransposon RIRE1. These are dispersed elements appearing in several hundred to several thousand copies haploid genome^{-1}.

Tnr1 was identified in the waxy gene in *Oryza glaberrima* and *O. sativa* (Umeda et al 1991). Tnr1, 230 bp in length, has terminal inverted repeat (TIR) sequences of 75

bp (Fig. 1). Copy numbers of Tnr1 in *O. glaberrima* have been determined to be about 3,500 haploid genome^{-1}(Tenzen et al 1994). Tnr1 is too small to encode transposase, suggesting that it is a defective form of an autonomous element. Here, we report the isolation and characterization of Tnr1 members including a possible autonomous element.

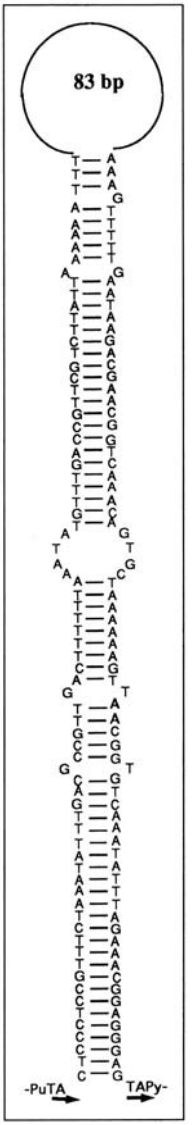

Fig. 1. Structure of an inverted repeat transposon, Tnr1, of 83 bp with 75 bp TIR.

Materials and methods

Total genomic rice DNA was isolated from rice plants as described previously (Ohtsubo et al 1991). Polymerase chain reaction (PCR) analysis was carried out in the Cetus buffer containing 2.5 units of AmpliTaq DNA polymerase (Perkin Elmer Cetus), as described previously (Tenzen et al 1994). Cloning of the PCR products was carried out by using a TA cloning kit (Invitrogen).

Results and discussion

Identification and characterization of Tnr1 members

We tried to isolate Tnr1 members located at various loci by amplifying the fragments containing end regions of Tnr1 and its flanking regions in *O. glaberrima* and *O. glumaepatula* by inverse polymerase chain reaction (IPCR) using primers that hybridize to the Tnr1 sequence and prime DNA synthesis toward the outside of Tnr1. The fragments amplified were cloned, and six clones with the end regions of Tnr1 (Tnr1 A, Tnr1B, Tnr1C, Tnr1D, Tnr1E, and Tnr1F) were identified by DNA sequencing (Fig. 2). The sequences flanking each Tnr1 member were 5'-PuTA-3' and 5'-TAPy-3', except for Tnr1F (Fig. 2). This suggests that Tnr1 transposed specifically to the sequence 5'-PuTAPy-3', duplicating the TA sequence used as target. The exception, Tnr1F, was flanked by 5'-PyCA-3' and 5'-TAPy-3'; the former may have been altered after

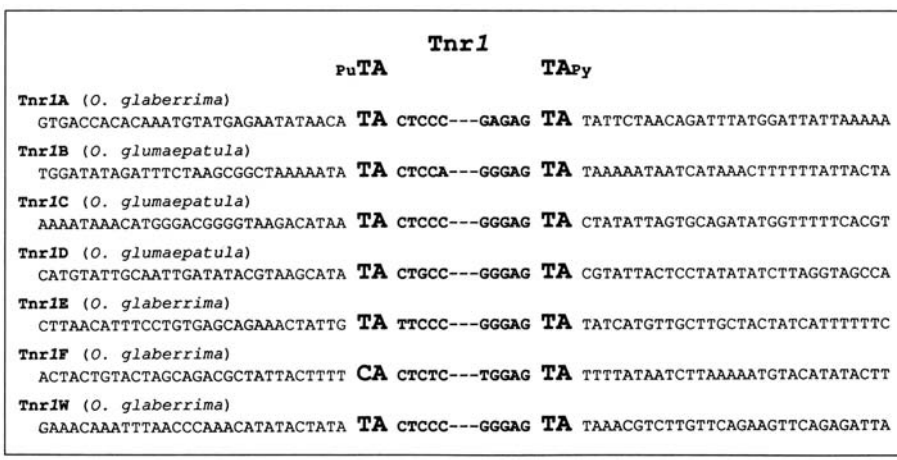

Fig. 2. Nucleotide sequences of the fragments obtained by IPCR. The sequences are oriented from 5' to 3'. Nucleotide sequences of the end regions of Tnr1 members are shown in boldface. Sequences 5'-PuTA-3' and 5'-TAPy-3' that are commonly seen at the junctions with Tnr1 members are shown. Tnr1A, Tnr1E, and Tnr1F were identified in *O. glaberrima,* while Tnr1B, Tnr1C, and Tnr1D were identified in *O. glumaepatula.* Tnr1W has been previously identified in the *waxy* gene in *O. glaberrima* (Umeda et al 1991).

duplication of the TA sequence generated upon transposition of Tnr1F into an original target site.

To determine the nucleotide sequences of the inner regions of the Tnrl members, we carried out PCR using primers that hybridize to the flanking sequences of each member (total genomic DNA of *O. glaberrima* or *O. glumaepatula* was used as template), and the PCR-amplified fragments were cloned. Nucleotide sequencing revealed that their sequences were similar in size and were highly homologous (more

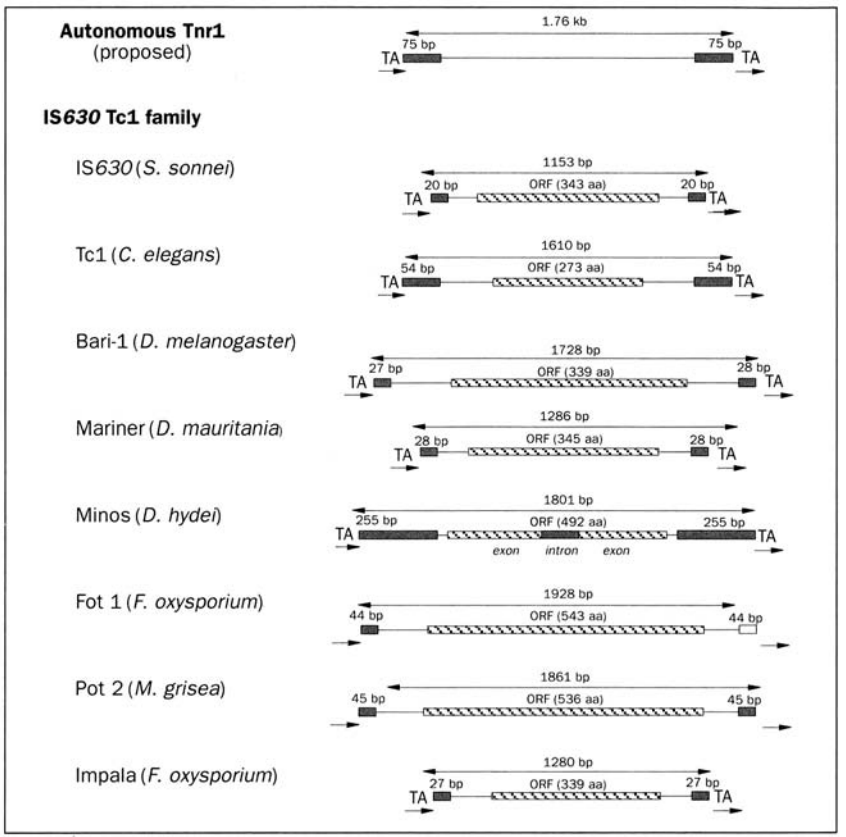

Fig. 3. Schematic representation of Tnr1 and IS*630*-Tc1 (Mariner) family transposons: hatched boxes indicate ORFs coding for transposase. Shaded boxes indicate TIRs of each element. Numbers above the lines with two arrowheads indicate the size of each element. TA duplications generated upon insertion of the elements are shown at both ends. IS*630*-Tc1 (Mariner) family elements are IS*630* (Matsutani et al 1987, Tenzen and Ohtsubo 1991), Tc1 (Moerman and Moerman 1989), Bari-1 (Caini et al 1993), Mariner (Hartl 1989), Minos (Franz and Savakis 1991), Fot1 (Daboussi 1992), Pot2 (Hachroo et al 1994), and Impala (Langin 1995).

than 80%) to the Tnr1 sequence (named Tnrl W) identified previously in the *waxy* locus in *O. glaberrima* and *O. sativa.* The consensus sequence of 235 bp derived from these sequences shows that Tnrl has TIRs of 75 bp. A family of elements, named Stowaway, which is homologous to Tnr1, is associated with the genes of both monocotyledonous and dicotyledonous plants (Bureau and Wessler 1994).

Recently, a number of transposable elements, which generate 5'-TA-3' duplications like Tnrl in rice, have been reported (Fig. 3). Those elements share rather simple structural organization: they have long TIRs and contain one open reading frame (ORF) coding for transposase (Fig. 3). These features are distinct from those of plant transposable elements so far identified, but resemble the so-called IS*630*-Tc1 (Mariner) family elements, such as IS*630* from *Shigella sonnei,* Tc1 from *Caenorhabditis elegans.* Mariner from *Drosophila,* and Fot1 and Pot2 from fungi (for references. see footnote, Fig. 3). A recent computer search using blocks of aligned transposase segments derived from the Tc1 family transposons has shown that their transposase share amino acid residues strongly conserved in the C terminal portion, including a fully conserved dipeptide DE and a block consisting of a fully conserved D residue and highly conserved E residue, separated by 35 (or 34) residues. This D35E motif is also homologous to the D3SE motif of the family of retroviral-retrotransposon integrases (Doak et al 1994).

A 128-bp transposonlike element named Tourist has been found in the maize chromosome (Bureau and Wessler 1992). Tourist has TIRs and appears as an interspersed repetitive DNA sequence, which can transpose preferentially to the sequence TAA. Tnr1 (Stowaway) and Tourist may be classified as another category of plant transposable elements.

Isolation of an autonomous element of Tnr1

The Tnr1 members do not share any nucleotide sequence homology with IS*630* and Tc1, but is likely to be another one of the relatives. Tnrl is, however, too small to encode transposase, suggesting that it is a defective form of an autonomous element. We isolated an autonomous element encoding the transposase by amplifying the fragments by PCR using a primer IR4 that hybridizes to TIRs and primes DNA synthesis toward the inside of the Tnrl sequence (Fig. 4). Nucleotide sequencing of four fragments (A to D in Fig. 4) obtained revealed a consensus sequence of Tnrl (1.76 kb in length). This sequence showed no obvious homology to those of the transposable elements belonging to the IS*630*-Tc1 family, but its length is similar to them.

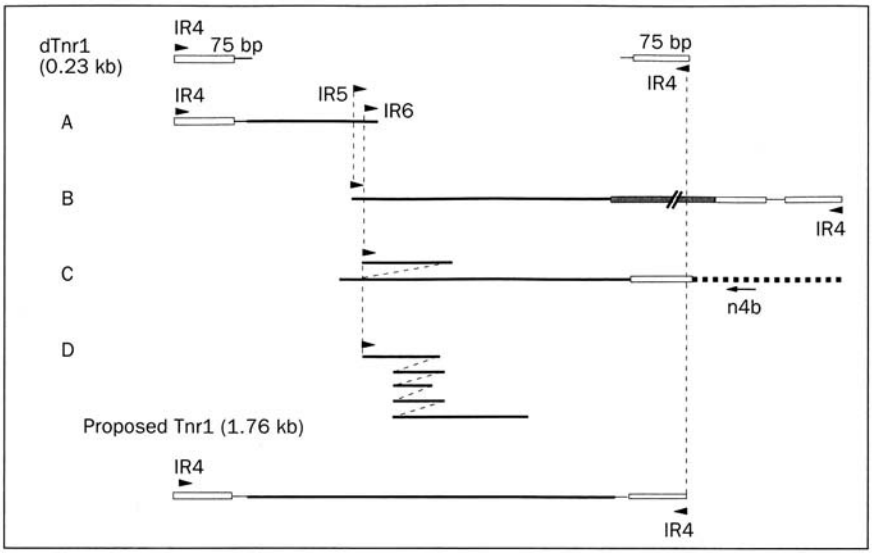

Fig. 4. Schematic representation of the PCR fragments that contain a portion of Tnr1. An original short Tnr1 sequence is shown as dTnr1. Fragment A (0.65 kb) contained a sequence from base-pair 1 to 83, almost the same as the consensus sequence derived from the dTnr1. We synthesized primers IR5 and IR6 and carried out PCR to extend DNA synthesis and obtained fragments B, C, and D. The structure of a consensus sequence is shown at the bottom. Open boxes indicate TIRs of Tnr1. Hatched box in fragment B and a broken line in fragment C are the regions of nonhomology with Tnr1. Closed triangles indicate positions and directions of primers used.

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Genetic and molecular analyses of sporulation and pathogenesis in the rice blast fungus

H. Leung, Z. Shi, Y. Shi, D. Fujimoto, J. Barroga, and D. Christian

Using chemical and insertional mutagenesis, we have identified six genes that control various steps of the sporulation pathway and four genes that control pathogenesis in rice blast caused by *Magnaporthe grisea.* We have established that the morphogenetic functions are directly related to pathogenesis. A fully functional set of developmental genes is essential for the fungus to colonize host tissue and eventually establish a sporulating lesion. Preliminary sequence analysis of two morphogenesis genes showed no homology with genes reported in the data base. These genes represent potential targets for intervention. Information about the gene products will be used in the design of ecologically sound compounds such as fungicides.

The disease cycle of rice blast caused by ***Magnaporthe grisea*** involves three distinct phases: infection, colonization, and sporulation. Interference with the development of the fungus in planta is a powerful approach to reduce the epidemiological potential of the fungus. Suppression of developmental processes during the post-infection phase is likely to be nonrace-specific, and hence minimizes the selection for wider virulence. To pursue a development-specific control strategy, we must first have a thorough understanding of the genetic basis of fungal development in the disease cycle. Our long-term goal is to gain a detailed understanding of the genetic and biochemical regulation of fungal development during host-pathogen interactions. We envision that by unraveling the genetic mechanisms regulating the disease cycle, we can identify critical steps or gene targets for intervention. Here, we summarize recent results in the identification and cloning of genes controlling sporulation and pathogenesis.

Tagging of developmental and pathogenicity genes by insertional mutagenesis

Insertional mutagenesis has allowed us to identify and isolate genes in *M. grisea* with high efficiency. We are in a position to identify a wide array of fungal genes that have not been previously known to be involved in pathogenesis. Our success in insertional mutagenesis is partly attributed to the application of restriction enzyme-mediated integration (REMI) transformation (Schiestl and Petes 1991, Kuspa and Loomis 1992, Shi et al 1995). The enhanced efficiency (5-10 fold) with REMI transformation has allowed us to generate a large number of transformants (> 1500 transformants in two strains, Guy11 and 2539) in a few experiments. From this collection of transformants, we recovered both sporulation mutants as well as mutants with reduced pathogenicity. The frequency of genetically tagged mutants was approximately 0.6%.

Morphogenetic genes involved in pathogenesis

Through a combination of chemical and insertional mutageneses, we have identified six genes (*con1, con2, con4, con5, con6,* and *con7*) that control various steps of the sporulation pathway (Leung and Shi 1994, Shi and Leung 1994, Shi and Leung 1996). The characteristics of these morphogenetic mutants are summarized in Table 1 and Figure 1. The most significant finding from the analysis of the sporulation pathway is that all the morphogenetic mutants are defective in pathogenesis (Table 2). Reduced pathogenicity in the *con2* and *con4* mutants is manifested as a reduction in lesion number and a prolonged latent period—suggesting that these two mutants have

Table 1. Characteristics of sporulation mutants of *Magnaporthe grisea.*

Strain	Origin of mutation	Phenotype	Spore morphology[a]	Radial growth[b]	Appressorium formation (%)[c]	Sporulation[d]
GTI	Insertional	Con1⁻	Elongated	72.5 c	0	2.0 c
Guy11-con2	Chemical	Con2⁻	Single or two-celled	95.6 ab	49.4 c	5.5 c
GT20	Insertional	Con4⁻	Three-celled, ellipsoid	92.3 b	74.3 b	65.2 b
GT399	Insertional	Con5⁻	Aconidial	100 a	na[e]	0
GT560	Insertional	Con6⁻	Aconidial	34.6 d	na	0
GT562	Insertional	Con7⁻	Mixture of abnormal spores	92.3 b	0	67.0 b
Guy11	Wild type	Wild type	Three-celled, pyriform	100 a	100 a	100 a

[a]See Figure 1 for detalls. [b]Radial growth was measured 9 d after incubation on oatmeal agar at 26 °C. Average of four replicates expressed as percent of Guy11. Values with the same letter are not significantly different at P=.05 level. [c]Percent appressorium formation was average of appressoria formed per germlings observed in three slide preparations. Approximately 100 germlings observed per slide and expressed as percent of Guy11. Values with the same letter are not significantly different at P=.05 level. [d]Sporulation assayed under illumination and expressed as percent of Guy11. Values with the same letter are not significantly different at P=.05 level. [e]na=not applicable.

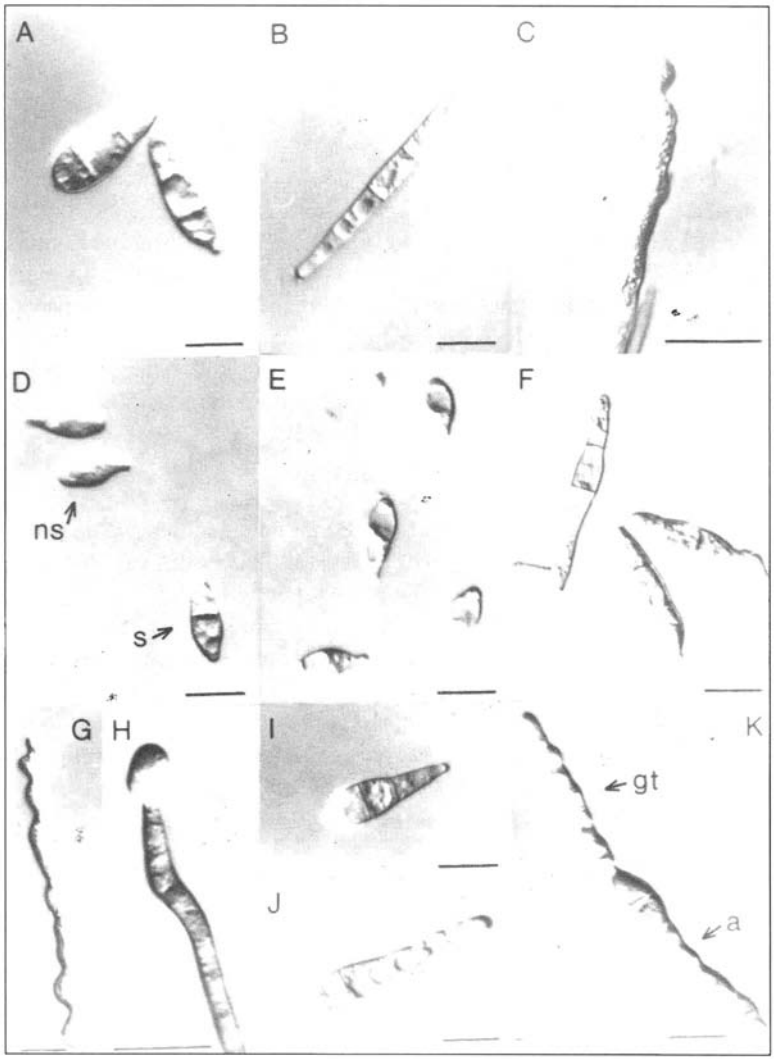

Fig. 1. Morphology of *Magnaporthe grisea* sporulation mutants. A) Wild type. Three-celled, pyriform conidia of wild type. B) Con1$^-$. Elongated conidia. C-D) Con2$^-$. Only conidium initial formed in the absence of direct illumination (C). Under continuous illumination, conidia with single (s) or no septum (ns) are formed (D). E) Con3$^-$. Conidia formed in the dark. Conidium morphology identical to that of Con2. F) Con4$^-$. Slightly elongated conidia with a narrowing of the basal cell. (G-H) Con6$^-$. Conidiophore only (g). Occasionally, a swollen hyphal tip resembling a conidium initial (H) is formed, but no mature conidia are produced. I-K) COn7$^-$. A variety of spore forms is produced: wild type-like conidium (I) and conidium with an attenuated tip (J). Conidium with an attenuated tip (a) produces a stout germ tube (gt) (K). Scale bar = 5 µm. Reproduced from Shi and Leung (1996) with permission from APS Press.

