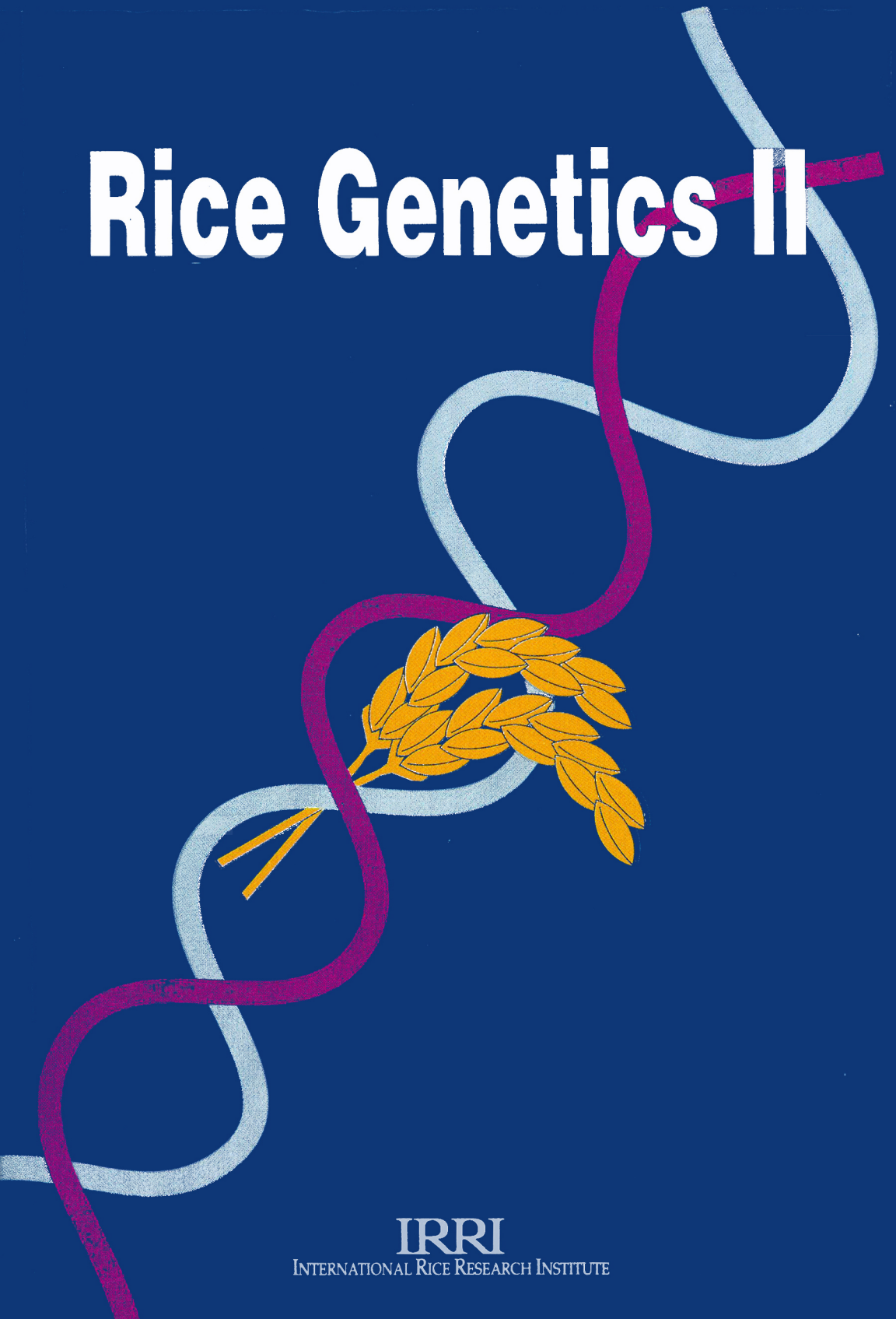
Rice Genetics II



IRRI
INTERNATIONAL RICE RESEARCH INSTITUTE

Rice Genetics II

Proceedings of the
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14-18 May 1990