Table 2. Pathogenicity of sporulation mutants of *Magnaporthe grisea* on rice line 51583.

Mutation	Inoculation method[a]		Latent period[b]
	Spray	Wounding	
Wild type	17.8 a	+	6
Con1-B$^-$	0	0	na[c]
Con2$^-$	1.2 b	+	8
Con4$^-$	3.3 b	+	8
Con7$^-$	0	0	na

[a]Plants were spray-inoculated with 5×10^5 spores. Average number of lesions per inoculated leaf. Data pooled from three experiments. About 18 plants were scored per mutant. Values with the same letter are not significantly different at P=.05 level. Wound inoculation involved injecting leaf sheath with a 10^5 spore ml^{-1} suspension. Only qualitative assessment of lesions was made: + = presence of lesions, 0 = no lesion. Experiments were repeated three times. [b]Days from inoculation to the appearance of a mature lesion (around 50 mm long). [c]na = not applicable.

impaired infection efficiency (more spores are required to produce lesions) and colonization ability (restricted or slow fungal growth in planta). Pathogenicity of the *con1* and *con7* mutants is completely lost, and cannot be compensated for by wounding—suggesting that the defects in pathogenesis occur after the penetration stage.

Epistatic relationships among most of the morphogenetic genes have been established by genetic crosses and gene replacement. Genetic analysis suggests that *con5* is epistatic to *con6* and *con7,* and *con2* is epistatic to *con1* (Leung and Shi 1994, Shi and Leung 1994, 1996). For mutants that are intersterile, interactions were determined using gene replacement techniques. Results indicate that morphogenetic loci interact at multiple levels affecting spore morphology, appressorium formation, and pathogenicity, and that epistatic relationships are trait-dependent (H. Leung, Z. Shi, and D. Christian, unpubl. data). From these analyses, we conclude that there is a central pathway *(con5 > con6 > con2 > con1)* determining the formation of the conidiophore and basic conidial structure. Genes in this central pathway interact with other genes *(con4* and *con7)* to determine morphogenesis and pleiotropy. Thus, morphogenetic genes in *M. grisea* appear to function in a genetic network rather than in a two-dimensional pathway (Loomis and Sternberg 1995).

Identification of genes controlling pathogenicity

Parallel to the analysis of sporulation, we searched for mutations that affect pathogenesis by directly screening integrative transformants on plants. Six hundred and fifty integrative transformants of strain 2539 were screened for reduced pathogenicity on weeping lovegrass. Weeping lovegrass has been long used because

it is highly susceptible to strain 2539, which facilitates the identification of genes that have quantitative effects on pathogenesis. Four mutants exhibiting reduced pathogenicity on weeping lovegrass were isolated and shown by genetic analysis to be tagged by plasmid insertion. Until the biochemical nature of these mutations is known, we tentatively designate the genes as path-531, *path-144, path-194,* and *path-361*.

Cloning of developmental and pathogenicity genes by plasmid rescue

Gene cloning is greatly facilitated by the genetic tagging of mutations. Plasmids containing flanking genomic sequences can be readily rescued from mutant genomic DNA. As of now, we have retrieved plasmids containing inactivated genes from two morphogenetic mutants (*con4, con3*) and three pathogenic mutants (*path-531, path-144, path-194*). The identity of four of these genes (except *path-194*) have been confirmed by gene replacement. Based on Southern blot analysis, all gene replacement mutants are the result of double crossovers by homologous recombination.

Using flanking sequences from the retrieved plasmids, we have isolated cosmid clones from a genomic library of Guy11 constructed in pAN7-2. To complement the insertional mutants (which are hygromycin-resistant), we have developed an alternative transformation system using the vector pBARGEM7-2 (Pall and Brunelli 1993), which contains the bar gene that confers resistance to phosphinothricin (Leung et al 1995). Transformants with wild-type spore morphology were recovered from *con4* and *con7* mutants transformed with the respective cosmids. Genetic analysis confirmed that these wild-type transformants are indeed mutants complemented by cosmids. We have further shown that the complementing cosmid restores full pathogenicity in the *con7* mutant. The evidence gathered thus far indicates that we have isolated the wild-type sequences of the *con4* and *con7* loci. Complementation experiments are in progress with putative cosmids corresponding to other morphogenetic and pathogenicity genes.

Using the DNA fragments of the *con4* and *con7* genes as probes, we recovered cDNA clones from a spore-specific cDNA library (provided by Dr. J. Hamer, Purdue University). The putative cDNA clones of the *con4* and *con7* genes contain inserts of approximately 2.5 kb. Preliminary sequence analyses of both cDNAs reveal no extensive homology with reported genes in the GenBank and EMBL databases, suggesting that *con4* and *con7* are possibly new genes. Computer analysis is in progress to locate functional motifs within the predicted gene products.

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Mapping avirulence genes in the rice blast fungus *Magnaporthe grisea*

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J.L. Notteghem, and M.H. Lebrun

The fungal pathogen *Magnaporthe grisea* is involved in specific interactions with rice cultivars. Fungal strains with avirulence genes are nonpathogenic to rice cultivars with the corresponding race-specific resistance genes. Unraveling the molecular basis of such interactions requires the cloning of both avirulence and resistance genes. We have identified three genetically independent avirulence genes in *M. grisea* (*Avr*MedNoï-1, *Avr*Irat7-1, *Avr*Ku86-1). Analysis of the segregation of avirulence toward a collection of rice cultivars suggests the occurrence of the same resistance gene in unrelated cultivars. To clone avirulence genes by chromosome walking, we constructed a partial genetic map using 77 random progeny from the backcross between isolates Guy11 and 2/0/3. This map includes 75 restriction fragment length polymorphism markers corresponding to either repeated, single-copy, or telomeric sequences and 25 random amplified polymorphic DNA (RAPD) markers. Two avirulence genes were mapped to chromosome tips *(Avr*MedNoi-1 and *Avr*Ku86-1). We also identified five RAPD markers tightly linked (1-4 cM) to these avirulence genes using bulk segregant analysis. Most of these linked RAPD markers were composed of repeated sequences adjacent to single-copy sequences, which could be used to initiate chromosome walks.

Breeding for resistance has shown that many rice cultivars have major genes conferring complete resistance toward specific races of the rice blast fungus *Magnaporthe grisea* (Kiyosawa et al 1986). This cultivar specificity is controlled on the pathogen side by avirulence genes corresponding to specific resistance genes in rice, in a gene-for-gene relationship that was first described in the pathosystem flax/*Melampsora lini* (Flor 1971). While significant progress has been made on the genetic basis of disease resistance in rice and avirulence in *M. grisea* (Zeigler et al 1994), the study of these interactions at a molecular level requires cloning of both resistance and avirulence genes.

Two main strategies were used to clone avirulence genes in fungi. The first strategy is map-based cloning, which implies isolation of a linked marker followed by identification of overlapping cosmids spanning the marker and the avirulence gene. This strategy allowed the cloning of two avirulence genes in *M. grisea*, *Avr*2-YAMO and *Pwl*2 (Sweigard et al 1995), whose functions are unknown up to now. The second strategy requires the isolation of an elicitor specific to the resistant cultivar, whose biosynthesis should be under the control of the avirulence gene. This strategy was successfully used in isolating peptide elicitors encoded by avirulence genes of the tomato pathogen *Cladosporium fulvum* (*avr*4 and *avr*9; De Witt 1992) and the barley pathogen *Rhynchosporium secalis* (*nip*1; Rohe et al 1995). To clone new avirulence genes in *M. grisea*, we used a map-based cloning strategy. We performed a genetic analysis of avirulence using *M. grisea* field isolates pathogenic to rice. Such crosses were used to identify molecular markers closely linked to avirulence genes. These markers will be the starting point of chromosome walks toward avirulence genes.

Genetics of avirulence in *M. grisea*

For a long time, genetic analysis in *M. grisea* was limited to hermaphroditic isolates that were pathogenic to grasses other than rice, since most of the isolates pathogenic to rice are female-sterile (Notteghem et al 1992). Two strategies were developed to obtain fertile *M. grisea* isolates that are pathogenic to rice (Zeigler et al 1994).

The first strategy involved crosses between hermaphroditic isolates pathogenic to hosts other than rice followed by crosses with isolates pathogenic to rice and leading to laboratory strains with improved fertility (Zeigler et al 1994). This strategy allowed the identification of avirulence genes either from the nonrice parental isolate or from the rice pathogen (Zeigler et al 1994).

The second strategy involved finding a few hermaphroditic isolates pathogenic to rice in a worldwide survey of *M. grisea* populations. Such fertile rice isolates were crossed with ricefield isolates leading to the identification of new avirulence genes (Zeigler et al 1994).

Using this strategy, we crossed a hermaphroditic isolate from South America, Guy11, with a female-sterile isolate from Africa, ML25 (Silue et al 1992a,b). To generate a large number of progeny, we focused on the more fertile backcross (2/0/3 × Guy11), in which three genetically independent avirulence genes were characterized: *Avr* MedNoï-1, *Avr* Irat7-1, and *Avr* Ku86-1. All progeny avirulent to rice cultivar MedNoï were also avirulent to cultivar Cica 8. Likewise, progeny avirulent to rice cultivar IRAT7 were also avirulent to cultivar DJ8-341 and Carreon. Such a cosegregation could be due to a strong genetic linkage between two avirulence genes. Alternatively, the same gene could be responsible for avirulence toward two different cultivars. This situation can be explained by a gene-for-gene relationship, already demonstrated in *M. grisea* (Silue et al 1992a). That is, one particular avirulence gene interacts specifically with its corresponding resistance gene present in different cultivars.

Mapbased cloning of avirulence genes in *M. grisea*

Mapping avirulence genes

To clone avirulence genes by chromosome walking, we constructed a partial genetic map using 77 random ascospores from the cross between isolates Guy 11 and 2/0/3. This map included 75 restriction fragment length polymorphism (RFLP) markers obtained with probes such as the repeated and dispersed sequences MGR583 and MGR586 (Hamer et al 1989), cosmids mapped (Sweigard et al 1993), and a telomere oligonucleotide (Farman and Leong 1995). We also mapped random amplified polymorphic DNAs (RAPDs) (Williams et al 1990), three avirulence genes, and the mating type locus *Mat*1.

Among 80 RAPDs generated by 280 operon primers, we identified five RAPDs linked to avirulence genes using bulk segregant analysis (Michelmore et al 1991). We also found one RFLP marker linked to avirulence gene *Avr*Irat7-1, which corresponded to cosmid A11D9 already mapped on chromosome 1 (Sweigard et al 1993). Overall, each avirulence gene is closely linked to at least one molecular marker (Fig. 1). Mapping telomeric RFLPs showed that two avirulence genes, *Avr*MedNoï-1 and *Avr*Ku86-1, were located near the chromosome tips (3-5 cM). Two other *M. grisea* avirulence genes were mapped near the chromosome tips (Sweigard et al 1993).

Overall, four of the eight mapped avirulence genes are subtelomeric. Such a chromosomal location might not be due to chance. In yeast, these regions are subject to frequent rearrangements (Louis et al 1994). This situation occurred at the subtelomeric *Avr*2-YAMO locus, where 70% of the spontaneous virulence mutations are due to deletions of the chromosome tip (Zeigler et al 1994).

Characterization of molecular markers linked to avirulence genes

The RAPD fragments linked to avirulence genes were cloned and characterized by their genomic hybridization patterns. OPG18 (0.7 kb), mapping 4 cM from *Avr*Irat7-

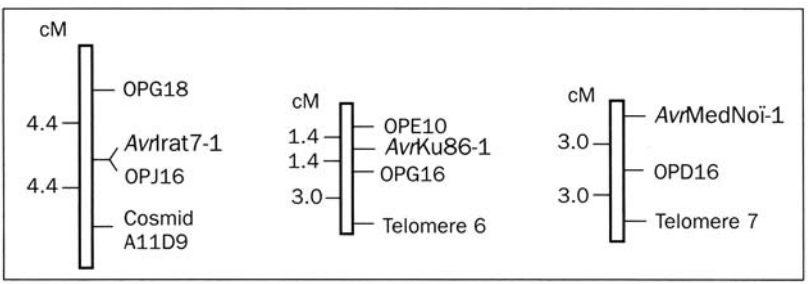

Fig. 1. Genetic maps of avirulence gene linkage groups. OPX refers to operon oligonucleotide number X, used for RAPD. Telomere Y refers to telomeric RFLP Y obtained with an oligonucleotide corresponding to three tandem repeats of the telomere sequence. Segregation analysis of molecular markers and avirulence genes was performed using 77 progeny from the cross Guy 11 x 2/0/3. Markers were mapped using MAPMAKER v2.0. Distances are in centimorgans (cM). Cosmid A11D9 is located on chromosome 1 of *M. grisea* strain 6043 (Sweigard et al 1993).

1, gave a pattern typical of a repeated sequence. OPJ16 (0.7 kb) was completely linked to *Avr*Irat7-1 and also gave a pattern typical of a repeated sequence. Subcloning of OPJ16 produced a 0.4-kb *Hin*dIII fragment giving the same pattern as the whole marker, while the 0.3-kb *Hin*dIII fragment revealed another repeated sequence.

The avirulence gene *Avr*Ku86-1 is located between the two RAPD fragments OPE10 (1.7 kb) and OPG16 (1.6 kb). OPE10 and OPG16 both correspond to distinct repeated sequences. When subcloning OPE10 with *Hin*dIII, we found that the smallest subclone (0.6 kb) corresponds to a single-copy sequence in parent 2/0/3. and to a duplicated sequence in parent Guyl1. Finally, the RAPD fragment OPD16 (0.3 kb), mapping 3 cM from avirulence gene *Avr*MedNoï-1, gave a pattern typical of a single-copy sequence. As this sequence was only detected in parent 2/0/3 (avirulent on cultivar MedNoï), we assume that it is deleted in parent Guy11 (virulent on cultivar MedNoï).

Overall, the majority (80%) of the RAPD fragments linked to avirulence genes correspond to repeated sequences. This situation could reflect that most of the polymorphisms between the two parental isolates involved repeated and dispersed sequences. It also reflects that avirulence genes might be located in chromosomal regions rich in repeated sequences, as already shown for *Pw12* (Sweigard et al 1995) and *Avr*2-YAMO loci (Zeigler et al 1994).

Conclusions

M. grisea field isolates pathogenic to rice and fertile in crosses are now available for genetic studies of avirulence. We have identified three avirulence genes in a backcross between the two isolates pathogenic to rice, Guy 11 and 2/0/3. Progeny of such crosses are useful for detecting resistance genes in rice cultivar using avirulence cosegregation analysis. These progeny are also needed for isolating avirulence genes using a map-based cloning strategy. Two avirulence genes are located near the chromosome tips, which may be highly variable regions. We found five RAPDs and three RFLPs linked to the three avirulence genes in segregating in our cross. Most of these markers corresponded to repeated sequences. However, subcloning single-copy junction fragments was possible for several linked RAPDs. Such single-copy fragments are the best candidates to initiate chromosome walks toward mapped avirulence genes.

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A giant gene cluster for an antifungal antibiotic and its potential relevance to rice biotechnology

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Genes for biosynthesis of a *Streptomyces* sp. FR-008 heptaene macrolide antibiotic with antifungal and mosquito larvicidal activity were cloned in *Escherichia coli* using heterologous DNA probes. The cloned genes were implicated in heptaene biosynthesis by gene replacement. The FR-008 antibiotic contains a 38-member, polyketide-derived macrolide ring. Southern hybridization using probes encoding domains of type I modular erythromycin polyketide synthase (PKS) showed that the Streptomyces sp. FR-008 PKS gene cluster contains repeated sequences spanning about 105 kb of contiguous DNA. Assuming about 5 kb for each PKS module, this is in striking agreement with the expectation for the 21-step condensation process required for synthesis of the FR-008 carbon chain. The methods developed for transformation and gene replacement in *Streptomyces* sp. FR-008 make it possible to genetically manipulate polyene macrolide production, and may later lead to the biosynthesis of novel polyene macrolides. A sequenced portion of the pathway is being tested for its potential expression in rice.

Most known antibiotics and many other bioactive molecules originate from members of the genus *Streptomyces* and related actinomycete bacteria. Antibiotics are low-molecular weight substances, requiring the action of many enzymes for their stepwise biosynthesis. Antibiotic biosynthetic genes of actinomycetes are in all known cases tightly clustered and usually located on the bacterial chromosome rather than on plasmids (Martin and Liras 1989). Antibiotics belong to many chemical families. One of these embraces the polyketides which are formed, similar to fatty acids, by the condensation of simple carboxylic acid units (typically residues of acetate, propionate, or butyrate) by polyketide synthases (PKSs) (O'Hagan 1991, Robinson 1991).

Several gene clusters encoding the enzymes of polyketide biosynthesis have been cloned partially or completely. Aromatic polyketides are synthesized by type II PKSs,

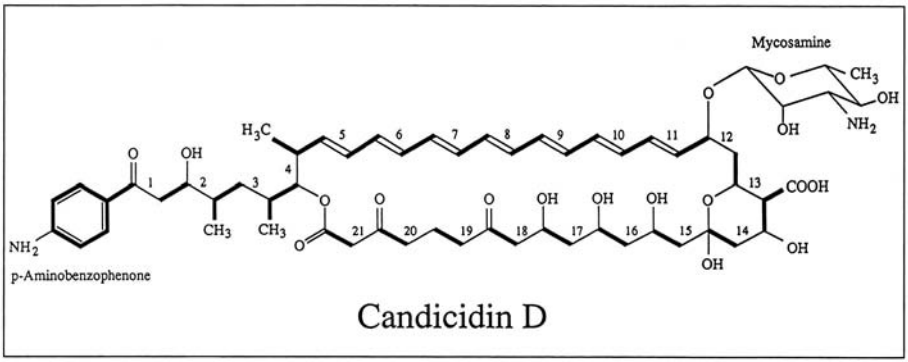

Fig. 1. Candicidin D (Omura and Tanaka 1984), a heptaene macrolide that differs from the FR-008 macrolide only by the sugar residue (mycosamine). The CoA ester of p-aminobenzophenone, the latter the product of the *pabAB* gene, is presumably the starter unit for polyketide synthesis. The 21 bonds presumably formed by the polyketide synthase are numbered and bold lines indicate the building units.

which are multi-enzyme complexes similar to the type II fatty acid synthases (FASs) of *Escherichia coli* and higher plants, and consisting of a series of polypeptides each used iteratively at every relevant step in carbon chain assembly and modification. In contrast, the polyketide chains of macrolides such as erythromycin and avermectin are synthesized by type IPKSs consisting of large multifunctional polypeptides similar to the type I FASs of vertebrates but consisting of repeated units or "modules" with each active site carrying out only one reaction in the assembly and modification of each carbon chain (Hopwood and Sherman 1990, Katz and Donadio 1993).

Polyene macrolides are a group of polyketides that interact with membrane sterols and are therefore active against fungi but not against bacteria (Hamilton-Miller 1973; Martin 1977, 1984; Gale 1984; Omura and Tanaka 1984). *Streptomyces* sp. FR-008 (Liang and Zhou 1987) produces a polyene macrolide with both potent antifungal activity and high toxicity for mosquito larvae. Yuan and Zhou (1990, 1991) found that this antibiotic (FR-008) is a heptaene macrolide with the same 4-aminoacetophenone-containing aglycone as candicidin D from *S. griseus* (Fig. 1). The precise structure of the carbohydrate moiety of FR-008 is unknown, but it differs from mycosamine and perosamine, found in other heptaene macrolides (Martin 1977).

Here, we describe the cloning of a large *Streptomyces* gene cluster involved in biosynthesis of the polyketide that forms the 38-member heptaene macrolide ring (aglycone) of FR-008 and show that it contains repeated PKS "modules." We also describe some preliminary work toward the expression of this gene cluster in rice.

Results

Cloning of *Streptomyces* sp. FR-008 heptaene macrolide biosynthetic genes in *E. coli*

Streptomycetes are naturally resistant to antifungal polyenes and so there was no possibility of cloning a resistance gene and no nonproducing mutants were available for cloning by complementation. Heterologous cloning of the whole pathway in *S. lividans* (Chen et al 1988) failed. Identification of genes by DNA hybridization therefore seems to be the best strategy.

Probing Southern transfers of total DNA of *Streptomyces* sp. FR-008 DNA with the labeled *pabAB* gene of *S. griseus* involved in biosynthesis of the starter unit for candicidin D assembly (Gil et al 1990) gave a strong signal at high stringency (0.1 × SSC, 68 °C). To isolate the hybridizing sequence, a genomic library of FR-008 DNA was constructed in the bifunctional cosmid vector pKC505. One cosmid, pHZ119 (Fig. 2), with a 34-kb insert of *Streptomyes* sp. FR-008 DNA, hybridized strongly to

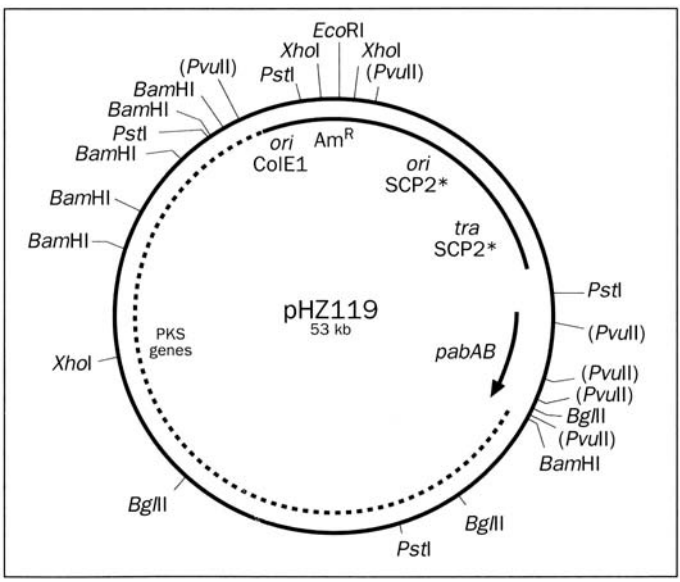

Fig. 2. pHZ119 and its derivative pHZ135. pHZ119 is a derivative of the cosmid vector pKC505 (indicated by continuous line) containing a 34-kb *MboI* (partial digestion) insert of FR-008 total DNA. Restriction sites are put in parentheses to indicate that not all the sites in the plasmid are shown. Am^R, apramycin resistance gene (also confers resistance to geneticin); ori, origin of replication; *pabAB,* gene involved in para-aminobenzoic acid biosynthesis, the orientation was deduced from the known orientation of the very similar *S. griseus pabAB* gene (Gil and Hopwood 1983); PKS, polyketide synthase; tra, transfer genes.

the pabAB probe at high stringency and was chosen for further analysis. The internal *Pvu*II fragments of the insert were the same size as the homologous *Pvu*II fragments of FR-008 total DNA, indicating that the cloned DNA was not (grossly) rearranged.

The CoA ester of para-aminobenzoic acid is the starter unit for candicidin synthesis (Gil et al 1980). It was thus hoped that gene(s) encoding the heptaene PKS might be close to the *pabAB* genes of strain FR-008. This was confirmed by hybridization of pHZ119 DNA with the labeled 10-kb fragment from the *DEBS2* erythromycin PKS gene. More than 20 kb of the pHZ119 insert downstream of *pabAB* hybridized with the ery probe (0.5 × SSC, 60 °C). Moreover, digestion of FR-008 total DNA with *Bam*HI+*Bgl*II and probing with the 10-kb ery probe gave multiple-hybridizing bands, indicating a large number of partially homologous PKS-encoding sequences. To clone DNA flanking that in pHZ119, a new cosmid library was generated in pHZ132. Two clones, pHZ138 and pHZ145 (Fig. 3), hybridized with the *S. griseus* pabAB probe and contained mainly DNA from upstream of the *pabAB* gene, which did not hybridize with the ery PKS probe. Two other cosmids, pHZ137 and pHZ151 (Fig. 3), largely

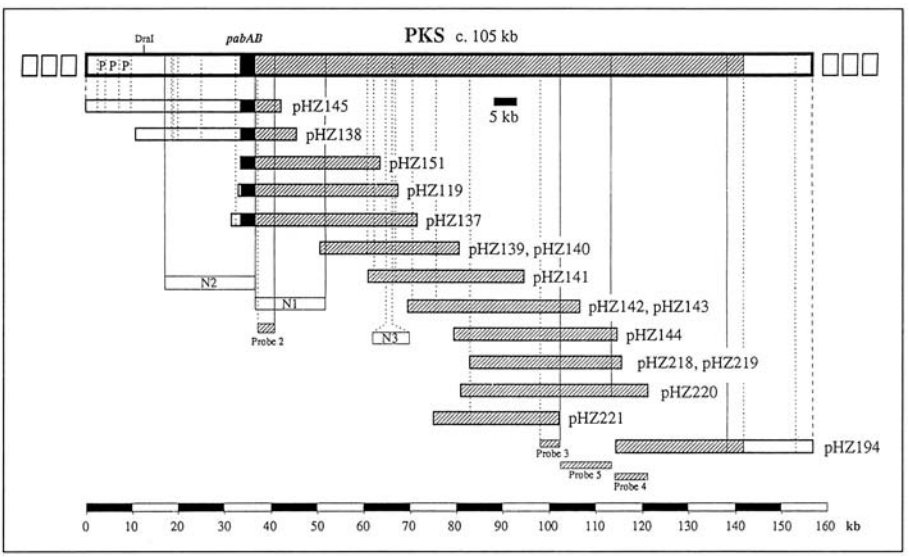

Fig. 3. Overlapping cosmids. Top: Representation of the part of the *Streptomyces* FR-008 genome-containing genes for the biosynthesis of the FR-008 heptaene macrolide. Vertical continuous and dotted lines indicate the positions of *Bgl*II and *Bam*HI sites. The boxes below indicate individual cosmid clones. Shading indicates *Bam*HI+*Bgl*II fragments hybridizing with the *Saccharopolyspora erythraea* ery PKS probes. Probes 2-4 were used to detect some of the clones in the gene libraries. Disruption of fragments labeled P did not affect FR-008 production; disruption or replacement of sequences labeled N abolished production. The following cosmid vectors were used: pKC505, pHZ119; pHZ132, pHZ137- pHZ144, pHZ194; plJ653, and pH2218 - pHZ221.

overlapped with pHZ119. Further clones were selected on the basis that they hybridized with the 10-kb ery PKS probe, but not (at high stringency) with the 3.8-kb *Bam*HI-*Bgl*II fragment labeled "probe 2" in Figure 3. These clones, pHZ139-pHZ144, partially overlapped with each other and formed a continuous overlapping series (a 'contig') with the other cosmids. Even the rightmost part of this contig hybridized with the ery probe, indicating that the end of the PKS cluster had not been reached.

Further cosmids were isolated by probing a pIJ653-based cosmid library with the labeled *Bam*HI-*Bgl*II fragment from pHZ144 (probe 3 in Figure 3). Restriction mapping showed that some of the cosmids thus obtained (pHZ218-pHZ221) extended the contig to the right, but still not beyond the PKS gene cluster. The rightmost part of pHZ220 (probe 4 in Figure 3) was used to isolate the pHZ132-derived cosmid pHZ194. At least 15 kb of the rightmost sequences of pHZ194 did not hybridize with the 10-kb ery PKS probe, suggesting that the end of the PKS gene cluster had been reached. The 3.3-kb *Bam*HI-*Bgl*II "end fragment" of the PKS gene cluster hybridized to probe 5, but not to probe 4. The hybridization studies were refined by using small probes encoding the ACP5, AT1, and KS1 domains of the ery PKS. All three probes gave multiple strong signals, indicating that there were 10 or more similar DNA sequences present in the DNA region covered by the overlapping cosmid clones, consistent with the hypothesis of repetitive PKS modules.

The whole contig consists of 16 cosmid clones and spans about 155 kb of *Streptomyces'* DNA. About 105 kb of continuous DNA hybridized to the ery PKS probes. The whole PKS gene cluster was probably contained by these cosmids because 35 kb to the left of the *pabAB* genes and 15 kb at the other end of the cluster did not hybridize to the ery PKS probes. Experiments involving gene disruptions and gene replacements had also been conducted to confirm that this gene cluster is indeed involved in heptaene biosynthesis.

Construct for the trial expression of PKS gene in rice

The 3 to 8 kb DNA immediately downstream of the *pabAB* genes was sequenced with the expectation to locate the beginning or the end of a multifunctional (type I) *PKS* gene. Sequence comparison with the protein data bases revealed part of a long open-reading frame with good similarity to the type I *PKS* genes (acyl transferase and ketosynthase functional units) involved in the biosynthesis of the antibacterial macrolide erythromycin (Bevitt et al 1992, Donadio and Katz 1992). This confirmed our hypothesis that the FR-008 antibiotic was synthesized by type I PKS. To see whether the *FR-008 PKS* gene can be expressed in rice, the sequence information was thus used to make a construct for expression in rice. Advised by Dr. Roger Hull and Dr. Paul Christou at the John Innes Centre, we chose a vector containing the CaMV35S promoter with the enhancer from ADH (alcohol dehydrogenase of maize) intron, a polyadenylation site from the *Agrobacterium* Ti plasmid nopalin synthetase gene, and a hygromycin resistance gene. The construct (Fig. 4) is now ready for introduction into rice. In parallel, the same PKS region (Fig. 4) has been cloned into *E. coli* pETI5b (T7 promoter expression system with amino-terminal His_6 tail for protein purification using nickel affinity columns). The *FR-008 PKS* had been overproduced in *E. coli*

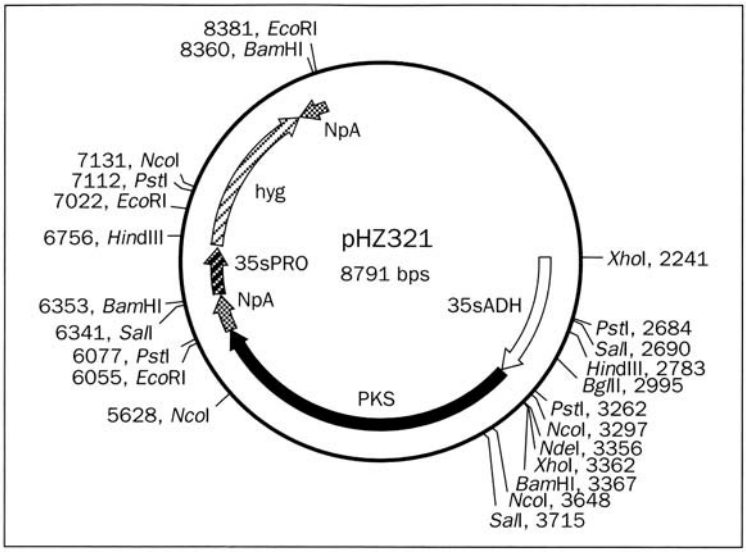

Fig. 4. A construct carrying *Streptomyces* sp. *FR-008 PKS* genes for transforming rice. 35S PRO, CaMV35S promoter; NpA, polyadenylation site from the *Agrobacterium* Ti plasmid nopalin synthetase gene; 35S ADH, enhancer for the 35S promoter from the ADH (alcohol dehydrogenase of maize) intron; *hyg,* hygromycin resistance gene. PKS, polyketide synthase from FR-008. PKS was cloned into WRG2410, a gift from Dr. P. Christou.

and is used to raise antibodies for the detection of *PKS* expression in rice. At this stage, we expect the introduced construct in rice neither to produce a functional enzyme nor to confer fungal resistance to rice, but it will give some indication whether the *Streptomyces PKS* gene, which has an extremely high G+C content (75%), can be expressed in rice.

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Biological and molecular characteristics of rice dwarf and tungro viruses in Nepal

G. Dahal, R. Hull, A. Druka, K. Murao, and I. Uyeda

Results of a recent survey in Nepal indicated the occurrence of rice tungro in some pockets of Janakpur and Bara districts and of rice dwarf in Kathmandu Valley. Both the rice tungro bacilliform virus (RTBV) and rice tungro spherical virus (RTSV) were isolated from rice tungro isolates and the rice dwarf virus (RDV) from rice dwarf isolates. The RTBV and RTSV were transmitted by leafhoppers *Nephotettix virescens* and *N. nigropictus* while RDV was transmitted by *N. nigropictus* only. Results of comparative analyses of viral proteins and nucleic acids of RTBV and RTSV with other tungro isolates from South and Southeast Asia by Western blot analysis and cross hybridization indicated that isolates from Nepal resembled more closely the various tungro isolates from the Indian subcontinent. A similar comparative analysis of RDV dsRNA with isolates from Japan, Korea, and the Philippines indicated that isolates from Nepal and the Philippines had a more distinct electrophoretic pattern than the Japanese and Korean isolates. Further characterization of genomes of both tungro and rice dwarf is in progress.

To date, only two viral diseases have been identified in Nepal—rice tungro, confirmed to be caused by rice tungro bacilliform virus (RTBV) and rice tungro spherical virus (RTSV) (Omura et al 1981) and rice dwarf caused by rice dwarf virus (RDV) (Omura et al 1982). Limited studies on transmission and screening of some local germplasm have been conducted for RDV but no reports are available on rice tungro. Recent studies indicate that there are two strains of rice tungro: one from South Asia and one from Southeast Asia (Fan et al 1995). This paper describes some biological characteristics of Nepalese isolates of rice tungro and preliminary molecular characteristics of the RTBV, RTSV, and RDV genomes.

Materials and methods

During 1992-94, surveys on rice viral diseases were conducted from major rice-growing areas in the southern plains. The incidence of these diseases was determined from randomly selected ricefields with viruslike symptoms. Random leaf samples were collected from the same area and a composite sample was indexed by latex serology (Dahal et al 1990), enzyme-linked immunosorbent assay (ELISA) (Bajet et al 1985), or dot-blot hybridization assay (Dahal et al 1992). Biological characteristics were determined based on transmission by leafhoppers, monitoring of disease in the field, and effect on yield. The virus transmission experiments were done by both test tube (one or three insects seedling^{-1}) and mass inoculation (five insects seedling^{-1}) methods using a colony of leafhoppers collected from Chitwan. The inoculated seedlings were scored visually and indexed by latex serology or ELISA.

For determining the molecular characteristics, the causal viruses were purified, and the viral proteins and nucleic acids of RTBV and RTSV were isolated as described by Jones et al (1991). The coat proteins (CPs) were detected by immunoblotting using antibodies against RTBV and the three CPs of RTSV (CP1, CP2, and CP3) (A. Druka et al, John Innes Centre, unpubl. data). The RTBV DNAs were analyzed by agarose gel electrophoresis, Southern blotting, cross hybridization. and restriction mapping. The RTSV nucleic acids were used to construct first-strand cDNAs by reverse transcriptase-polymerase chain reaction (RT-PCR) using primerpairs from 5' nontranslating region (NTR), the three CPs, and the region downstream of the CPs (DS) of RTSV genome of the Philippine isolate. RDV dsRNAs were analyzed by sodium dodecylsulfate-polyacrylamide gel analysis (Murao et al 1994).

Results and discussion

Occurrence and distribution

Results of surveys in 1993 and 1994 and indexing of composite leaf samples for RTBV and RTSV indicated that rice tungro was distributed in some isolated pockets of Janakpur (Hardinath farm and some areas near the Janakpur town area) and Bara (Parwanipur farm) districts, and rice dwarf was found in the Kathmandu Valley. During 1994, incidence of rice tungro in Parwanipur and Hardinath ranged from about 4 to 32%, while the incidence of rice dwarf in Kathmandu Valley ranged from 0.1 to 20%.

Biological studies

Transmission by leafhoppers. Both the RTBV and RTSV were transmitted by *Nephotettix virescens* and *N. nigropictus,* while the RDV was transmitted by *N. nigropictus* and not by *N. virescens.* RTBV and RTSV were acquired and inoculated in 30 min on adult *N. virescens* and had an incubation period of 7-10 d in rice plants.

Tungro reaction to selected rice cultivars. The cultivars Gampai 30-12-15, TKM6, Utri Rajapan, and Utri Merah had low infection with both RTBV and RTSV but relatively high RTBV infection. Doubly infected plants of cultivars Gampai 30-12-15, TKM6, and TN1 had yellow-orange discoloration, stunted growth, interveinal chlorosis, and leaf twisting, while those of ARC11554, Balimau putih, and Utri Merah

Table 1. Reaction of 15 selected popular rice cultivars to RTBV and RTSV as detected by latex serology in Nepal. IAAS, 1994.

Cultivar	Seedlings tested (no.)	Seedlings (no.) that reacted with	
		RTBV + RTSV	RTBV
Amjhutte	10	4	1
Anadi	6	2	0
Bam Morcha	11	4	1
Chaite 2	11	6	3
Chaite 4	9	6	0
Janaki	9	2	6
Masuli	10	3	2
Pant 4	3	2	0
Pakhe Masuli	6	1	2
Radha 7	3	0	1
Radha 9	10	4	3
Radha 17	10	4	3
Sabitri	10	4	2
Sarju 52	10	3	3
TN1	26	8	10

had slight reduction in height and no leaf discoloration. Plants infected with RTBV alone had milder symptoms than those of doubly infected plants. In the mass inoculation, most of the popular rice cultivars in Nepal were highly infected (Table 1) and very few cultivars had infection of less than 40%.

Disease development and their effect on yield. In the field studies, rice tungro symptoms appeared only during the main rice season (September) and the disease spread at the rate of 0.1 to 0.55 hills d^{-1} and attained about 32% terminal incidence, and reduced grain yield by 54 g $panicle^{-1}$. The RDV symptoms appeared during August, spread at the rate of 0.05 to 0.41 hills d^{-1} and attained 20% terminal incidence, and reduced yield by 83 g $panicle^{-1}$.

Purification and electron microscopy (EM)

Crude unfractionated preparations of RTBV and RTSV and RDV were obtained as described by Jones et al (1991) and Uyeda and Shikata (1982), respectively. When observed under EM (Roberts and Harrison 1979), the unfractionated preparation from tungro contained both RTBV and RTSV particles, while RDV particles were predominant in the preparations from rice dwarf plants. The diameter and length of the RTBV particles ranged from 24 to 38 nm (average 29.2 nm), and from 124 to 238 nm (average 166 nm), respectively. The diameter of the RTSV particles ranged from 28 to 40 nm (average 34 nm), whereas that of RDV ranged from 60 to 81 nm (mean about 70 nm).

Molecular characteristics

Viral proteins. In the immunoblots, the CP of RTBV reacted with the antiserum against RTBV, giving bands of 37 kDa similar to the RTBV CPs of a Philippine isolate of tungro. A comparative analysis of the CPs of Nepalese and Philippine RTSV

preparations by Western blot analysis indicated no difference in relative intensity and size distribution of signals for different CPs.

Viral nucleic acids—RTBV DNA. In comparative Southern blot analyses of virion DNA of RTBV from Nepal (RTBV DNA-N) with that of RTBV from India using a unique cutting restriction enzyme, *Bam*HI, both the genomes were found to have similar sizes (about 8 kbp). Further comparison of RTBV DNA-N by PCR and hybridization analyses revealed that the RTBV DNA-N was comparable more with the South Asian isolates (Bangladesh, India, Sri Lanka) with a deletion and hybridized more strongly with a probe made from RTBV (Indian, Delhi isolate) than from the Philippine isolate. The RTBV genome was cloned onto plasmid pUC18, and the approximate position of four restriction endonucleases (*Bam*HI, *Eco*RI, *Hin*dIII, and *Pst*I) with respect to a published map of the Philippine isolate was mapped (Fig. 1). Comparative studies are in progress to further characterize the genome.

Viral nucleic acids—RTSV RNA. In the PCR analysis, all the primer sets amplified RTSV RNA from both Nepalese and Philippine isolates and the expected sizes of products were obtained. The results revealed no significant variation between the two isolates. For further characterization, the CP regions from both isolates have been cloned in pBluscript t-tailed vector and their nucleotide sequencing is in progress.