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IRRI
INTERNATIONAL RICE RESEARCH INSTITUTE
P. O. Box 933, Manila 1099, Philippines

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Posters

Foreword

The First International Rice Genetics Symposium held at IRRI in May 1985 was attended by 200 scientists from 32 countries. The proceedings of the symposium, published in 1986 under the title *Rice Genetics,* contains an excellent summary of the knowledge then available concerning different aspects of rice genetics. One important outcome of the symposium was the decision to establish the Rice Genetics Cooperative (RGC) for promoting international cooperation in rice genetics. The RGC has played an important role in developing and promoting the use of standardized gene symbols and has been updating the linkage maps. It has assumed responsibility for publishing the Annual *Rice genetics newsletter* and for the maintenance and dissemination of rice genetic stocks.

During the First International Rice Genetics Symposium, it was decided to hold similar symposia periodically. The RGC established an international committee to organize the Second International Rice Genetics Symposium, held 14-18 May 1990. IRRI welcomed the opportunity of again hosting the symposium. Most of the preparatory work at IRRI was done by G. S. Khush, who served as organizing secretary with Y. Futsuhara. H. I. Oka, T. Kinoshita, and G. Toenniessen contributed advice and support in the preparation and implementation of symposium plans. The proceedings were edited by Dr. Stephen J. Banta, assisted by Mrs. Gloria S. Argosino.

The second symposium was attended by 300 scientists from 24 countries. About half the papers dealt with the molecular and cellular genetics of rice. This shows the increasing status of more fundamental studies on rice. It also emphasizes the progress made in biotechnology. An important outcome of this symposium was the decision to adopt a unified system of numbering rice chromosomes for linkage maps. This basic information on the Mendelian and molecular genetics of rice will be extremely useful in developing more productive rice cultivars for the 21st century.

IRRI will make all effort to be a partner to those who feel committed to use the latest knowledge and tools of biological science, specifically biotechnology, for the benefit of our ultimate target groups: the rural and urban poor whose staple food is rice.

Klaus Lampe
Director General

Opening remarks

Klaus Lampe

Modesty is one of IRRI's guiding principles. I like to think of it as one of mine. But it is difficult for me this morning not to get overexcited.

I firmly believe that this symposium—which IRRI has the great honor to host —is a collection of the best minds in the world able to deal with the genetic improvement of a plant that one-third of the world's population depend on daily.

Revolution is the term used to describe the production increases of the 1960s and 1970s. Revolutions are much more closely related to destruction and conflict than to the production of food. I see our work and our symposium more as a contribution to peace than as preparation for another revolution.

The East-West conflict, which we are learning to overcome by dialogue, is no longer seen as a threat to peace. But are we aware that a new—perhaps even more threatening—conflict can be seen on the horizon?

Almost daily we read, see, and hear warnings about population growth rates, urbanization, destruction of mega-environments and habitats, water shortages, and all kinds of climate changes. Collectively, they represent a far greater threat to society than any that civilization has faced before.

What actions are being taken beyond the oral expression of what should be done? Again, I have difficulty staying within the bounds of modesty. This community of biologists and geneticists, who have decided to spend this week together, belongs to the far too small brain force committed to the search for solutions.

Presentations at the Rockefeller Foundation's meeting on rice biotechnology last week showed that our knowledge of the molecular and cellular genetics of rice is expanding rapidly. Our understanding of rice genetics—such as chromosome maps and availability of genetic stocks—has facilitated research on rice biotechnology. Until a few years ago, no organization coordinated international collaboration in rice genetics. Consequently, recognized rules for gene symbolization and nomenclature for rice did not exist. Several systems for numbering rice chromosomes were followed. There was no coordinated effort to collect and preserve the seeds of rice mutants.

The First International Rice Genetics Symposium, held here in 1985, led to the birth of the Rice Genetics Cooperative. The cooperative has done a commendable job of enhancing the cause of rice genetics. A system of gene nomenclature has been agreed upon. Two stock centers for the preservation of rice mutants and chromosomal variants have been established. A *Rice genetics newsletter* is published annually. You will have ample opportunities to interact informally and through committee meetings in the next few days. I hope that interaction will lead to more international collaboration in the genetics of this most important food crop.