Viral nucleic acids—RDV RNA. The RDV dsRNA isolated dwarf rice plants and a rice weed (*Echinochloa crus-galli*) from Kathmandu Valley had 12 segments similar

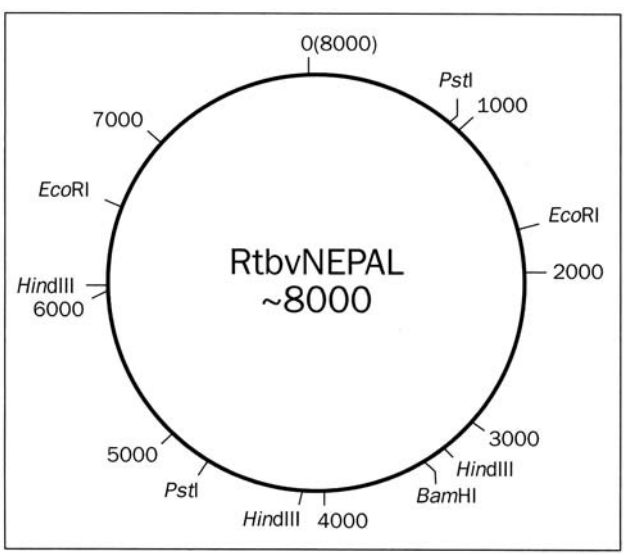

Fig. 1. A restriction endonuclease map of an RTBV DNA clone from a tungro isolate (Hardinath). The genome was cloned onto pUC18 on *Pst*I site and approximate position of restriction endonuclease sites was mapped in relation to the published map of the Philippine isolate.

to those reported for RDV dsRNA from Japan. A comparative analysis of genomic profile of RDV dsRNA with isolates from Japan, Korea, and the Philippines indicated that isolates from Nepal and the Philippines had more distinct electrophoretic profiles characterized by distinctly slower mobility of segment 8 (S8) than those of the Japanese and Korean isolates. For further characterization, cloning of S8 of RDV dsRNA and the analysis are in progress.

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Detection and analysis of genetic polymorphism in *Xanthomonas oryzae* pv. *oryzicola* using repetitive sequence-based polymerase chain reaction

A.K. Raymundo, M.T. Perez, and J.E. Leach

Repetitive sequence-based polymerase chain reaction was used both for identification and for analysis of genetic diversity of *Xanthomonas oryzae* pv. *oryzicola (Xcola),* the rice bacterial streak pathogen. Using ERIC primers, genetic polymorphism was detected among strains of *Xcola* and *X. oryzae* pv. *oryzae (Xoo),* a related bacterium that is the causal organism of bacterial leaf blight of rice. These two strains have banding patterns distinct from strains of other *Xanthomonas* pathovars. Using BOX and REP primers, a lesser degree of polymorphism was detected among the strains, but the two primer sets allowed distinction of *Xcola* and *Xoo* from each other and from other pathovars of *Xanthomonas.* BOX primers were most discriminating among pathovars. Patterns after amplification of DNA with primers based on the repetitive element R41, which was isolated from *Xcola,* also allowed differentiation of *Xcola* and *Xoo* by a simple banding pattern.

Bacterial leaf streak (BLS), which was first described by Reinking in 1918, is now one of the major bacterial diseases of rice in tropical and subtropical Asia. In the Philippines, BLS causes an estimated yield loss of 8% in susceptible cultivars (Mew 1988) and in China, yield losses were as high as 30-40%. The bacterium causing the disease is *Xanthomonas oryzae* pv. *oryzicola (Xcola)* (Swings et al 1990).

An important aspect of disease management is detection and monitoring of the pathogen. Conventional bacteriological procedures are not capable of detecting very low levels of *Xcola,* nor can they differentiate *Xcola* from the closely related rice pathogen, *X. oryzae* pv. *oryzae* (*Xoo*). Lack of sensitive detection and identification techniques for the pathogen has hampered the understanding of the epidemiology of BLS.

The development of the polymerase chain reaction (PCR) technology has allowed an increasing number of plant pathogens, both bacterial and fungal, to be detected in

plant parts or in the environment (Henson and French 1993). PCR is an in vitro method of amplifying specific DNA sequences by repeated cycles of heat denaturation of template DNA, annealing of the primers to complementary sequences and extension of the annealed primers by a DNA polymerase (White et al 1989). PCR-based assays are sensitive, rapid, and versatile, allowing for detection of very few bacteria. For example, amplification of DNA from the plant pathogen *Erwinia amylovora* with plasmid sequence-based oligonucleotides yielded a single 0.9-kb product even with only 1-pg DNA (around 100 bacteria) as a template (Bereswill et al 1992). The amplification products were either larger or smaller than those obtained for plant-associated bacteria and other plant pathogens such as *E. herbicola, Agrobacterium tumefaciens, E. carotovora* subsp. *atroseptica, E. carotovora* subsp. *carotovora, E. chrysanthemi,* and *P. syringae,* and were thus diagnostic for *E. amylovora.*

In this paper, the PCR method with primers based on the repetitive elements, REP, ERIC, and BOX, collectively known as rep-PCR (Martin et al 1992, Louws et al 1994) was used to determine the utility of these primers in detection and genetic analysis of *Xcola.* In addition, amplification patterns from primers based on a repetitive element, R41, an element isolated from *Xcola* (Raymundo et al 1995), were compared with those of the rep elements for ability to distinguish *Xcola* and *Xoo.*

Materials and methods

Sources of strains and media used

Xcola and *Xoo* isolates were obtained from the Entomology and Plant Pathology Division at IRRI. The isolates were maintained either in slants of peptone sucrose agar (PSA) (Ou 1972) or modified Wakimoto's medium (Karganilla et al 1973) at 4 °C, or in 5% skim milk at -20 °C. Different pathovars of *X. campestris* and other plant pathogenic bacteria were provided by the Department of Plant Pathology, Kansas State University.

DNA isolation

Xcola and *Xoo* isolates, grown on PSA slants for 2 d, were used to inoculate 30 ml of nutrient broth (Difco) and then incubated in a shaker (100 rpm) overnight at room temperature. DNA was isolated following a modified method of Wilson (1987).

PCR amplification

Amplification reactions using ERIC, REP, and BOX primers listed below (Stern et al 1984, Martin et al 1992) were done to generate genomic fingerprints of different strains of *Xcola, Xoo,* and pathovars of *X. campestris.* The primers, synthesized by Operon Technologies, Alameda, CA, were:

ERIC IR 5' ATGTAAGCTCCTGGGGTGAGCG 3'
ERIC 2 5' AAGTAAGTGACTGGGGTGAGCG 3'
BOX AIR 5' CTACGGCAAGGCGACGCTGACG 3'
REP IR-1 5' IIIICGICGICATCIGGC 3'
REP 2-1 5' ICGICTTATCIGGCCTAC 3'

The reaction mix consisted of 1x Gitshier buffer (Kogan et al 1987), 0.16 mg bovine serum albumin, 10% dimethylsulfoxide (DMSO), 2.5 mM dNTp, 50 pmol of each primer, 0.5 µl of Taq polymerase, 50-100 ng of template and distilled water, in a 25 µl final volume (Louws et al 1994). Amplification was performed using the programmable thermal controller or PTC-100 Model 60 (MJ Research, Inc., Watertown Massachusetts) using the following cycles: an initial denaturation at 95 °C for 7 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at 52, 53, and 44 °C for 1 min with ERIC, BOX, and REP primers, respectively, extension at 65 °C for 8 min and a final extension at 65 °C for 15 min.

Primers R41 PR4 (forward) and R413 (reverse), which are based from the repetitive probe, R41 (Raymundo et al 1995), were synthesized by Operon Technologies, Almeda, CA, and are as follows:

R41 PR4 5' CTCCGCGGCGCAGGGTCGA 3'

R413 5' CCGTGGATCTGTTAGCTG 3'

The reaction mix consisted of l x PCR buffer, 2.5 mM $MgCl_2$, 0.7 mM dNTP, 0.5 µl Taq polymerase, 50 pmol of each primer, 50-100 ng template and distilled water. The amplification conditions were 5 min at 95 °C (initial denaturation), 30 cycles of 2 min at 94 °C, 2 min at 56 °C, 2 min at 72 °C, and 10 min at 72 °C.

Agarose gel electrophoresis

The PCR products were detected by horizontal gel electrophoresis through 1.5% agarose gel in 0.5 x tris-borate-EDTA (TBE) buffer. For rep-PCR gels (REP, BOX, ERIC), Synergel (Diversified, BIOTECH, Inc., MA) was incorporated at an equal proportion with agarose.

Results and discussion

The total genomic DNA of different *Xcola* and *Xoo* strains was isolated and used as template for rep-PCR. The rep-PCR reactions utilized primers based from either ERIC (ERIC IR and ERIC 2), REP (REP IR- 1 and REP 2- l), or BOX (BOX AIR) sequences.

Amplification reactions using these primers yielded multiple PCR products for both pathovars. The resulting PCR products ranged from 200 to 4000 bp with ERIC primer. Four bands, which were common among the six *Xcola* strains tested, distinguished this pathovar from *Xoo.* The fingerprints that the ERIC primers generated for the different *X. campestris* pathovars tested (*X. campestris* pv. *holcicola, X. campestris* pv. *sojense, X. campestris* pv. *cerealis, X. campestris* pv. *carotae, X. campestris* pv. *vasculorum, X. campestris* pv. *pelargonii, X. campestris* pv. *gumisudense, X. campestris* pv. *pheipatensis, X. campestris* pv. *hordei, X. campestris* pv. *heroderae*) and other plant pathogenic bacteria (*Pseudomonas syringae, P. andropogonis, Agrobacterium tumeficiens,* and *Erwinia herbicola*) were distinct from the patterns for the *X. oryzae* pathovars. Not too many common bands were evident among the *X. campestris* pathovars; however, fingerprints generated were distinguishable from the *X. oryzae* pathovars.

Amplification of *X. oryzae* genomic DNA with REP primers produced bands of 100-3200 bp. The approximate number of bands for the six strains of *Xcola* and *Xoo* were 23 and 18, respectively. Of the 23 bands from *Xcola,* three were unique to *Xcola* distinguishing it from *Xoo.* Two of these bands were of low molecular weight (100-500 bp). Nine bands were common in *Xcola* and *Xoo,* two of which were distinct from the *X. campestris* pathovars and other plant pathogenic bacteria.

Amplification with BOX primers yielded approximately 8-12 bands ranging from 200 to 4000 bp for *Xcola* DNA and 14-15 bands with a size range of 200-2500 bp for *Xoo.* The patterns generated were relatively simpler in that there were very few faint bands; the bands were mostly distinct. Three bands were unique to *Xcola.* The genomic fingerprints generated for *X. oryzae* were clearly distinguishable from the *X. campestris* pathovars tested.

The genomic fingerprints of the different strain earlier defined by RFLP and *Pst*I analysis (Raymundo et al 1995) generated using the BOX primers were nearly identical, that is, only a few different patterns were observed. ERIC and REP primers differentiated more patterns between strains of *Xcola* than did BOX. Comparisons of groupings based on the rep-PCR primers and those defined by RFLP and *Pst*I analysis have not been completed. However, since rep-PCR allows both detection of genomic diversity among strains of *Xcola* and distinction of the pathogen from other pathovars and species, the technique is a useful tool for detection, diagnosis, and analysis of genomic diversity of the pathogen. The study confirmed the results of Louws et al (1994) in that the rep-PCR technique was able to discriminate among related but distinct bacterial strains. Likewise, deBruijn (1992) demonstrated that the REP/ERIC PCR method is useful for the identification and classification of bacterial strains.

Another primer set, R41PR4 (forward) and R413 (reverse), which is based on the repetitive probe R41 (Raymundo et al 1995), yielded four distinct PCR products for five *Xcola* strains tested. For the several strains of *Xoo* tested, three PCR products were obtained with sizes similar to those bands generated in *Xcola.* The fourth and uppermost band in *Xcola* strains was consistently missing in the *Xoo* strains tested, hence this distinguished *Xcola* from *Xoo.* Based on this initial test, the R41 primer set is a potential tool for rapid identification of *Xcola.* The advantage of the R41 primers as a diagnostic tool is that the pattern generated is simple, whereas those generated by rep-PCR is complex in terms of the large number of bands produced. Furthermore, the buffer used is much simpler and a lower concentration of the dNTPs is used, thus reducing the cost and the time for amplification (4 h) than when using rep-PCR primers (6 h). However, a large number of isolates or strains and other related bacteria need to be tested to establish reproducibility and applicability of the R41 primers.

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Notes

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Molecular genetic studies of the bacterial leaf blight pathogen of rice in India

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R. Ramanan, A.P.K. Reddy, and R.V. Sonti

Xanthomonas oryzae pv. *oryzae* (*Xoo*) causes bacterial leaf blight, one of the serious diseases of rice in India. Conditions for gene transfer by electroporation as well as by conjugation with donor *Escherichia coli* cells have been standardized for a strain that belongs to the dominant pathotype of *Xoo* in India. A genomic library of this strain, with an average insert size of 30 kb, has been constructed in the broad host range cosmid vector pUFR034. Restriction digestion and Southern hybridization of two randomly selected clones from the library suggest that the cloned DNA is not rearranged when reintroduced into *Xoo.* This library is being used to clone genes encoding virulence functions by complementation of *Xoo* mutants, which exhibit substantially reduced virulence. Evidence has also been obtained for transposition of particular Transposon 5 (Tn5) derivatives in *Xoo.* To understand the population structure of *Xoo* in India, RFLP studies were performed on *Xoo* isolates from 20 different locations in India using *Xoo* repeat element probes. These results suggest that clonal selection for isolates belonging to a single lineage has occurred within the *Xoo* population in India; bacteria belonging to this group can be isolated from widely separated locations in India.

Xanthomonas oryzae pv. *oryzae* (*Xoo*) is the causal agent of bacterial leaf blight, which is one of the serious diseases of rice in India. A research program has been initiated in our laboratory to identify virulence functions of *Xoo* using molecular genetic methods. The strategy is to identify bacterial mutants that exhibit reduced virulence and to clone genes that are defective in these mutants, by functional complementation with clones from a genomic library of the wild-type bacterium. The progress made in the isolation of these mutants and the development of necessary protocols and libraries are described here.

Materials and methods

The *Xoo* strain BXO1 , collected from the Chinsuria, was used as our laboratory wild type. Genomic DNA isolation for library construction was as described by Leach et al (1990), except for the introduction of the extraction with cetyl triethylammonium bromide in order to remove polysaccharides that inhibit ligation reactions during the library construction. Otherwise standard molecular techniques were used.

Results

Gene transfer into an Indian isolate of *Xoo* by electroporation and conjugation

Gene transfer into BXO1 has been obtained both by electroporation and conjugation (bi- and triparental matings) with donor *E. coli* strains. Broad host range plasmids pUFR027 and pUFR034 (DeFeyter et al 1990) and pHM1 have been introduced into BXO1. The protocol for gene transfer by electroporation is as described by Choi and Leach (1994). The protocol for conjugation was a modification of that described by Hopkins et al (1992). To determine if plasmid transfer had occurred into BXO1, plasmids were isolated from donor, recipient, and putative transconjugant strains. The plasmid profile of a BXO1 transconjugant shows presence of the endogenous plasmid and the introduced plasmid pUFR027 (Fig. 1). The composite plasmid profile indicates that pUFR027 has been transferred into BXO1.

Construction of a genomic library of BXO1

A genomic library of BXO1 was constructed in the cosmid vector pUFR034 using DNA that was partially digested with *Eco*RI. The library consists of 2,000 independent clones. The average insert size in this library is 30 kb (Fig. 2). The *Xoo* genome should be represented about 12 times in this library assuming that the *Xoo* genome is 5,000 kb (about the size of *E. coli*).

Clones in the BXO1 genomic library are not rearranged when reintroduced by conjugation into BXO1

Previous studies with *Xanthomonas campestris* pv. *malvacearum (Xcm),* which causes disease on cotton, had indicated that cloned genomic DNA can be subject to substantial rearrangement when reintroduced into *Xcm* (DeFeyter et al 1990). Gross rearrangements of cloned DNA upon reintroduction into BXO1 would interfere with our strategy of identifying virulence functions by functional complementation of virulence mutants. Therefore, two randomly selected clones (pLR1 and pLR2) from the library were reintroduced into BXO1 and the structure of the insert DNA was analyzed by restriction digestion and Southern hybridization of plasmid DNA isolated from the transconjugants.

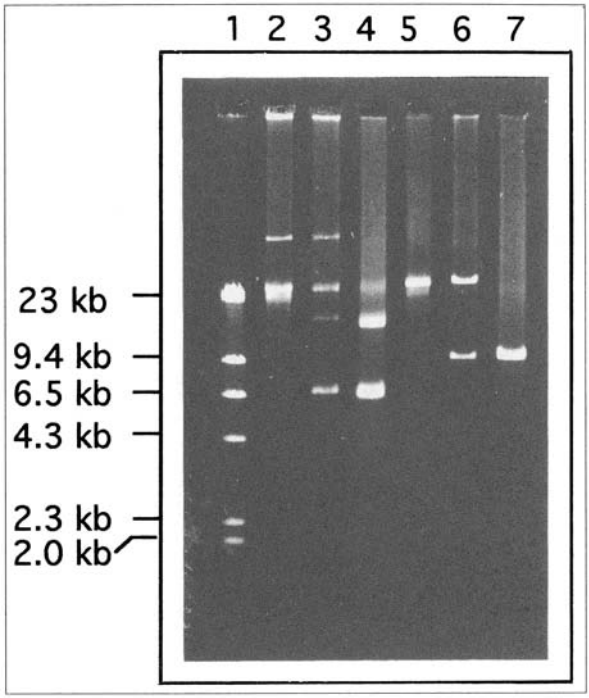

Fig. 1. Plasmid transfer from *E. coli* to *Xanthomonas oryzae* pv. *oryzae* (BXO1). Plasmid DNA was electrophoresed on a 0.7% agarose gel and stained with ethidium bromide. Lane 1: l *Hin*dIII DNA marker; lane 2: endogenous plasmid DNA from BXO1; lane 3: total plasmid DNA from *E. coli* from BXO1/pUFR027 transconjugant; lane 4: plasmid DNA from *E. coli* donor strain (pUFR027); lane 5: *Bam*HI digest of BXO1 endogenous plasmid; lane 6: *Bam*HI digest of plasmid DNA from the BXO1 transconjugant; lane 7: *Bam*HI digest of pUFR027.

Isolation of *Xoo* mutants that exhibit reduced virulence

Two different classes of *Xoo* mutants that exhibit reduced virulence have been isolated and characterized. One class consists of spontaneous mutants that produce reduced levels of extracellular polysaccharides. These mutants were first described 25 yr ago (Goto and Okabe 1967) but the genetic basis of the phenotype has not been characterized. A pigment-deficient mutant isolated after ethyl methanesulfonate mutagenesis represents a second class of mutants that exhibit reduced virulence.

Transposition of Tn5 (Transposon 5) in *Xoo:* TnS-Gus derivatives (obtained from Dr. Kate Wilson, CAMBIA) have been introduced into BXO1 using biparental matings with donor *E. coli* strains in which the transposon is on a suicide plasmid. One of these Tn5-gus derivatives (Tn5*gus*A11) carries the entire *gus* operon of *E. coli* and

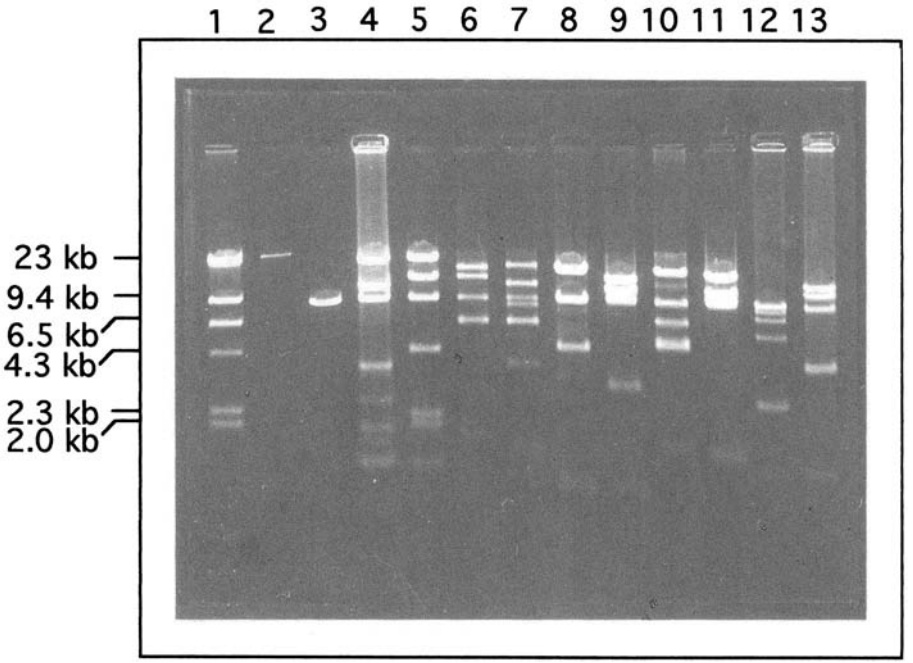

Fig. 2. Unique inserts are present in randomly selected clones of the genomic library of BXO1. Plasmid DNA was digested with *Eco*RI, electrophoresed on a 0.7% agarose gel and stained with ethidium bromide. Lane 1: l *Hind*III DNA marker; lane 2: undigested l DNA; lane 3: cosmid pUFR034 linearized with *Eco*RI; lanes 413: plasmid DNA isolated from 10 randomly picked clones of the BXO1 genomic DNA library and digested with *Eco*RI. The digestion pattern of each of the inserts is unique (lanes 4-13) and the average size of the insert DNA is 30 kb.