With all that optimism there is, however, no reason to become euphoric or complacent. Biological research, especially in the industrialized countries, is confronted with a growing number of very critical—sometimes even aggressive—observers. They do not depend on rice as a source of income or as their staple food. They are not without gainful employment. Most of them have not felt the pain of an empty stomach or empty pockets. But their influence is growing. The impact of our work may be dramatically affected if—and for as long as—the application of genetic research is questioned, opposed, or restricted to an extent that endangers its economical use.

There are at least two key problems that are likely to remain controversial, even beyond the next rice genetics symposium. They are the ethical basis of our work and potential legal restrictions.

First, let us consider the ethical basis of modern biology and biotechnology. To what extent are we allowed to manipulate nature? And what are the possible biological consequences and associated risks?

We understand breeding as a selection process, almost as old as agricultural production. For hundreds of generations, it was an unquestioned—even highly respected—social and scientific function. Today, breeding—undersrood as an artificially induced gene selection and recombination process—has become a hot political and social issue with complicated state-controlled mechanisms monitoring research.

Even more important are the public awareness, the social acceptability, and the public support our work needs to become ultimately effective.

We need a very carefully planned and conducted dialogue with all interested groups. Absolute transparency and an active information system will be required to provide increased understanding on the part of all groups that form and influence public opinion. The speed with which biotechnology is proceeding is, for many of our partners and ultimate beneficiaries, literally breathtaking. Our experiences in the development of nuclear technology and medicine should serve as a warning to us.

The second concern is seen mostly as a legal one. How can intellectual rights in biotechnological research be protected and at the same time be commercially exploited? What patent rights can be or should be invested in single genes? What protection, if any, should accrue to human-engineered plants? Should inbred cultivars derived from the genetic pool of wild species receive patent protection?

Many agree that these are predominantly ethical, not legal, questions. Research is progressing so fast, however, that discussion of questions such as what kinds of rights should and could be protected, and when and under what circumstances, is left behind.

As a result, we have to deal with yet another dimension of uncertainty, which might even affect conferences such as ours. How can you openly exchange know-how as long as property rights and proper handling are not clearly understood?

The problem has for years been on the agenda of international organizations. Some national governments have taken actions that are strongly opposed by others. We all know that without an international understanding, without an acceptable code of conduct, international cooperation in breeding will become extremely complicated. The free exchange of improved or unimproved genetic material may become almost impossible.

That situation serves the interests of no one. It will dramatically affect our research, and it must be avoided by all means. Scientists who have chosen rice, the poor man's staple, as their crop are best qualified to seek solutions.

The resolution of these ethical and legal questions should not be left to lawyers and policymakers alone. If we do not take the initiative, then others will decide finally—and perhaps unwisely—on the application of our research efforts.

International cooperation in rice genetics

G.S. Khush

Rice is the principal food of nearly half of mankind. Yet, until recently, our knowledge of rice genetics lagged behind that of other food crops such as wheat, maize, barley, and tomato. However, several developments that took place during the last decade have resulted in rice becoming one of the best known crops genetically and a model plant for molecular genetic studies:

- association of linkage groups with cytologically identifiable chromosomes in 1984
- publication of the *Rice genetics newsletter* (RGN) annually, starting with volume 1 in 1984
- publication of proposed rules for gene symbolization in rice and a list of known genes with suggested gene symbols
- initiation of an International Program on Rice Biotechnology by the Rockefeller Foundation in 1985
- holding of the First International Rice Genetics Symposium at IRRI in May 1985
- establishment of the Rice Genetics Cooperative during the Symposium for promoting international cooperation in rice genetics

I shall briefly recount here some of the historical events that have led to the present high level of international cooperation in rice genetics.