Xoo strains that inherit this transposon become GUS^+ and are therefore uniquely marked. Another Tn5-Gus derivative (Tn5*gus*A40) in which the *gus* gene lacks a promoter has been introduced into *Xoo* and is being used to make fusions of the reporter *gus* gene to bacterial promoters. A library of bacterial strains in which the *gus* gene has been inserted at different genomic locations is being built up to identify bacterial genes that are induced by plant defense molecules or other signals that emanate from the rice plant.

RFLP studies of Indian isolates using an *Xoo* repeat element probe

To understand the population structure of *Xoo* in India, we have initiated RFLP studies on 40 *Xoo* isolates collected from 20 different locations across India. One of the probes used in this study is avrXa10, a member of an *Xoo* avirulence gene family (Hopkins et al 1992). A dendrogram obtained from analysis of these data (Fig. 3) indicates that isolates from 13 of the 20 locations sampled belong to a single lineage (at the level of 80% similarity). These results suggest that clonal selection for isolates in this lineage has occurred within the *Xoo* population in India.

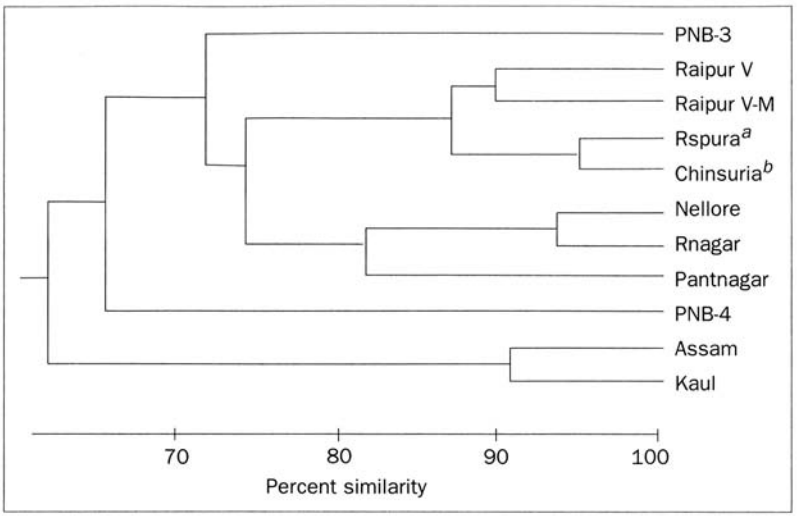

Fig. 3. Dendrogram of Indian isolates of *Xoo* derived from RFLP studies. The dendrogram was constructed using PHYLIP on the basis of RFLP data obtained with avrXa10 as a probe (see Materials and Methods). Forty isolates of *Xoo* were collected from 20 different locations in India.
[a] An isolate from Kurukshetra has an identical haplotype to the isolate from Rspura. [b] Isolates from Cuttack, Kapurthala, Karnal, Maruteru, Punjab-1, Pusa, Raigad, and Titabar have a haplotype identical to that of the Chinsuria strain.

Discussion

We have described the development of molecular genetic methods to understand virulence functions of *Xoo.* Our strategy is to isolate mutants that exhibit reduced virulence and to clone the genes that are defective in these mutants by functional complementation. By analysis of the cloned genes, we hope to understand the role of particular gene products in bacterial pathogenesis. The ultimate objective would be to use the knowledge generated to devise strategies for limiting, or possibly eliminating, yield losses due to this pathogen. The necessity to develop protocols for gene transfer and establishing genomic libraries for an Indian isolate was caused by quarantine regulations that limit the use of Philippine *Xoo* isolates in India. Initially, the protocols followed were those established for the Philippine *Xoo* isolates. Modifications to these protocols were made, when necessary, either due to the unique nature of the strains that we have been using or due to differences in the reagents that were available. A major advance in methodology that we have established is the introduction of Tn5-Gus derivatives into *Xoo.* The results from the DNA fingerprinting studies suggest that a single lineage consisting of closely related individuals has been selected within the *Xoo* population in India.

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Assessment of genetic diversity of *Xanthomonas oryzae* pv. *oryzae* in Nepal

T. Adhikari, R.J. Nelson, T.W. Mew, and J.E. Leach

Two DNA clones from *Xanthomonas oryzae* pv. *oryzae (Xoo), IS1112,* an insertion sequence, and *avrXa10,* a member of a family of avirulence genes, were used as probes to analyze genomes of the 45 strains of *Xoo,* collected from different rice-growing ecosystems in Nepal. Thirty haplotypes were detected when the data from both pJEL101 and pBSavrXa10 were combined. Haplotype diversity was 0.98, suggesting high genetic variability within populations of *Xoo* in Nepal. Virulence of the randomly selected strains was determined by inoculating five differentials and 10 near-isogenic lines. Most of the strains were virulent to cultivars containing the bacterial blight resistance genes, while a majority of the strains was avirulent to *Xa21.*

Bacterial blight, caused by *Xanthomonas oryzae* pv. *oryzae (Xoo),* is a major biological constraint to rice production in Nepal. Major epidemics of the disease have been reported from Nepal in 1979 and in 1987 (IRRI 1979, Adhikari and Shrestha 1990).

Although host plant resistance is an inexpensive and environment-friendly means of disease control, it has often proven unstable. Instability of resistance could be due to several factors, including inadequate screening, such that the released variety is susceptible to preexisting pathogen populations, and/or changes in the pathogen population such that new, virulent populations evolve. Thus, knowledge of pathogen population structure is essential for formulating long-term disease management strategies. Information on pathogen diversity can be used in identifying and characterizing resistant germplasm, and can provide the best strategy for deployment of resistance. In addition, knowledge of the spatial distribution of pathogen subpopulations, for example, can aid in the selection of disease resistance sources for a regional crop breeding program. Unfortunately, such information on pathogen populations and study of molecular variation in Nepal have not been sought out previously. In this study, the population structure of *Xoo* collected from major rice-growing districts of Nepal was as-

sessed by using restriction fragment length polymorphism (RFLP) and virulence analyses.

Materials and methods

Bacterial strains

Sixty-one bacterial strains collected from 18 districts of Nepal during the 1987-94 period were included in this study. The strains were isolated on peptone sucrose agar (PSA) and preserved in 15% glycerol at -80 °C.

DNA probes

Two DNA clones, pJEL101 and pBSavrXa10, were used as probes in this study. Probe pJEL101 is plasmid pUCl8 with a 2.4-kb *Eco*RI-*Hin*dIII fragment that carries all but three nucleotides of insertion element *IS1112* (Leach et al 1990, Yun 1991). Probe pBSavrXa10 contains a 3.1-kb *Bam*HI fragment, which is internal to an avirulence gene from *Xoo* in vector pBluescript II (Hopkins et al 1992).

RFLP analysis

Genomic DNA of 45 bacterial strains was extracted by the lysozyme-sodium dodecyl sulfate lysis method. DNA pellet was dissolved in 40-100 µl TE (100 mM Tris, 1 mM EDTA, pH 8.0). Bacterial DNA was digested to completion with *Bam*HI or *Eco*RI (2 U/µg of DNA) at 37 °C for 2-3 h. Electrophoresis, nick translation, and autoradiography were done according to the procedure of Leach et al (1990).

Each distinct RFLP banding pattern produced by the combined data from both probes was regarded as a "haplotype." Clustering statistics were analyzed by SAS (SAS 1989) and haplotype diversity was calculated by the approach of Nei and Tajima (1981).

Virulence analysis

Virulence of the 45 strains of *Xoo* was assessed by inoculating five differential rice cultivars, IR8 (with the bacterial blight resistance gene *Xa11*), IR20 (*Xa4*), Cas209 (*Xa10*), IR1545-339-2-2 (*xa5*), and DV85 (*xa5, Xa7*) and 10 near-isogenic lines (IRBB1, IRBB2, IRBB3, IRBB4, IRBB5, IRBB8, IRBB10, IRBB11, IRBB14, and IRBB21) each containing a single gene for resistance. The experiment was conducted twice. Plants were grown in plastic pots (8.9 × 8.9 cm). Six fully expanded leaves of each cultivar per pot were clip-inoculated 50 d after sowing. Lesions were measured 14 d after inoculation. Plants having lesions of 0-3 cm were classified as resistant, and those longer than 3 cm were rated as susceptible.

Results and discussion

Two probes, an avirulence gene (*avrXa10*) and an insertion sequence (*IS1112*), were used to analyze genomes of the 45 strains of *Xoo* collected from diverse locations in Nepal. Results of the RFLP analysis are summarized in Table 1. When the data from

Table 1. Haplotype, pathotype, and cluster of *Xanthomonas oryzae* pv. *oryzae* strains in Nepal (Adhikari et al 1995).

Type of strain for each haplotype	Strains per haplotype[a] (no.)	Pathotype[b]	Cluster[c]
NX0101	1	1	5
NX0194	1	4	5
NX0228	2	6	5
NX0334	1	6	5
NX0157	6	6	5
NX0159	2	4	5
NX0149	3	6	5
NX0195	1	4	5
NX0160	1	3	5
NX0161	1	7	5
NX0237	1	6	5
NX0174	3	6	5
NX0196	1	6	5
NX0245	2	6	5
NX0275	1	6	5
NX0282	1	6	5
NX0151	2	6	5
NX0198	1	4	5
NX0200	1	6	5
NX0201	1	6	5
NX0215	2	6	5
NX0256	1	7	5
NX0260	1	6	5
NX0355	1	6	5
NX0347	1	1	5
NX0356	1	3	5
NX0331	1	1	5
NX0240	2	6	5
NX0259	1	7	5

[a] Haplotype was determined from banding patterns observed after hybridization of *Bam*HI-digested DNA with pBSavrXa10 and *Eco*RI-digested DNA with pJEL101. [b] Pathotype was determined by inoculation to the rice differential cultivars IR8 (*Xa11*). IR20 (*Xa4*), IR1545-339 (*xa5*), DV85 (*xa5* and *Xa7*), and Cas 209 (*Xa10*). [c] Cluster is based on analysis of RFLP data after hybridization with both pJEL101 and pBSavrXa10.

both pJEL10l and pBSavrXa10 were combined, a total of 30 haplotypes were detected. On the basis of consensus of three clustering statistics (SAS 1989), the 45 strains constituted a single cluster (Adhikari et al 1995). Haplotypic diversity was relatively high (0.98). The high genetic diversity in Nepal may reflect the relatively broad range of environmental conditions under which rice is grown and the wide use of diverse traditional rice cultivars.

When the virulence data on five IRRI differential rice cultivars were considered as qualitative information (reaction classified as resistant or susceptible), five pathotypes (1, 3, 4, 6, and 7) were detected (Table 1). Of these, pathotype 6 was virulent on all IRRI differential cultivars, while the other pathotypes were incompat-

Table 2. Representative strains of *Xanthomonas oryzae* pv. *oryzae* used for virulence analysis.[a]

	IR24	IR20	IR1545	Cas 209	DV85	IRBB1	IRBB2	IRBB3	IRBB4	IRBB5	IRBB8	IRBB10	IRBB11	IRBB14	IRBB21
NX0377	S	S	S	S	R	S	R	S	S	S	S	S	R	S	R
NX0384	S	R	S	S	R	S	S	S	R	R	S	S	S	S	R
NX0400	S	R	S	S	R	S	S	S	R	R	S	S	S	S	R
NX0402	S	R	S	S	R	S	S	S	R	R	S	S	S	S	R
NX0386	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R
NX0401	S	R	R	S	R	S	S	S	R	R	S	S	S	S	R
NX0374	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R
NX0392	S	S	S	S	R	S	S	S	S	S	R	R	S	S	R
NX0382	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R
NX0398	S	S	S	S	R	S	S	S	S	S	R	S	S	S	R
NX0365	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R
NX0387	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R
NX0407	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R
NX0380	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R
NX0397	S	S	S	S	R	S	S	S	R	R	S	S	S	S	R
NX0381	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R

[a]Six fully expanded leaves of each cultivar per pot were clip-inoculated 50 d after sowing. Lesions were measured 14 d after inoculation. Plants having lesions of 0·3 cm were classified as resistant (R) and those with lesions longer than 3 cm were rated as susceptible (S).

ible with at least one of the hosts (Adhikari et al 1995). Pathotype 7, which consisted of a few weakly virulent strains, was likely an artificial group that had lost virulence in culture. It is intriguing that pathotypes 2 and 5 were not detected in Nepal. A majority of the strains were avirulent on IRBB21 (Table 2).

Our results show that, although populations of *Xoo* in Nepal are genetically very diverse, they are not virulent to the *Xa21* gene for resistance. Thus, this information provides a preliminary basis to design strategies to use and deploy varieties with the *Xa21* gene for resistance to the bacterial blight pathogen in Nepal.

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Population structure of *Pyricularia grisea* in two IRRI blast nurseries: implications and applications

D.H. Chen, R.S. Zeigler, and R.J. Nelson

The population structure of *Pyricularia grisea* was analyzed at two field sites used by IRRI for evaluating blast resistance in rice. During 1992, 1,516 monoconidial isolates of the pathogen were collected from 38 rice cultivars and lines from the blast nursery of the International Rice Research Institute (IRRI-BN) and from the upland screening site at Cavinti, Laguna, Philippines. Each isolate was subjected to DNA fingerprinting and phenetic analysis using the probe MGR586. Nine lineages, or groups of isolates sharing >80% DNA similarity and inferred to be related by descent, were detected at Cavinti, while four lineages were found at the IRRI-BN. Subpopulations of the pathogen were differentiated by site, by season, and by host. The rice lines could be grouped according to the spectrum of lines infecting them in the field. Inoculation studies were conducted to more thoroughly define the resistance spectra of selected rice genotypes. Although a complex relationship between lineage and pathotype was observed, a characteristic virulence spectrum could be discerned for each pathogen lineage, and a characteristic resistance spectrum could be discerned for each host genotype. Based on this information, an experiment on line and cultivar mixtures was conducted at Cavinti in 1994. "Good" line/cultivar mixtures (those with components having complementary resistance spectra to the known line/cultivar such that no line/cultivar could infect all components) and "poor" line/cultivar mixtures (those for which one or more line(s)/cultivar(s) could infect all the mixture components) were designed for CO 39 near-isogenic lines and rice cultivars. Leaf blast severity (diseased leaf area, or %DLA) was reduced for the good line mixture relative to the mean of the mixture components, but not for the poor line mixture. For the cultivar rnixtures, both good and poor mixtures showed significantly reduced %DLA in later plant growth stages. These results suggest that knowledge of pathogen population structure can be useful for making decisions both for breeding and gene deployment.

Rice blast, caused by *Pyricularia grisea* (Cooke) Sacc. [*P. oryzae* Cav., the anamorph of *Magnaporthe grisea* (T.T. Hebert) Yaegashi & Udagawa], is the most important fungal disease of rice worldwide. Improved resistant rice cultivars have been the preferred, "environment-friendly'' way of managing the disease; however, these typically are not durable (Kiyosawa 1982). Extreme pathotypic variation, inadequate screening of breeding lines in blast nurseries, and widespread monoculture of single or closely related rice genotypes all contribute to the rapid breakdown of resistance.

For developing and sustainably managing the durable blast-resistant rice cultivars, it is necessary to thoroughly understand the population structure of *P. grisea* in the breeding/screening nurseries and the host-pathogen interaction (Zeigler et al 1994, Chen et al 1995). Molecular marker analysis of the asexual *P. grisea* populations (Leung et al 1993) suggests that these populations generally consist of clonal lineages (Levy et al 1991, 1993; Xia et al 1993; Chen et al 1995; Zeigler et al 1995). In seeming contrast to the reported hypervariable nature of the pathogen, lineages appear to encompass a limited number of pathotypes (Levy et al 1991, Levy et al 1993, Zeigler et al 1995). In some cases with complicated relationships between lineage and pathotype, nonrandom association of virulence in a given lineage with host genotypes has been demonstrated (Chen et al 1995, Zeigler et al 1995). Information on pathogen population structure and associated virulence characteristics is useful for deciding the breeding strategy (e.g., lineage exclusion strategy, Zeigler et al 1994), designing the host genotypes (resistance gene combinations), and wisely deploying resistant genotypes.

In this paper, we describe the population structure of *P. grisea* in the two screening nurseries used at IRRI for evaluating germplasm materials and breeding lines for rice blast resistance and explore the application of knowledge of the pathogen population structure and the relationship between host genotype and virulence characteristics in lineages to blast resistance breeding and deployment.

Population structure of *P. grisea* in the two blast nurseries

The IRRI lowland blast nursery (IRRI-BN) has been used for many years and is surrounded mainly by indica rice cultivars. The screening site for blast evaluation of mainly japonica rice materials at Cavinti is an upland site, relatively isolated from the commercial rice production fields and 40 km away from the IRRI-BN. High blast levels are observed in both sites. Although pathotypic diversity of *P. grisea* has been studied in both sites, little is known about the pathogen population structures and the relationship between phenotypic and genotypic diversities.

In 1992, 1,516 monoconidial isolates were systematically collected from the 38 genetically diverse rice genotypes (Chen et al 1995) at the two nurseries in the dry (DS) and wet (WS) seasons and were subjected to DNA fingerprinting using probe MGR586 (Hamer et al 1989). The phylogenetic relatedness among individuals was analyzed (Rohlf 1989) and the robustness of the groups formed in the cluster analysis was statistically tested by bootstrapping using the WINBOOT program (Nelson et al 1994).

One hundred and thirty haplotypes were detected in the three collections. These were clustered in 10 high robust groups (putative lineages) at 80% DNA profile similarity (Fig. 1) (Chen et al 1995). Four lineages were found in the IRRI-BN, each present in both DS and WS; three of which and an additional six lineages were detected

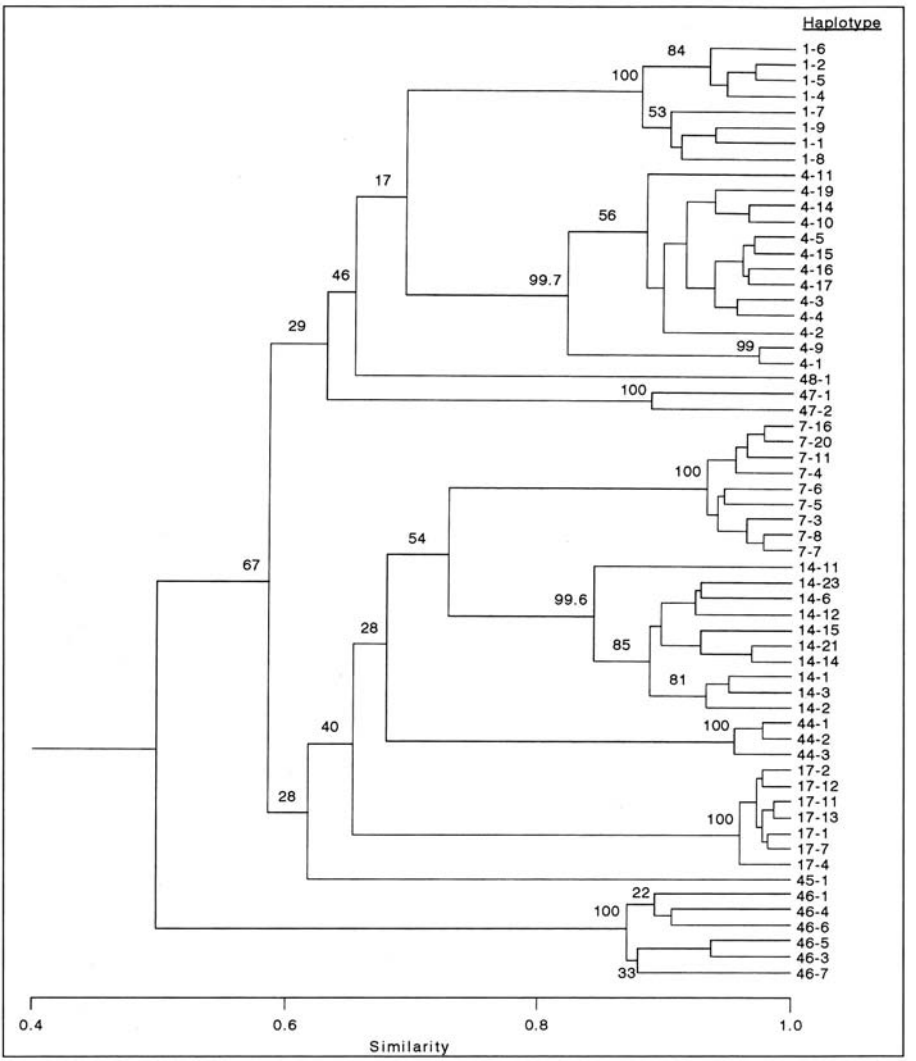

Fig. 1. Phenogram derived by unweighted pair-group method with arithmetic mean (UPGMA) based on DNA band data obtained using the probe MGR586, depicting similarities of isolates representing different lineages of *Pyricularia grisea* collected from the blast nursery and the upland screening site at Cavinti, Laguna, Philippines, in 1992. Values on the branches of the clusters represent the results of bootstrap analysis (the percentage of times the group occurred during 2000 iterations). Lineage number is indicated by the first letter of the haplotype designation.

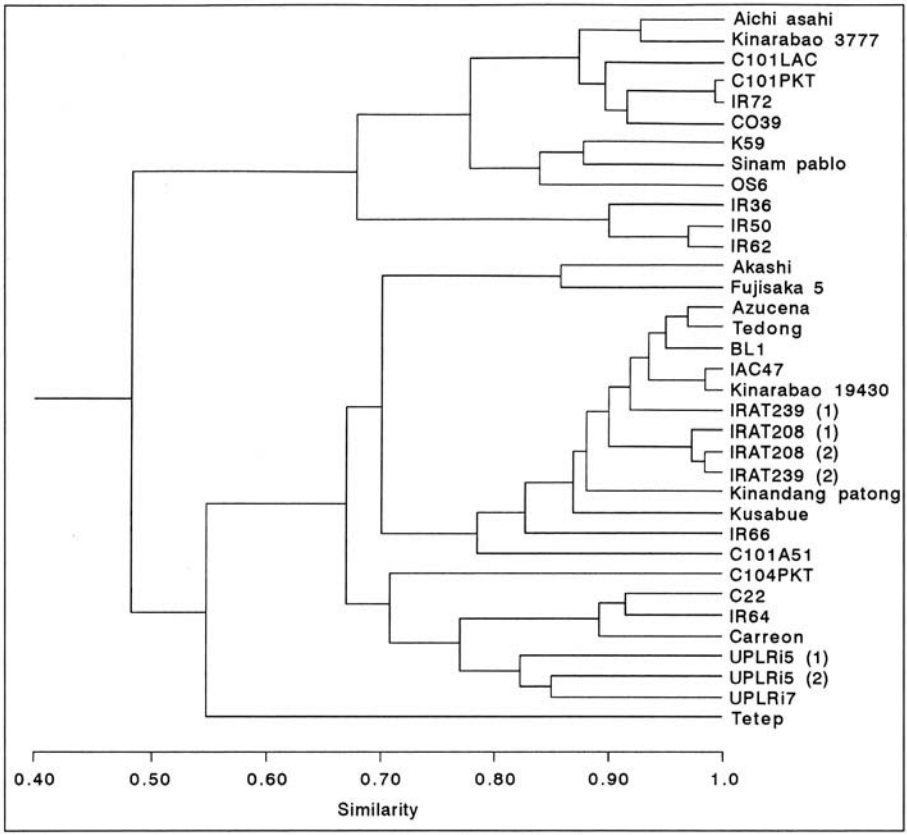

Fig. 2. Phenogram derived by UPGMA based on lineages collected from each host in three collections.

in Cavinti. The observed lineage diversity of the Cavinti population was the greatest, followed by the IRRI-BN/DS and the IRRI-BN/WS populations.

The subpopulations of *P. grisea* were geographically and temporally differentiated. Although the same lineages were present in the IRRI-BN/DS and IRRI-BN/WS, their frequencies varied from DS to WS. For instance, lineage 14 was predominant in the IRRI-BN/DS but ranked second in the WS. Most haplotypes were unique for one of the three collections. Approximately 19% of the overall lineage variation among the three collections was attributable to differences among locations and seasons based on the estimation of the genetic differentiation coefficient (Nei 1973, Crow 1986).

Most hosts were infected by multiple haplotypes, and many were infected by multiple lineages. Two hosts were unique: C101A51, an isogenic line carrying the blast resistance gene *Pi2(t)* (Mackill and Bonman 1992, Inukai et al 1994), was infected only by lineage 44; Tetep, a donor cultivar for blast resistance widely used in breeding programs, was infected only by lineage 17. Greenhouse inoculations confirmed the compatibility of the two lineages with their respective host of origin (Chen 1993).

Lineage 44 was recovered only from C101A51 at Cavinti in the 1992 collection and was characterized with extreme narrow virulence spectrum (Chen et al 1993, Zeigler et al 1995). Although lineage 44 was compatible with other hosts, e.g., C104PTK [*Pi3*(t)], no isolates of lineage 44 were detected among 20 isolates collected from C104PKT at Cavinti in 1992, possibly due to an extremely low frequency of lineage 44 in the population. This suggests that lineage 44 may not have been detected if C101A51 had not been included in the trap nursery.

Based on lineage data from individual hosts, 10 clusters were formed over the 38 hosts, with genetically similar host genotypes being grouped together (Fig. 2). When lineages from the same hosts were pooled from the three collections for partitioning of genetic variation by host, a high genetic differentiation coefficient (0.39) was obtained. This high coefficient, coupled with the nonrandom distribution of lineages on individual hosts based on the chi-square test using the Monte Carlo option data set, indicated that the host exerts a strong selection on structuring pathogen population.

Virulence characteristics of *P. grisea* lineage in the two blast nurseries

A simple relationship between pathotype and lineage of *P. grisea* was reported in the United States (Levy et al 1991). Multiple related pathotypes within lineage were demonstrated in a blast resistance breeding farm in Colombia (Levy et al 1993, Correa-Victoria et al 1994). To more thoroughly define the virulence structure of lineages in the IRRI breeding nurseries, a greenhouse inoculation of a subset of isolates from the collections was conducted on sets of near-isogenic lines (NILs) (Mackill and Bonman 1992, Inukai et al 1994, Ling et al 1995). Multiple pathotypes were associated with all the lineages and a complex relationship between lineage and pathotypes was observed (Chen 1993, Chen et al 1995, Zeigler et al 1995). Each lineage showed a defined virulence spectrum, likewise each host genotype exhibited a characteristic resistance spectrum. The NIL carrying resistance gene *Pi2(t)* in C101A51 was only compatible with isolates in lineage 44 from among more than 570 isolates in 19 lineages tested (Chen et al 1996). Resistance gene *Pi1(t)* in C101LAC conditioned resistance to more than 100 isolates in lineage 4. Lineage 7, collected from 31 out of 38 cultivars, showed compatibility with 10 major genes in two sets of NILs. *Pi-ta*^{2} in NIL F128-1 and *Pi2(t)* in NIL C101A51, however, conferred resistance to all 38 isolates of lineage 7 tested (Chen et al, unpubl. data). Combination of the well-characterized genes with complementary resistance to different lineages in a rice genotype could yield resistance to different lineages of the pathogen (Zeigler et al 1994). Deployment of resistance genes based on such information might provide a way for managing blast.

Cultivar/line mixtures in managing blast in the Cavinti screening site

With the well-characterized population structure of *P. grisea* at the Cavinti screening site (Chen et al l995) and the defined relationship between lineage and host genotype

(Zeigler et al 1995), a "good" mixture and a "poor" mixture of cultivars/lines can be designed for deployment against *P. grisea*. The good line mixture should confer resistance to all the pathogen subpopulations (lineages), while at least one lineage could infect all the mixture components in the poor mixture. Two mixtures (cultivars and NILs) and pure plots of their components (Table 1) were tested in the 1994 WS and evaluated for diseased leaf area (%DLA) throughout the season in Cavinti.

Leaf blast severity in the good line mixture was significantly ($P = 0.05$) lower than in the poor line mixture throughout the season. In the last (ninth) observation, %DLA in the good line mixture was 53% of that in the poor line mixture. In comparison with the average %DLA of the mixture components, six of the nine observations showed significantly lower %DLA in the good line mixture than the average of the mixture components, while in no case was the %DLA in the poor line mixture lower than the average of the mixture components. The relative area under disease progress curve (RAUDPC) for the good line mixture was significantly lower than that of the susceptible components but was no different from the resistant components. Meanwhile, the RAUDPC for the poor line mixture showed no significant difference from the susceptible mixture components.

For the rice cultivar mixtures, significant %DLA differences between the good and the poor mixtures were observed only in the last three observations. In comparison with the average %DLA of the mixture components, both good and poor cultivar mixtures showed significant disease reduction in late plant growth stages. The final %DLA on the good cultivar mixture was only 31% of the poor cultivar mixture. The

Table 1. Reactions of cultivars/lines to different lineages based on greenhouse inoculation tests.[a]

Mixture	Cultivar/line	Lineage			
		1	4	7	44
Good line mixture[d]	C101LAC[*Pi1(t)*]	All R (71)[b]	All R (151)	29% S (115)	All R (16)
	C101A51[*Pi2(t)*]	All R (71)	All R (151)	All R (151)	All S (16)
	CO39	All R (71)	All S (151)	All S (115)	All S (16)
Poor line mixture[e]	C101LAC[*Pi1(t)*]	All R (71)	All R (151)	29% S (115)	All S (16)
	C101PKT[*Pi4^a(t)*]	All R (71)	26% S (151)	95% S (115)	All R (16)
	CO39	All R (71)	All S (151)	All S (115)	All S (16)
Good cultivar mixture	IAC165	58% S (12)	All R (53)	All R (16)	...[c]
	C22	69% S (36)	73% S (59)	All R (41)	All R (11)
	Carreon	All R (34)	All R (59)	13% S (46)	27% S (11)
Poor cultivar mixture	IAC47	78% S (36)	All R (59)	All R (41)	All R (11)
	IAC165	58% S (12)	All R (53)	All R (16)	...
	C22	69% S (36)	73% S (59)	All R (41)	All R (11)

[a]Data of Zeigler et al (1995) were included. [b]Number inside the parentheses is the total apparent virulent isolates tested. [c]No isolates from the lineage were tested on the cultivar. [d]Good mixture = the mixture components confer resistance to all lineages in population. [e]Poor mixture = at least one lineage could infect all the mixture components.

RAUDPCs for both good and poor cultivar mixtures were not significantly different from their respective susceptible mixture components. An early season drought (21 d) may have prevented sufficient epidemic development to allow differences to be detected.

These preliminary results suggest that knowledge of pathogen population structure and the relationship between host and pathogen lineages may help in designing gene deployment schemes.

Diversification of pathogen genotypes in the blast nursery

Diversification and maintenance of pathogen genotypes (lineages) in a blast nursery should improve the exposure of breeding materials to the broad pathogen genotypes and should reduce the risk of "disease escape." In the blast nurseries, "spreader rows'' are often used for multiplying pathogen inocula (Correa-Victoria and Ziegler 1993), and selection and management of their components are key to their effectiveness. Spreader row cultivars are usually chosen based on their field susceptibility. Highly susceptible cultivars are most often used as spreader row components.

Moreover, among 38 rice cultivars/lines, in no case were all the haplotypes or lineages recovered from a single cultivar/line. IR50, IR72, and IR442-2-58 have been used singly as spreaders at IRRI-BN in the Philippines. Each was infected by only 2-3 lineages in all the three collections (Chen et al 1995), suggesting that a single cultivar is not sufficient for amplification of diverse pathogen genotypes. The diversity of the initial inocula multiplied by spreader rows determines, to a great extent, the diversity of the pathogen population during the study. The observed genetic diversity (ODv_{lin} = 0.35 - 0.50) at the IRRI-BN in this study would have been far from it had all the types of the pathogens been equally represented (potential diversity or PDv_{lin} = 0.75). We propose that selection of spreader row cultivars should be based on the spectra of pathogen genotypes amplified by the cultivar, as well as its overall field susceptibility.

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Studies on the genetic diversity of *Pyricularia grisea:* a molecular approach for management of rice blast

R. Sivaraj, S.S. Gnanamanickam, and M. Levy

The MGR-DNA fingerprints (*Eco*RI restriction fragment profiles that hybridize to the homologous dispersed repeated sequence MGR586) were used to determine the genealogical relationships among field isolates of the blast fungus in southern India. The genetic diversity in the sampling indicates that the pathogen population in southern India is composed of 29 distinct clonal lineages (A to C, E to Z, and 1 to 4). Of these, lineage I was predominant in the samples so far analyzed. Lineage I also was found in Bangladesh. In plant inoculations onto a set of international and Indian rice cultivars, 38 isolates were screened for pathotype diversity. From the differential disease reactions, 14 pathotypes of *Pyricularia grisea* were identified and categorized into the international race groups IA, IB, IC, and ID. Data revealed a partial relationship between virulence and phylogeny. Lineages D, E, G, H, J, and K formed separate pathotypes and had distinct disease reaction patterns on the differential rice cultivars. However, lineages A, B, C, F, H, I, and J shared more than one pathotype. Forty isolates were inoculated onto seedlings of seven rice isolines (IL-1, IL-6, IL-8, IL10, IL-17 (subset), IL-23, and 11-21) that differ from each other by one or more blast resistance genes. Data on disease reactions on these isolines suggest that the blast resistance gene present in IL-1 could be used to develop a durably blast-resistant rice cultivar for southern India.

Breeding resistant rice cultivars is a principal tool for blast management. Major efforts have been made to understand and describe pathotype diversity. There is a lingering controversy over the degree of pathotype instability in the blast fungus. Great pathogenic variations have been reported from single lesions and monoconidial subcultures (Ou and Ayad 1968, Ou et al 1970) while other studies have shown isolates to be pathogenically stable (Latterell 1975, Bonman et al 1986). Molecular techniques offer new approaches for the critical evaluation and analysis of genetic variation in

the blast fungus. A family of dispersed repetitive DNA sequence was reported by Hamer et a1 (1989), and this is being widely used for fingerprinting and phylogenetic analysis of P. grisea (Levy et al 1991, 1993; Borromeo et al 1993; Chen 1993; Hans et al 1993; Shajahan et al 1993; Xia et al 1993; Zeigler et al 1995).

In our study, an attempt has been made to analyze the blast fungus population in southern India, to describe its pathotype organization, and to identify rice line(s) that exclude the fungus with the intent of developing cultivars with durable blast resistance.

Materials and methods

Collection of samples

Rice tissues showing typical leaf, neck, collar, and panicle blast symptoms were collected on different dates during the 1991–93 period from different locations in six states of southern India: Tamil Nadu, Kerala, Andhra Pradesh, Karnataka, Maharashtra, and Orissa.

DNA isolation and MGR-DNA fingerprint production

Isolates were propagated first in oatmeal agar medium. Then, mycelia grown in yeast extract glucose (YEG) liquid medium were used for isolating DNA. M. *grisea* DNA was digested with *Eco*RI, fractionated on 0.8% agarose gel, and transferred to Hybond-N-hybridization membrane (Amersham International). DNA blots were hybridized with subclone PCB586 which was radioactively labeled by the random primer method. Following hybridization, the DNA blots were washed at high stringency at 65 ºC in 0.1% SDS, 0.1% ppi, 0.2X SSPE (1X SSPE .018 M NaCl, 1 mM EDTA, 10 mM sodium phosphate, pH 7.4) and were exposed to X-ray film.

Inoculum preparation

For pathotype analysis, the inoculum was prepared according to the method of Mackill and Bonman (1986).

Pathotype analysis

A random selection of 38 isolates of P. grisea was subjected to pathotype analysis on a set of international rice cultivars. Susceptibility of the test plants was determined by examining the leaves for blast symptoms and by assessing disease severity based on a standard evaluation system developed by IRRI.

Blast trap nursery

To determine the population structure and associated virulence properties of the rice blast fungus, a blast trap nursery was set up in a hot spot location with 75 international rice lines and 25 local commercial rice cultivars. Standard nursery procedure was followed for planting. Monoconidial isolates of P. *grisea* obtained from susceptible cultivars were further analyzed by MGR-DNA fingerprinting method.

Use of rice isolines (NILs) for identifying genes for blast resistance

Mackill and Bonman (1992) developed a set of NILS each containing a single dominant gene. Forty isolates subjected to analysis were pathotyped using isolines IL-1, IL-6, IL-8, IL-10, IL-17 (subset) IL-23, and IL-21 under controlled conditions.

Results

MGR-DNA fingerprint in the sample indicates that the blast pathogen population in southern India had 29 distinct clonal lineages (A to C, E to Z, and 1 to 4). Isolates within a lineage generally exhibited more than 90% similarity in RFLP pattern and isolates of different lineages had less than 70% similarity.

Seven lineages (A, I, U, V, X, 3, and 2) were found in Tamil Nadu. Lineage I had the widest host selection and distribution in parts of Tamil Nadu.

The isolates from Kerala showed six lineages (M, G, I, J, H, and K). Another set of seven lineages (A, B, I, O, T, P, and Q) was identified in Pattambi, the hot-spot location where the trap nursery was set up.

Fingerprinting of the Karnataka isolates revealed six different lineages (L, A, B, E, F, and H). Isolates from Andhra Pradesh belonged to three lineages or groups (I, H, and Y). Samples analyzed from Maharashtra showed that two lineages (R and S) exist. The lineages present in Orissa were H, Z, 4, and 1.

When 37 of the monoconidial isolates of *P. grisea* were inoculated onto each set of eight cultivars, there were 11 differential disease reaction patterns observed. On the basis of these patterns, the isolates were classified into the international race groups A-97, C- 1, D-3, D-8, C-17, A-48, A-61, A-64, A-28, and B-46.

The test conducted with a set of IRRI isolines has shown that IL-1 was resistant to the south Indian isolates screened. Two isolates belonging to the predominant lineage I were able to produce an intermediate reaction in IL-1, while none of the other south Indian isolates that belonged to lineages A to N of *P. grisea* were able to induce blast reaction

Discussion

Lineage analysis with the use of MGR-DNA fingerprinting method shows 29 lineages of *P. grisea* in the Indian population examined thus far. These were given code names (lineages A-Z and 1-4). They represent the genetic diversity in the *P. grisea* population in southern India. Lineage I was detected in IR50 grown in Bangladesh (Shahjahan et al 1993). Lineage C found in Karnataka state is identical to a fingerprint found in a pathogen population in the Philippines. Thirty-seven isolates of *P. grisea* were classified into 14 pathotypes. A pathotype was formed by isolates of some of the lineages (D, E, G, J, H, and K) while others were formed by isolates of more than one lineage. At least 5 of the 14 pathotypes have not been reported previously. The test conducted with IRRI isolines against the blast population of southern India (lineages A-N) showed

that IL-1 is resistant to almost all the southern Indian *P. grisea* population. We consider this information significant and it will be useful in the development of a blast-resistant cultivar.

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Population structure of *Magnaporthe grisea* in the traditional Himalayan rice system

J. Kumar, R.J. Nelson, and R.S. Zeigler

Fairly simple clonal population structures of the rice blast fungus *Magnaporthe grisea* have been observed in the Philippines, the Americas, and Korea. A study of Himalayan *M. grisea* reveals a population that is far more diverse. The *M. grisea* population samples were collected from the central Himalayan Hills of India, where traditional rice and millet have a long history of cocultivation. MGR586 homologous DNA profiles ('fingerprints') of 225 isolates from rice revealed 46 distinct lineages. Both traditional and modern cultivars contributed equally to genetic diversity in the *M. grisea* population. Among 26 locations for which rice isolates were analyzed, the site at the village of Matli showed exceptional diversity, with 20 distinct lineages detected from among 47 rice-derived isolates over 2 consecutive yr. An epidemic population of 56 isolates analyzed during the third year revealed eight distinct lineages, two of which dominated the population. We considered the hypothesis that recombination might have contributed to the high genotypic diversity observed. For each lineage observed at Matli, we used low-copy restriction fragment length polymorphism markers to assess linkage disequilibrium and observed random association of alleles at independent loci. In a related study, we determined the fertility of *M. grisea* isolates from rice, millet, and weeds, and found these to have high mating competence and cross fertility. These results support the hypothesis that recombination, possibly sexual, could be active in the life cycle of *M. grisea* at one site in the Himalayas.

The ascomycete *Magnaporthe grisea* Barr (anamorph: *Pyricularia grisea* Sacc) causes the blast disease in rice and other Graminae species and is organized into a number of host-specific forms (Ou 1985, Rossman et al 1990). Isolates from cultivated millet (e.g., *Eleusine, Echinochloa,* and *Setaria* spp.) are fertile, producing abundant ascospores in culture while fertility among isolates from rice is rare (Kato et al 1976).