Gene symbolization in rice

In the absence of any rules for assigning gene symbols, different symbols were assigned to the same genes. As an example, symbols *m, U, am, gl,* and *wx* were all assigned to the gene for glutinous endosperm (Kihara 1964). Similarly, the same gene symbol was assigned to entirely different genes. Symbol *gl,* for example, was used to designate glutinous endosperm as well as glabrous leaves. K. Ramiah of India was the first geneticist to advocate the standardization of gene symbols in rice. Kadam and Ramiah (1943) published a review of the use of gene symbols and suggested rules for gene symbolization that adopted many conventions used in maize, cotton, and *Drosophila.* The recommended symbols, along with those used by various authors and

relevant references, were given. The recommendations were accepted by scientists in some countries, but not in others.

Gene symbolization was discussed by the International Rice Commission (IRC) Working Party on Rice Breeding at its Sixth Session in Penang, Malaysia, in 1955. The Working Party suggested that unified system of gene nomenclature be developed, and appointed a committee for this purpose, with N.E. Jodon of the U.S. as convenor and S. Nagao of Japan and N. Parthasarathy of India as members.

In the meantime, the International Committee for Genetics Congress nominated a small committee to draft rules for genetic symbols and nomenclature for all organisms. The report of the International Committee was accepted by the Tenth International Genetics Congress held in Montreal, Canada, in 1959. The report was timely, because it provided the rice committee with guidelines in preparing rules for gene symbolization in rice and listed suggested gene symbols. The report of the rice committee was accepted by the IRC Working Party on Rice Production and Protection in 1959 and published in the *IRC newsletter* (Anonymous 1959). The rules for gene nomenclature and gene symbols were reviewed during the Symposium on Rice Genetics and Cytogenetics held in 1963 at IRRI and were accepted by the participants. Unfortunately, no mechanism existed for monitoring the use of symbols, and very few authors followed the rules for symbolization.

To promote cooperation and adoption of uniform gene symbols for rice in Japan, an Interim Committee of Japanese scientists was organized in 1979. The committee was named the Japanese Committee on Rice Gene Nomenclature and Linkage Groups in 1981 and was renamed the Japanese Rice Genetics Information Committee in 1984. The committee did significant groundwork in reviewing the IRC-recommended gene symbols.

During 1981 and 1982, there was considerable correspondence between IRRI scientists (M.S. Swaminathan and G.S. Khush) and officials of the Japanese Committee on Rice Gene Nomenclature and Linkage Groups (H. I. Oka, T. Kinoshita, and Y. Futsuhara) about the status of rice genetics. On the basis of this correspondence, the decisions were made to publish the RGN annually for exchanging information among rice geneticists and to hold an International Rice Genetics Symposium. The first volume of the RGN was published in 1984; it contained proposed rules for gene symbolization (RGN1:2-3) and a list of known genes with proposed gene symbols. The First International Rice Genetics Symposium was held at IRRI in May 1985. On the basis of discussions among participants it was decided to organize the Rice Genetics Cooperative (RGC) to promote international cooperation in rice genetics and to publish the RGN on an annual basis. One of the standing committees of the RGC coordinates and monitors new gene symbols for adherence to the rules of gene nomenclature; the new gene symbols are published annually in the RGN.

Chromosome numbering system

Various systems for numbering rice chromosomes have been proposed. Shastry et a1 (1960) studied the chromosome complement of rice at the pachytene stage of meiosis.

Chromosomes were numbered in decreasing order of length—the longest being chromosome 1 and the shortest being chromosome 12. Nishimura (1961) assigned numbers I to XII to the rice chromosomes involved in translocations, following the order in which the translocations were discovered. Thus the two chromosomes involved in the first translocation were numbered I and II, and the chromosomes involved in the second translocation were numbered III and IV. Later on the Roman numerals were changed to Arabic numerals. This system was arbitrary and was not based on cytological identification of the chromosomes. Kurata and Omura (1978) numbered the chromosomes on the basis of length at somatic prophase. The longest was designated K1 and the shortest K12. However, somatic chromosomes are difficult to identify because size differences are minor. During the First International Rice Genetics Symposium, a committee under the chairmanship of C. M. Rick of the University of California, Davis, California, USA, recommended that the chromosome numbering system based on the length of pachytene chromosomes as proposed by Shastry et al (1960) be followed. This recommendation was accepted by the RGC.