Magnaporthe grisea is notorious for its pathotypic diversity and for its capacity to overcome varietal resistance, the preferred disease management tool. This could be due to the shift in frequency of formerly rare pathotypes or the frequent occurrence of genetic changes in new virulent forms, or both, compounded by inadequate disease screening methodology (Correa-Victoria and Zeigler 1995). Analysis of pathogen populations can provide insights into the genetic variation among individuals in the population, their phylogenetic relationship, and virulence dynamics (Levy et al 1991, 1993; Chen et al 1995; Zeigler et al 1995). DNA fingerprinting and phylogenetic analysis using the MGR586 element as a probe (Hamer et al 1989) have recently contributed significantly to a better understanding of the population biology of the blast fungus than is possible from pathogenicity assays alone. Population structure inferred from virulence data may not reflect the true genetic diversity and evolutionary history of the isolates since the assay is environmentally sensitive and is somewhat arbitrary. Based on DNA fingerprinting, *M. grisea* rice pathogen populations from the USA, Colombia, and the Philippines are organized into a few distinct groups inferred to reflect clonal genetic lineages derived from a common ancestor (Hamer et al 1989; Levy et al 1991, 1993; Chen et al 1995). Recovery of representatives of the same lineage over different years/seasons/regions suggests an exclusively clonal mode of reproduction in *M. grisea* (Levy et al 1991, 1993; Xia et al 1993; Chen et al 1995). The generalization of simple clonal population structure can have important implications for rice blast resistance breeding programs (Zeigler et al 1994), yet it is based on population analyses of breeding fields and/or areas almost exclusively planted to relatively few modem cultivars.

We asked if traditional rice-growing areas show population structures similar to those observed in the modem rice breeding and production fields. We further asked whether any effect on population structure could be detected from the recent introduction of modern cultivars. We chose as our study area the Uttar Pradesh Hills in the central Himalayas of India.

The traditional Central Himalayan rice system

This region is considered to be one of the centers of diversity for cultivated rice. Rice is cultivated as an irrigated transplanted crop in well-managed terraces in isolated valleys and, to a limited extent, on terraces on hills, and as a direct-seeded, rainfed upland crop on hill slopes. Traditional cultivars predominate. Rice is typically grown in association with traditional cultivars of millet, which are also potential hosts for *M. grisea.* Because of the antiquity of rice cultivation in the region, the range of blast-conducive microenvironments, and the diverse traditional cropping systems and germplasm, the *M. grisea* population structures in the region could provide insights into early relationship between host and pathogen.

Genetic variation of *M. grisea* in the traditional Himalayan system

Collections were made from farmers' fields in the rice-growing areas from valleys at mid- and high altitudes. About 440 samples (281 from rice and 158 from millet and grasses) were collected over 3 yr from 36 locations in 7 contiguous geopolitical regions. Fungal culture, DNA extraction, restriction fragment length polymorphism and DNA fingerprinting analysis, and lineage designation were conducted as reported elsewhere (e.g., Chen et al 1995).

MGR-based group assignment

Isolates from rice typically had high copy number (around 60-70 bands ranging from 1.1 to 23 kb) of MGR586 hybridizing bands, with only about 3% of isolates having <10 bands. Most isolates from millet and grasses had low copy number of MGR sequences, and only approximately 4% had copy number similar to that in rice isolates. Identical haplotypes were recovered from more than one monocot species, suggesting that although *M. grisea* forms are largely host-limited, closely related isolates can infect different species. In all sample sites, a wide array of host genotypes was present. Phenetic analysis on a set of isolates representing each visually determined group based on similar DNA profile yielded considerable overlapping among groups and poorly resolved trees. On the basis of 70% DNA profile similarity, 122 isolates collected from rice during 1992 were clustered into 24 groups (consensus value based on 2,000 bootstrap replications), and 103 collected during 1993, were similarly clustered into 26 groups or lineages—a diversity far greater than those observed in other studies. At the 80% similarity level, 27 and 31 groups could be resolved from the 1992 and 1993 collections, respectively. Each lineage usually contained more than one haplotype. No obvious relationship was detected between lineage and origin from traditional and modem cultivars. The abundant lineages were shared between the two types of cultivars and those found only on traditional and modern cultivars were the rare ones.

Genetic diversity and population distribution

DNA fingerprint data were used to calculate Nei's measure of genetic diversity (Nei 1973) in the Himalayan blast population. The total diversity (H_T) was 0.92 during 1992 and 0.91 during 1993. Of all locations analyzed for the pathogen population structure, four showed very high genetic diversity although most showed a unique population structure. The *M. grisea* population was genetically differentiated across the region. Approximately 35% of overall genetic diversity (G_{ST} = 0.35) was due to differences among sites. Individuals from 12 lineages were found in multiple locations in the region, despite the mountainous terrain that separates different rice-growing ecosystems, suggesting active gene flow in the region. Individuals from two lineages found in multiple locations were also highly diverse in the Himalayan population—that is, 35 haplotypes out of 47 isolates representing the two lineages could be detected.

Himalayan *M. grisea* populations do not show a lineage structure as strong as reported elsewhere (Levy et al 1991, 1993; Xia et al 1993; Chen et al 1995; Zeigler et al 1995). Among the high-diversity sites, the population structure at Matli was

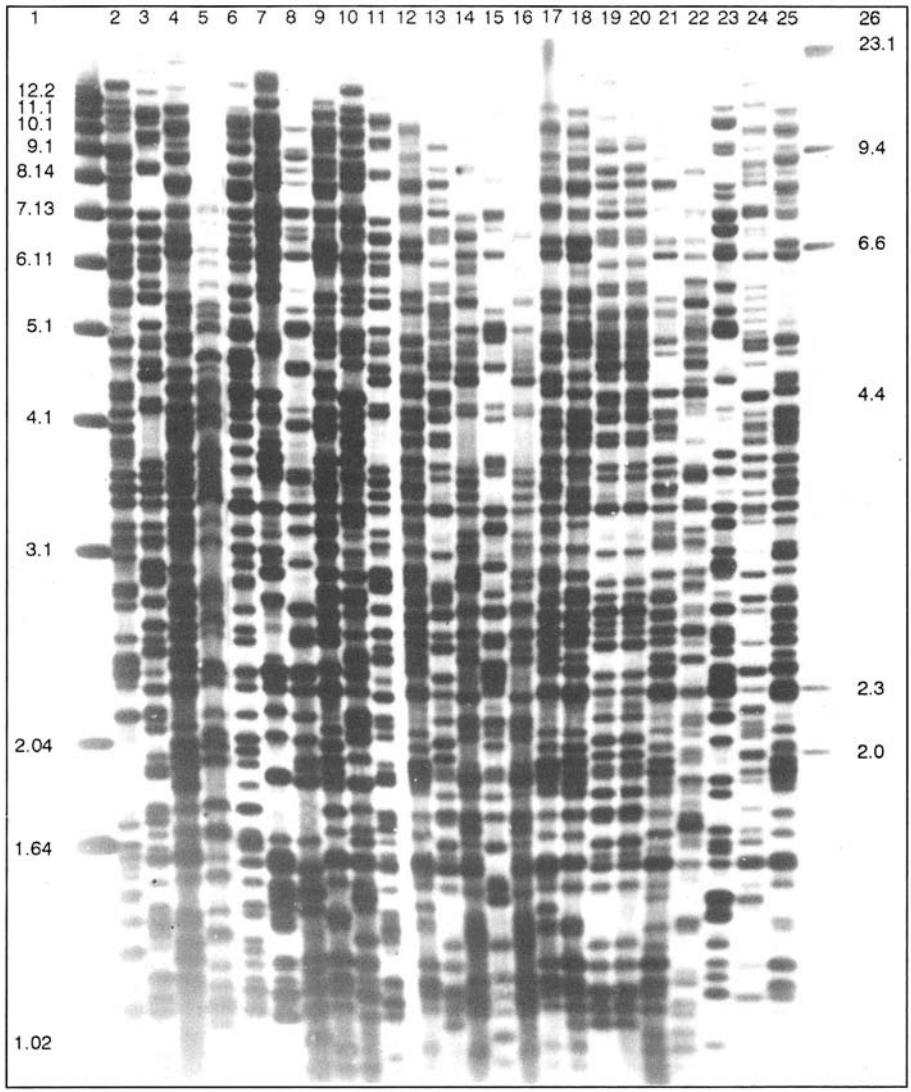

Fig. 1. MGR586 fingerprints of a set of *M. grisea* isolates from rice from Matli. Isolate (year and isolate no.): 1,1-kb ladder; 2, H92-18-3; 3, H92-27-2; 4, H92-29-3; 5, H92-34-2; 6, H92-37-3; 7, H92-32-4; 8, H92-19-2; 9, H92-49-4; 10, H92-38-3; 11, H92-27-3; 12, H93-58; 13, H93-88A-1; 14, H93-741; 15, H93-87-1; 16, H93-64-1; 17, H93-59; 18, H93-72-1; 19, H93-85; 20, H93-82-1; 21, H93-76; 22, H93-337; 23, H94-20; 24, H94-26-1; 25, H94-408-3; 26, λ *Hin*dIII.

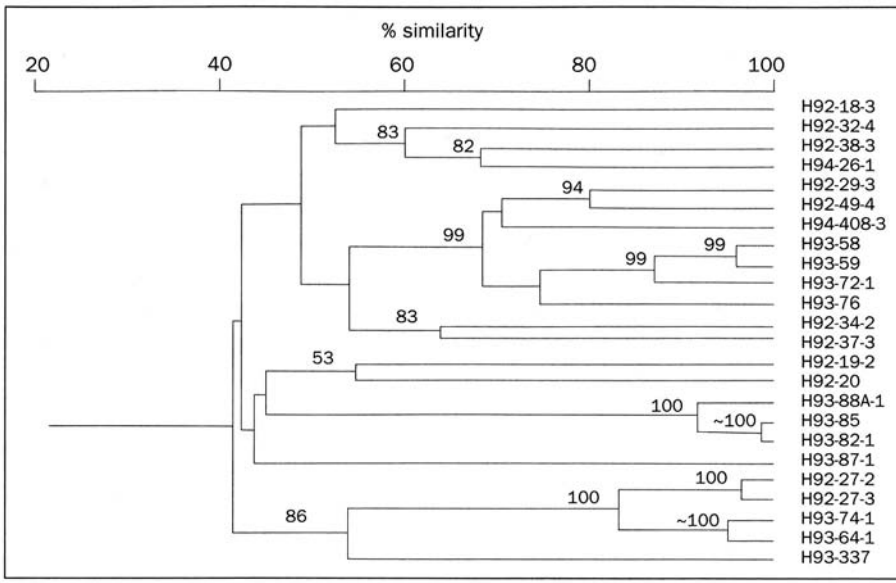

Fig. 2. Phenogram derived by UPGMA cluster analysis. Values on the branches of clusters represent the results of bootstrap analysis.

particularly fluid over 3 consecutive yr (Fig. 1 and 2). During the first 2 yr, only three lineages were found in common from the 20 lineages identified from among 47 isolates. During the third year, three unique lineages were found among the eight identified from 56 isolates. It is noteworthy that disease incidence was low during the first 2 yr, but very high in the third year. Lineages IHR2 and IHR3 appeared at low frequency during the first 2 yr, but constituted 86% of the sample during the third year. Thus, the lower diversity in the epidemic year may be due to particularly fit genotypes within a few lineages becoming dominant in the population (Maynard Smith et al 1993). Approximately 20% of the overall fingerprint group differentiation (G_{ST} = 0.2) among three collections was attributed to differences between collections, indicating a substantial variation within each subpopulation.

Gametic phase equilibrium among the Himalayan blast subpopulation

Population genetic methods can be used to infer mode of reproduction of microorganisms in nature (Tibayrenc et al 1991, Burt et al 1996). We analyzed each clone-corrected lineage from the Matli population for gametic phase equilibrium using low-copy DNA probes (provided by S.A. Leong and H. Leung; Skinner et al 1993). In di-locus comparisons for all the possible pairs of the most informative loci (most common alleles at each locus), all four genotypes were observed in the population. Analyzing all the loci simultaneously using a multilocus analysis (Brown et al 1980, Maynard Smith et al 1993), the null hypothesis of independence at the level of locus pairs could not be rejected. Considering that gametic phase disequilibrium is strongly favored by most evolutionary factors (selection, genetic linkage, clonal propagation)

affecting the life cycle of *M. grisea*, the random association among polymorphic loci is consistent with the hypothesis that genetic recombination plays or has played a role in the generation of observed genotypic diversity.

Magnaporthe grisea has been considered to be an exclusively asexually reproducing and clonal pathogen, despite its high pathogenic variability (Levy et al 1993, Zeigler et al 1994). However, *M. grisea* isolates in the Himalayan blast population are fertile. Both mating types of the fungus have been found from rice and were easily observed from other monocots that are traditionally cocultivated with rice in the Himalayan cropping system. Because of the wide host range of the fungus, production of perithecia could occur on several cultivated species. Thus, the observed genetic diversity may in part result from sexual recombination. Recent evidence for parasexual genetic exchange among isolates from different lineages (R. Zeigler et al, IRRI, unpubl. data) offers another source of recombination that could explain the observed gametic phase equilibrium.

Evidence for recombination is not inconsistent with predominant asexual propagation, by which a few successful recombinant genotypes can come to dominate a population due to relative fitness and a high reproductive capacity. Some members of the populations may be capable of generating a severe epidemic when host and/or environmental conditions are suitable. At this time, it is not clear whether the observed genetic diversity is a historical relic of a recombining population, preserved by an array of diverse compatible hosts, or the result of continuing genetic recombination. We conclude that Himalayan blast populations are highly diverse and are not exclusively clonal, as inferred from other studies. *M. grisea* from rice and from other cultivated and wild Graminae species are not as genetically isolated and host-limited as suggested by others. Genetic recombination may be a significant mechanism for generating genotypic variability of *M. grisea* in the Himalayas. Modern cultivars do not appear to act as bottlenecks that reduce pathogen diversity in the region.

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Rice recommendation domains and implications for biotechnology initiatives in Nepal

H.K. Upadhyaya

This study identified and characterized rice recommendation domains and suggested biotechnology initiatives for Nepal. Data were obtained through an extensive survey of 401 villages from 45 districts representing all physiographic and development regions of the country. These sample districts account for 91% of the total rice area and 92% of the total rice production of the country. The specific biotechnology agenda for Nepal should include development of rice varieties that are resistant or tolerant to blast and bacterial leaf blight, armyworm, gundhi bug, caseworm, and borer. These recommendations take into account the importance of the medium- and high-rainfall tarai and the high-rainfall foothill domains, which cover most of the country's rice area.

Nepal is a small, but uniquely diverse, country. The climate ranges from subtropical to arctic. Physiography includes the vast alluvial plains of the tarai in the south and the permanently snow-covered mountain peaks in the north. Mean annual soil temperatures range from less than 0° to nearly 30 °C. Annual rainfall ranges from less than 300 to more than 5,000 mm. Agriculture is practiced in a wide range of landscapes and altitudes, from near sea level to 4000 m above sea level, with cropping intensities ranging from one crop in 2 yr to three crops in 1 yr. Within a physiographic region, large variations exist from east to west, especially in terms of amount and distribution of rainfall, humidity, and landforms.

Rice research has generally considered the three broad physiographic regions—mountains, hills, and tarai (including inner tarai)—as the recommendation domains. However, it is clear that such a broad, macro-level, classification of rice-growing areas is not very meaningful since, within each of these regions, there exists large biophysical, environmental, and socioeconomic diversities, leading to significant variation in the demand for suitable technologies. Consequently, technologies developed

so far have been largely supply-oriented rather than demand-driven, resulting in their low adoption or nonadoption at the farm level.

Eighty-five percent of rice cultivation in Nepal occurs on alluvial landscapes (most of which lie in the tarai and some parts in the hills). The remaining 15% of the rice cultivation occurs on level land and bench-terraces that have soils that developed from the weathering of rocks of different geologic origins.

The objectives of the study were to identify and characterize rice recommendation domains and to suggest biotechnology initiatives for Nepal. Data were obtained through an extensive survey of 401 villages from 45 districts representing all physiographic and development regions of the country. These sample districts account for 91% of the total rice area and 92% of the country's total rice production.

Identifying the recommendation domains

Factor analysis was conducted on all the observed variables. The results indicated that seven factors—rainfall, altitude, rice intensity, population density, irrigation, tarai, and fertilizer—cumulatively explain about 90% of the observed variability. In the context of Nepal and given the need for capturing both the north-south and the east-west variability within the country, rainfall and altitude factors (together explaining nearly one-third of the variability) were considered for delineating the recommendation domains. Besides being observable and interpretable, these factors will help delineate recommendation domains that can be compared with other countries having similar ecosystems.

Hence, the study areas were grouped into three different rainfall regimes: <200 mm, 200-500 mm, and >500 mm based on the past 5-yr record of average monthly rainfall during the rice-growing period (June-October). Similarly, four altitude groups were identified: 400 m, 500-1000 m, 1000-1500 m, and >1500 m. Thus, 12 different domains were delineated based on the combination of the two factors. However, since the study sample did not include areas with high rainfall (i.e., >500 mm) in both the 1000-1500 m and > 1500 m altitude groups, the actual number of domains was 10.

The Kathmandu Valley appeared to be a distinctly different domain, not explained well by the above two factors. Although a part of the hills region, this valley—which consists of three districts, i.e., Kathmandu (the national capital), Bhaktapur, and Lalitpur—has the highest levels of input use and yields and the widest access to modern technology and infrastructure in the country. Vegetables are most intensively cultivated with extensive use of chemicals and pesticides, which are not commonly used in other parts of the country that fall into similar altitude and rainfall regimes. Agriculture is largely commercial and mechanized. For these and several other reasons, the Kathmandu Valley is considered a separate domain. Thus, 11 recommendation domains were identified based on the sample survey.

The altitude ranges used to delineate the domains can also be interpreted in terms of the popular physiographic regions. The low-altitude (400 m) range covers the entire tarai and inner tarai and a few areas of the foothills. As such, more than 85% of the country's rice areas fall into this altitude range. The medium-altitude (500-1000

m) range covers the foothills, including river valleys and fans or tars. The high-altitude (1000-1500 m) range covers the commonly called the hills region. The very high-altitude (> 1500 m) range covers the rice areas of the high hills or the mountains. For simplicity, these four altitude ranges can be generally interpreted as tarai (including inner tarai), foothills, hills, and high hills (or mountains).

Within each altitude group, the three rainfall regimes distinguish rice areas horizontally along the east-west direction. As such, all these domains can be conveniently located physically on the map and used for planning rice research and development in the country.

Characterizing the recommendation domains

Land use pattern

Table 1 presents the comparative land use patterns in the identified domains. In general, the proportion of cropped area is higher in the medium-rainfall range than in the low- or high-rainfall ranges, within a given altitude range. As expected, the tarai (and also Kathmandu Valley) has the highest proportion of cropped area compared with other regions. As proportions of cropped area, rice area constitutes 100% in the high-rainfall tarai and 76% in the high-rainfall foothills.

In general, cropping intensities increase as the rainfall increases from low to medium and then decreases as the rainfall increases further from medium to high. The overall average (average of irrigated and rainfed environment) cropping intensity is lowest (125%) in the low-rainfall mountains. Rainfed rice cultivation is most common in the medium- and high-rainfall areas of the tarai and the Kathmandu Valley, and is not practiced in the high-rainfall foothills and medium-rainfall hills of Nepal.

Rice - wheat is a dominant cropping pattern in Nepal, occupying about 43% of the rice areas and is most common in the foothills and the hills of Nepal, including Kathmandu Valley. In the mountains, the pattern is practiced in the medium-rainfall area. In the tarai, rice - fallow is a major cropping pattern in the high-rainfall area, and covers about 56% of the rice area. The rice - maize rotation is more common in the high-rainfall tarai area. Double rice cropping, which covers only 2% of the rice area, is mainly practiced in low- and medium-rainfall areas of the tarai and the foothills where irrigation facilities are available.

Technology, input use, and yields

The level of modern variety (MV) adoption (all MVs combined) ranges from zero in the low-rainfall mountains to 61% in the medium-rainfall tarai and high-rainfall foothills (Table 1). MV adoption rate tends to increase with increase in the amount of rainfall, especially in the tarai and the foothills, which account for most of the rice areas of the country. The overall MV adoption level in Nepal is estimated at 53%.

Although the number of MVs introduced to Nepal for general cultivation has reached 41, Masuli—one of the oldest varieties released two decades ago—remains the single most planted rice variety in Nepal.

Table 1. Selected rice production characteristics of the identified recommendation domains.

Detail	Domain											
Altitude (m)	<500	<500	<500	500-	500-	500-	1000-	1000-	>1500	>1500	KTM	Nepal
Rainfall (mm)	<200	200-500	>500	1000 <200	1000 200-500	1000 >500	1500 <200	1500 200-500	<200	200-500	Valley	
Land use												
Cropped area (as % of total area)	75	87	75	53	65	66	44	52	34	62	83	74
Rice area (as % of cropped area)	43	86	100	36	52	76	43	52	47	58	72	74
Annual cropping intensity (%)												
Total cropping intensity	174	181	154	174	187	176	157	161	125	180	171	177
Rice cropping Intensity	46	89	102	42	59	72	39	54	49	56	71	74
Rice cropping pattern (% of rice area covered)												
Rice - wheat	34	45	14	65	65	71	64	61	0	49	67	43
Rice - fallow	3	21	56	5	12	14	20	19	10	20	7	25
Rice - maize	2	12	28	5	3	6	0	14	0	12	0	14
Rice - rice	8	2	0	1	4	0	0	0	0	0	0	2
Adoption of modern varieties (MVs)												
Total MV adoption (%)	27	61	58	17	34	61	24	13	0	9	50	53
Mashuli adoption (%)	1	27	49	2	6	28	0	2	0	0	1	23
Input use and yield												
Fertilizer use (kg NPK ha^{-1})	61	72	52	78	88	65	50	60	36	68	187	80
Yield (t ha^{-1})	3.3	2.9	2.6	2.9	3.0	3.3	2.3	1.9	2.1	2.0	4.9	2.9
Crop losses (as % of average yield)												
Flood loss	7	15	9	5	18	11	10	14	3	17	6	14
Drought loss	13	25	33	26	19	15	26	20	25	5	21	23
Diseases loss—irrigated	5	10	16	21	15	9	25	11	20	17	8	12
Diseases loss—rainfed	2	10	48	3	2	–	–	6	–	1	3	7
Insect loss—irrigated	10	18	10	24	23	7	25	14	1	41	6	18
Insect loss—rainfed	4	16	31	7	3	–	–	13	–	7	3	11

The rate of fertilizer application to rice increases as the rainfall increases from low to medium and then decreases as the rainfall increases further from medium to high. In all domains, fertilizer use is the highest in the medium-rainfall range.

Rice production problems and crop losses

Drought and flood are the most serious abiotic constraints to high rice yields on Nepal. The extent of flood losses is relatively higher in the medium-rainfall range, mainly because such areas are generally river basins and are more prone to river flooding. Drought is much less severe in the medium-rainfall mountains than in other domains since mountain rice is mostly irrigated.

Except in isolated cases, crop losses to diseases and insects are larger under irrigated than under rainfed production environments in all domains. Diseases appear less severe in the low-rainfall tarai and more severe in the low-rainfall hills than in other areas under irrigated conditions where, as under rainfed conditions, they appear to be most severe in the high-rainfall tarai, causing a crop loss of 48%. Insect losses also are generally high under irrigated conditions in all domains, except in the case of the high-rainfall tarai, where such losses are much higher under rainfed conditions. Under both production environments, insects appear relatively less severe in the high-rainfall foothills, low-rainfall mountains, and the Kathmandu Valley.

Table 2. Major rice diseases, by recommendation domain.

Domain		Major rice diseases[a]	
		Irrigated	Rainfed
Altitude (m)	Rainfall (mm)		
<500	<200	BLB[b] (3); leaf spot(1); blast (1); others(0)	BLB(2); others(0)
<500	200-500	Blast (3); BLB(5); zinc(2); others (1)	BLB(5); blast(2); zinc(1); others 1
<500	>500	BLB(12); blast(3); false smut(1); others(0)	BLB(40); blast(8); others (0)
500-1000	<200	BLB(8); blast(7); leaf spot(4); others(1)	Blast(1); leaf yellow(1) BLB(1); others(0)
500-1000	200-500	Blast(7); BLB(5); leaf spot (2); others (2)	Leaf spot(1); others(1)
500-1000	>500	BLB(6); blast(2); false smut(1); others(1)	None
1000-1500	<200	Blast(19); leaf spot(6); others(0)	None
1000-1500	200-500	BLB(4); blast(4); leaf spot(1); others(2)	Blast(3); leaf spot(1); others(1)
>1500	200	Blast(16); foot rot(1); sterility(2); others(0)	None
>1500	<200-500	Blast(9); BLB(3); leaf spot(3); footrot(1); others(0)	BLB(1); others(0)
Kathmandu Valley (Kathmandu, Lalitpur, Bhaktpur)		Zinc(2); blast(2); BLB(2); alse smut(1); others(1)	Leaf spot(1); BLB(1); others(0)
Nepal		Blast(5); BLB(4); leaf spot(1); zinc(1); others(1)	BLB(3); blast(2); zlnc(1); others(1)

[a]Figures in parentheses indicate percentage yield loss. [b]BLB = bacterial leaf blight.

Table 3. Major rice insects, by recommendation domain.

Domain		Major rice insects	
		Irrigated	Rainfed
Altitude (m)	*Rainfall (mm)*		
<500	<200	Bug(5); white grub(3); borer(2); leaffolder(1); others(0)	Bug(1); borer(1); leaffolder(1); others (1)
<500	200-500	Bug(5); borer(4); caseworm(3): grasshopper(2); others(4)	Borer(4); caseworm(3); bug(3); others(6)
<500	>500	Bug(6); borer(2); caseworm(1); grasshopper(1); others(0)	armyworm(11); caseworm(7); bug(7); borer(6); others(1)
500-1000	200	Bug(6); leaffolder(5); borer(4): mole cricket(2); others(6);	Bug(1); white grub((1); others(6)
500-1000	200-500	Bug(7); borer(5); caseworm(4); leaffolder(2); others(5)	Borer(1); bug(1); seedbed beetle(1); others(1)
500-1000	>500	Borer(2); leaffolder(2); caseworm(2); others(2)	None
1000-1500	<200	Leafhopper(10); borer(5); bug(3); white Grub(2); others(6)	None
1000-1500	200-500	Bug(4); leaffolder(2); armyworm(2); others(5)	White grub(4); mealy bug(4); bug(2); Armyworm(2); others(2)
21500	<200	Armyworm(1); bug(2); others(0)	None
>1500	200-500	Leaffolder(12); armyworm(8); bug(7); borer(6); others(8)	Leaffolder(2); bug(2); termite(1); others(2)
Kathmandu Valley (Kathmandu, Lalitpur, Bhaktpur)		Bug(2); cutworm(1); hispa(1); others(3)	Hispa(2) bug(1); others(0)
Nepal		Bug(5); borer(4); caseworm(2): others(7)	Bug(3); borer(2); caseworm(2); others(5)

The distribution of major diseases and insects affecting rice with corresponding percentage yield losses is shown in Tables 2 and 3, respectively.

Conclusions and implications for biotechnology initiatives

Due to low levels of irrigation and water control facilities, the rice cultivation in Nepal is significantly influenced by the amount and distribution of rainfall during the rice-growing period. The proportion of rice area, technology adoption, cropping intensities, and yields are all positively related to the amount of rainfall. In terms of rice cultivation, three recommendation domains—the medium- and high-rainfall tarai and high-rainfall foothills —appear to be the most important ones. Major rice production problems in these regions are bacterial leaf blight, rice blast, armyworm, gundhibug, caseworm, and borer. Drought and flood are the common abiotic stress factors in all domains.

Given that more than 75% of rice area and production in the country are in the medium- and high-rainfall tarai, the specific rice production problems of these two

domains will have important implications for rice research priorities and biotechnology initiatives in Nepal. Physiographically, in terms of landform, about one-third of Nepal's rice area is located in the intermediate position of tarai, which is similar to most parts of the eastern Indian states. The rainfed rice cultivation is most common in the high-rainfall areas of tarai, where Masuli rice is extensively grown. The popularity of Masuli in these areas is also attributed to its usual escape from rice bug infestation, which is a major rice insect in this domain. Moreover, this variety gives fairly stable yields under rainfed production environments with high average monthly rainfall during the rice-growing period.

Hence, availability of any higher yielding variety that can tolerate this insect and preferably has medium grain quality is likely to replace Masuli and raise average rice yields in Nepal. Since gundhibug is also a major rice insect in the eastern Indian states, collaborative biotechnology initiatives may be a more efficient strategy toward producing a variety with gundhibug resistance. In the context of Nepal, the specific biotechnology agenda should include development of rice varieties that are resistant or tolerant to blast, bacterial leaf blight, armyworm, gundhi bug, caseworm, and borer.

Notes

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Priority setting for rice research in southern India

C. Ramasamy

Rice production in southern India has developed slowly in recent years due to many constraints. The prospects for higher productivity in rice appear gloomy in view of the fact that the farmers' yields on irrigated land are about to reach the potential of existing technologies in this environment. A breakthrough is possible only by a revolution of another kind in rice production by way of increasing biological efficiency through hybridization of rice varieties, and by unfolding new frontiers of production technologies with biotechnology and wide hybridization. This paper identifies the major constraints to rice production in southern India and suggests that rice biotechnology research will provide the answers to solving these problems.

It is important that we identify the constraints that keep rice yields significantly below their maximum potential and to channel efforts to increase yield by solving the constraints particularly in the "low-yield'' regions and on the farms with low yields. The approach should be to identify the nature of the yield gap between the potential and what farmers get. Once the magnitude and nature of the yield gap is established, the second aspect is to identify biological, physical, and socioeconomic factors that explain the gap. This study broadly adopts the procedure suggested by Herdt and Riely (1987) in ranking the constraints and in setting the priorities.

Estimating yield gaps

Yield gaps will vary from one environment to another. They can be attributed to biological, physiological, and socioeconomic constraints. These constraints, combined, account for a yield gap in a particular environment. A gap can be composed of the difference between an experiment station's maximum yield and an on-farm experiment's maximum yield (yield gap I) and the difference between actual farm yield and the yield attained in on-farm experiments (yield gap II). Yield gap II, which is the

concern of this study, reflects biological, physiological, and socioeconomic constraints. This study quantifies the contribution of each major constraint to yield gap II and estimates the benefits that might accrue by overcoming the constraints.

In southern India, the maximum rice yields obtained in experimental stations under irrigated conditions vary from 6.0 t ha^{-1} at Kerala to about 8.6 t ha^{-1} in Tamil Nadu and Andhra Pradesh. The average yields in farmers' fields are less than half of these amounts—varying from 3.3 t at Kerala to 5.2 t at Tamil Nadu. Most of these gaps are due to environmental factors that cannot be managed in farmers' field.

The yield achieved in experimental stations is substantially lower under rainfed conditions than under irrigated conditions.

Yield losses

One hundred and twenty scientists engaged in rice research and an equal number of extension personnel provided estimates of yield losses due to each constraint for their respective agroclimatic zones (Tables 1-3). Not all these constraints are major or occur simultaneously. They may occur in the most severe form in any one of the regions during a certain season. The yield losses indicated are when the constraints occur beyond economic threshold levels and are at least moderately severe.

Problem areas targeted for research

The 15 problem areas targeted for research on the basis of the estimated loss of production are shown in Table 4. Since the ranking is ordinal, it is difficult to compare the severity of constraints when the relative ranking changes across states.

Table 1. Estimates of yield losses (kg ha^{-1}) from insects and diseases, by state.

Constraint	Andhra Pradesh	Tamil Nadu	Karnataka	Kerala	South India
Leaffolder	42	64	27	36	44
Earhead bug	34	50	25	30	35
Stem borer	31	34	29	31	32
Gall midge	22	34	22	30	25
Brown planthopper	23	22	22	24	23
Green planthopper	19	18	17	23	19
Thrips	13	18	18	21	16
Rodents	13	18	18	21	16
Others	4	0	11	4	3
Total losses from insects	201	258	189	220	213
Blast	28	37	30	30	30
Bacterial leaf blight	18	21	17	23	19
Sheath blight	18	18	16	29	19
Brown spot	16	23	25	23	19
Sheath rot	16	18	17	27	18
Tungro	15	20	18	24	17
Grain discoloration	11	18	19	23	15
Total losses from diseases	122	155	142	179	137

Table 2. Yield losses (kg ha^{-1}) from abiotic stresses, adverse soils, and management practices, and socioeconomic circumstances, by state.

Constraint	Andhra Pradesh	Tamil Nadu	Karnataka	Kerala	South India
Scarcity of irrigation water	23	37	24	28	26
Drought	18	23	18	0	18
Cold temperature at anthesis	0	6	14	0	4
Lodging	28	28	17	28	26
Low light intensity	0	3	11	0	3
Total losses from biotic stresses	69	97	84	56	77
Soil salinity	23	22	22	27	23
Low fertility	17	29	18	18	20
Zinc deficiency	15	25	23	0	18
Acid soils	0	9	10	27	6
Alkalinity	0	12	0	0	3
Iron toxicity	0	6	0	0	2
Total losses from adverse soils	55	103	73	72	72
Weeds	25	30	25	10	25
Imbalanced use of fertilizer	19	41	26	0	24
Aged seedlings	7	7	0	0	5
Varietal problems	0	0	26	28	7
Total losses form management practices	51	78	77	38	61
Socioeconomic circumstances	39	64	111	142	66

Table 3. Rice production losses in southern India, by ecosystem.

Ecosystem	Area (000 ha)	Actual farm yield (t ha^{-1})	Actual production (000 t)	Production loss (t h a^{-1})	Potential production (000 t)
Irrigated					
Andhra Pradesh	3,531	3.8	13,264	0.49	15,014
Karnataka	1,066	3.7	3,910	0.63	4,580
Kerala	425	2.6	1,111	0.69	1,405
Tamil Nadu	1,742	4.5	7,782	0.75	9,083
Rainfed upland					
Andhra Pradesh	216	2.2	468	1.28	746
Karnataka	106	2.5	263	1.15	386
Kerala	11	1.5	15	0.59	22
Tamil Nadu	50	2.5	126	0.55	154
Rainfed lowland and deepwater					
Andhra Pradesh	75	1.2	90	1.20	121
Karnataka	192	1.9	360	0.64	482
Kerala	128	1.7	218	0.85	326

Table 4. Ranking of rice yield constraints, by state.

Rank	Andhra Pradesh	Karnataka	Kerala	Tamil Nadu
1	Leaffolder	Rice blast	Leaffolder	Leaffolder
2	Stem borer	Yellow stem borer	Yellow stem borer	Earhead bug
3	Yellow stem borer	Leaffolder	Earhead bug	Fertilizer imbalance
4	Lodging	Fertilizer imbalance	Rice blast	Rice blast
5	Rice blast	Varietal problem	Gall midge	Water management
6	Weeds	Earhead bug	Sheath blight	Yellow stem borer
7	Brown planthopper	Weeds	Varietal problem	Gall midge
8	Salinity	Water management	Lodging	Weeds
9	Water management	Zinc deficiency	Water management	Low fertility
10	Gall midge	Brown planthopper	Salinity	Lodging
11	Green leafhopper	Gall midge	Acid soils	Zinc deficiency
12	Fertilizer imbalance	Salinity	Sheath rot	Thrips
13	Bacterial leaf blight	Brown spot	Brown spot	Drought
14	Drought	Grain discoloration	Brown planthopper	Brown spot
15	Sheath blight	Thrips	Rice tungro virus	Salinity

Based on the magnitude of crop losses due to these constraints, it is possible to identify a research agenda. As seen in Table 4, the major constraints identified are found in all four states studied. Mechanisms of tolerance for pests at all stages of crop growth are crucial to increased productivity. Genetic resistance to insects and diseases has a twofold advantage: it serves to increase yields and reduces the dependency of farmers on pesticides, thereby addressing environmental concerns. Significant losses from leaffolder, earhead bug, yellow stem borer, thrips, brown planthopper, green leafhopper, rice blast, brown spot, bacterial leaf blight, sheath rot, rice tungro virus, and sheath blight demonstrate the urgent need for genetic resistance and biotechnological research.

Lodging tolerance is crucial since many areas experience periodic cyclones and floods. Elongating varieties tend to lodge after floodwaters recede. So, the ability to withstand short-term inundation through genetic mechanisms could prove only very beneficial to large areas of southern India.

Some mechanisms to reduce losses due to weeds are clearly necessary. Although there are many conventional methods available, they are not successful enough to prevent yield losses. Early seedling vigor would help rice to compete with weeds for light nutrients. Selective herbicide resistance would allow rice to survive while nonrice plants are eliminated with herbicides.

Drought tolerance is crucial since many areas in southern India experience periodic drought stress during the summer season and premonsoon periods. Salinity and acid soils are other constraints for which there are many conventional solutions, but some of them are costly and take many years to reclaim soils. Hybridization embraces a wide range of technical possibilities and may help ameliorate yield losses with adverse soil complexes. In this regard, transferring of genes for tolerance for salinity and acid soils is desirable.

Important constraints for which chemical and cultural methods are the solutions include unbalanced use of fertilizers, low fertility, water management, and zinc deficiency.

Discussion

Great imbalances in both rice production and productivity have been observed among the production environments within a region/state. Despite adoption of improved cultivation practices, rice yields are stagnating in almost all production environments. Rice scientists are highly concerned about this trend since there is an ever increasing rice-eating population. In addition, many dietary surveys conducted in southern India reveal that people switch to a rice-based diet from millet and other coarse grains as their standard of living increases. The only way to match this growing demand for rice is to increase its production. However, the limiting factors are availability of land particularly with assured irrigation. In fact, due to urban industrial and infrastructure expansion, availability of land for cultivation has been decreasing. So increasing rice production will only be realized through growth in productivity in existing farming areas.

Recent developments indicate that current technologies seem to be inadequate to boost rice production further. It is argued that the wide gap between the potential yield (experiment stations) and farm yield is yet to be closed. Socioeconomic factors at the farm level are also inhibiting the efforts to narrow the yield gap. Most of modern rice technologies are resource- or input-intensive and put the small-scale farmers of southern India at a disadvantage. Hence, there must be a thrust on designing technologies that are scale-neutral and cost-effective.

At this juncture, biotechnologies with their inherent low-cost and resource-neutral characteristics could be a viable technology alternative to small-scale farmers to help them attain the yield potentials of modern rice varieties. Apart from their cost effectiveness, biotechnologies have another strong case in their favor—they are best suited for an ecology-friendly farming system that is essential for ensuring sustainable agriculture.

The objective of a rice biotechnology program would be to evolve high-potential varieties that are more responsive to biological inputs. Recombinant DNA technology and protoplast fusion may help to achieve genetic diversity, which is essential to obtain plant types that utilize soil nutrients, resist damage by pests and diseases, withstand a wide range of soil toxicities and deficiencies, and allocate energy more efficiently. Rice biotechnology research, therefore, deserves a high priority. The genetic resistance mechanism when they substitute high-cost inputs and synthetic technologies could be a great incentive to the resource-poor rice farmers in southern India. Because, in this part of India, high doses of chemical inputs (above the national average) are being used to obtain maximum rice yields. The observations of Widawsky and O'Toole (1990), with respect to the contributions of rice biotechnology research in eastern India, could very well apply to southern India. As they stated, "the returns to research in biotechnology may be realized through higher yields at low costs, costs which

would otherwise be incurred through input procurement and distribution, and training programs in proper input use. Such innovations are, particularly, important to resource-poor rice farmers."

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Report of the committee on gene symbolization, nomenclature, and linkage

Achievements to date

- Uniform system of numbering rice chromosomes has been universally accepted.
- One hundred and twelve new genes were registered during the 1987-95 period.
- Guidelines for designating isozyme loci, cloned genes, and blast resistance genes were finalized and published in issues 7, 8, and 11 of the *Rice genetics newsletter*, respectively.
- Committees have been appointed to prepare guidelines for the following traits where some confusion still exists: dwarfness, embryo mutants, cytoplasmic male sterility, grain protein mutants, cloned genes for isozymes, designation for restriction fragment length polymorphism and polymerase chain reaction markers, and designation of quantitative trait loci.

New rules

- The hyphen has been removed between the gene symbol and numeral, e.g., *xa21* instead of *xa-21*.
- The allele designation is now at the same level as the gene symbol instead of a superscript and is separated by a hyphen, e.g., *Amp4-1* instead of *Amp-4*^{1}.
- As soon as the arm location of a gene is known, the locus numbering should be revised to reflect that information. The short arm of each chromosome is designated as the left arm and the zero position is at the left end of the short arm.

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