Linkage map of rice

The first report of linkage in rice was by Parnell et al (1917). Morinaga and Nagamatsu (1942) and Ramiah and Rao (1953) reported additional linkages between genes. Jodon (1956) proposed 7 linkage groups, and Nagao and Takahashi (1963) postulated 12 (I-XII). When the independence of these linkage groups was tested by Iwata and Omura (1975,1976) through trisomic tests, the 12 linkage groups were reduced to 9. However, 3 additional linkage groups were soon discovered; by 1980, 12 independent linkage groups had been established.

Associating linkage groups with chromosomes

Several workers have established primary trisomic series of rice. However, only two of the trisomic series have been used in associating linkage groups with respective chromosomes. Primary trisomics of rice variety Nipponbare were utilized for associating linkage groups with chromosomes at Kyushu University, Japan, by N. Iwata and associates (Iwata et al 1984). Similarly, primary trisomics of IR36 rice were produced and used by G.S. Khush and associates at IRRI, Philippines (Khush et al 1984). The extra chromosomes of trisomics of Nipponbare were identified at somatic prophase, and those of IR36 at the pachytene stage of meiosis. When the chromosome-linkage group associations found in these independently conducted studies were compared, the results did not agree.

Toward a unified system of numbering rice chromosomes and linkage groups

To remove the discrepancies between the results of Iwata et al (1984) and Khush et al (1984), it was decided to reexamine the extra chromosomes of trisomics of IR36 at pachytene. Consequently, R.J. Singh (representing the IRRI group) and N. Kurata (representing the Kyushu University group) met in June 1986 at IRRI and examined the extra chromosomes of IR36 trisomics. Identifications of extra chromosomes of six

trisomics were confirmed. The remaining six trisomics were examined by N. Kurata and H.K. Wu of Taiwan. On the basis of these results and discussions during several meetings, a tentative agreement has been reached to change the designations of four trisomics of IR36. It is planned to change the designation of triplo-4 to triplo-3, triplo-3 to triplo-6, triplo-6 to triplo-12, and triplo-12 to triplo-4. These recommendations will be reviewed by a committee appointed by RGC, and the results of discussions will be presented to the general body on the last day of this symposium. It is hoped that an acceptable system of numbering rice chromosomes and linkage group will emerge as an outcome of the symposium.

Rice genetics newsletter

The RGC has assumed responsibility for the publication of the RGN on an annual basis. To date, six issues have been published. The RGN is an excellent medium for exchanging information on the latest findings in rice genetics. We need your cooperation and contributions to future issues of the RGN.

Rice genetics symposia

As mentioned earlier, the First International Rice Genetics Symposium was held at IRRI in May 1985. We are assembled here today to participate in the Second International Rice Genetics Symposium. Major advances in rice molecular and cellular genetics have taken place during the five intervening years. I am sure much new information will be presented during this symposium and that the participants will have the opportunity to have informal discussions with other colleagues and explore areas for research collaboration. I am grateful to the members of the organizing committee for their full support in preparing for the symposium. This is the largest of all the symposia and conferences held at IRRI to date, and many colleagues at IRRI have given their support to make this a successful event. The Third International Rice Genetics Symposium will be held in 1995, and we hope future symposia will continue to be held at regular intervals.

Rockefeller Foundation's International Program on Rice Biotechnology

This is a unique network that supports upstream research on molecular and cellular biology for solving downstream problems of rice production. Through carefully identified laboratories in developed countries, the Rockefeller Foundation is supporting research that should eventually lead to the production of transgenic rices with novel genes.

During less than five years of the existence of the network, protocols for transformation of rice have been developed, useful genes for transformation have been identified. a restriction fragment length polymorphism (RFLP) map of 320 markers has been prepared, and efforts are under way for tagging genes of economic importance with RFLP markers. Thanks to the advances in molecular and cellular biology under the Rockefeller Foundation Program, rice has become model cereal crop for further research on fundamental and applied genetics.

Establishment of the Rice Genetics Cooperative

One of the important outcomes of the First International Rice Genetics Symposium was the birth of the RGC for enhancing international collaboration in rice genetics. The RGC has played a key role in monitoring gene symbols, publishing the RGN, and sponsoring discussions aimed at evolving a unified system for numbering rice chromosomes and linkage groups. The RGC has also established two genetic stock centers for collecting, conserving, and distributing seeds of genetic markers and chromosomal aberrations. We hope the RGC will continue to serve the cause of rice genetics for years to come.

With this brief introduction to the history of international collaboration in rice genetics, I add my words of warm welcome to those already expressed by our Director General, Klaus Lampe. I hope you will have fruitful discussions during the symposium and an enjoyable stay at IRRI. We at IRRI are looking forward to your inputs to the symposium and to enhanced collaboration in rice genetics.

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Abbreviations and acronyms

A

ABA = abscisic acid
AC = amylose content, analysis of correspondence
ACP = acid phosphatase
ADH = alcohol dehydrogenase
AMP = aminopeptidase
azaC = 5-azacytidine

B

6-BA = 6-benzyladenine
BAP = benzylaminopurine
BB = bacterial blight
BC = backcross
BI = blast
BPH = brown planthopper

C

CaMV = cauliflower mosaic virus
CAT = catalase
CHEF = contour-clamped homogeneous electric field
CMS = cytoplasmic male sterile
CTAB = cetyltrimethyl-ammonium bromide
ctDNA = chloroplast DNA

D

2,4-D = 2,4-dichlorophenoxyacetic acid
d_{jk} = Euclidean distance
DEAE = diethyl amino ethyl
DHFR = dihydroxyfolate reductase
DMRT = Duncan's Multiple Range Test
DMSO = dimethyl sulfoxide
DS = dry season

E

ECS = embryogenic cell suspension
EDTA = disodium ethylene diamine tetraacetate
EGMS = environment-sensitive genetic male sterility
ELISA = enzyme-linked immunosorbent assay
EMS = ethyl methanesulfonate
ER = endoplasmic reticulum
EST = esterase

F

FAC = factor analysis of correspondence
FDA = fluorescence diacetic acid
FOFIFA = Centre National de la Recherche Appliquée au Developpement Rural

G

GCA = general combining ability
GLH = green leafhopper
GMS = genetic male sterile
GOT = glutamate oxaloacetate transaminase
GUS = b-glucuronidase

H

HAC = hierarchical ascending classification
HMG-CoA = 3-hydroxy-3-methylglutaryl coenzyme A

HMGR = 3-hydroxy-3-methylglutaryl coenzyme A reductase
HPGMR = Hubei photoperiod-sensitive genetic male sterile rice
HPT = hygromycin phosphotransferase
HYV = high-yielding variety

I

IAA = indole-3-acetic acid
ICD = isocitrate dehydrogenase
IRBN = International Rice Blast Nursery
IRRI = International Rice Research Institute

K

KIN = kinetin
KPR = Kao's protoplast regeneration (medium)

L

LHCP = light-harvesting chlorophyll a/b-binding protein
LS = Linsmaier and Skoog (medium)

M

MAAL = monosomic alien addition line
m^5C = 5-methylcytosine
MCDV = maize chlorotic dwarf virus
MMS = modified Murashige and Skoog's medium
MNU = N-methyl-N-nitrosourea
mRNA = messenger RNA
MS = male sterile, Murashige and Skoog's medium
MSYo = Murashige and Skoog's semisolid medium
mtDNA = mitochondrial DNA
4-MUG = 4-methyl umbelliferyl glucuronide

N

NAA = naphthaleneacetic acid
NIL = near-isogenic line
NK58 = Nongken 58
NPTII = neomycin phosphotransferase II

P

PAL = phenylalanine ammonia lyase
PB = protein body
PCA = principal component analysis
PCR = polymerase chain reaction
PEG = polyethylene glycol
PGD = phosphogluconate dehydrogenase
PGI = phosphoglucose isomerase
PGMS = photoperiod-sensitive genetic male sterility
PMC = pollen mother cell

R

rDNA = ribosomal DNA
RER = rough endoplasmic reticulum
Rf = relative mobility
RFLP = restriction fragment length polymorphism
RIA = radioimmunoassay
Rsea/b = residual salt-extractable albumin subfraction
RTBV = rice tungro bacilliform virus
RTSV = rice tungro spherical virus

S

SCA	=	specific combining ability
SDH	=	shikimate dehydrogenase
SDS	=	sodium dodecylsulfate
SDS-PAGE	=	sodium dodecylsulfate polyacrylamide gel electrophoresis
SM	=	streptomycin
SSD	=	single seed descent

T

TGMS	=	thermosensitive genetic male sterile
tRNA	=	transfer RNA
TS	=	transgressive segregants
TTC	=	triphenyl tetrazolium chloride

W

WA	=	wild abortive
WBPH	=	whitebacked planthopper
WCV	=	wide-compatibility variety
WS	=	wet season

X

Xoo	=	*Xanthomonas oryzae* pv. *oryzae*

SESSION 1

Varietal Differentiation and Evolution

Association between *Pox-1* variation and seed productivity potential in wild rice

H. Morishima

Alleles at the *Pox-1* locus are differentially distributed in cultivated rice species and their wild relatives. Seed-propagating taxa are nearly monomorphic with allele 2A, while vegetatively propagating ones carry allele 4A at high frequency. This association between allozyme and life history was consistently found over broad areas at the species level as well as at the population level. A cross between an annual (2A2A) and a perennial (4A4A) strain of Asian wild rice *Oryza rufipogon* was extensively studied to elucidate the genetic mechanism of this association. In the F_3, 2A2A plants showed higher reproductive allocation, shorter plants, earlier flowering, and shorter anthers, which are characteristics of annual life history. When the hybrid population was selected for early flowering, allele 2A increased. When it was naturally grown under conditions favorable for vegetative propagation, allele 4A increased. In the isogenic pairs of 2A vs 4A at the *Pox-1* locus, association between allozyme and characters mostly disappeared. The above results suggest that some of the quantitative trait loci for these life-history traits are clustered on a chromosome segment marked by *Pox-1,* but their linkages are not tight. There must be certain selective interactions between those genes including *Pox-1* to maintain this gene block in nature.

Populations of the Asian common wild rice *Oryza rufipogon* Griff. tend to be differentiated into two ecotypes, annual and perennial (Barbier 1989, Morishima et al 1984, Oka and Morishima 1967). The annual type is characterized by a typical monocarpic life history, high seed productivity, high seed dispersing ability, early flowering, and high selfing rate. In contrast, the perennial type (polycarpic) is characterized by low seed productivity, high regenerating ability, late flowering, and high outcrossing rate. The annuals are found in shallow swamps that are parched in the dry season; the perennials are found in deep swamps that are relatively stable and retain soil moisture in the dry season. The contrasting characteristics and differential habitat preference of the two types represent intraspecific differentiation in adaptive strategy in *O. rufipogon.*

Fitness characters are often associated with each other in the coadapted manner in nature, characterizing the adaptive strategy of taxa. The genetic mechanism of the

development and conservation of such an adaptive syndrome has not been fully explored, although it has long attracted the interest of evolutionists. This is probably because the characters relevant to fitness or adaptation are controlled mostly by multiple genes, which cannot easily be analyzed for their location and organization in the genome. The recent availability of biochemical markers, however, has presented a new way to resolve quantitative traits into Mendelian factors (Kahler and Wehrhahn 1986, Paterson et al 1988, Weller 1987).

In the isozymic study of *O. rufipogon* and its related taxa, it was noted that, among a number of isozyme loci, *Pox-1* seemed to show a unique allele distribution associated with variation in seed vs vegetative propagation. This paper aims to elucidate the mechanism of preserving this association between allozyme and life history in this plant group. I will first present allelic variation at the *Pox-1* locus observed in cultivated rice species and their wild relatives having different propagating systems. Then I will report on the results of a series of experiments with a cross between an annual and a perennial strain of *O. rufipogon.*

Materials and methods

A number of accessions, each representing natural populations belonging to AA genome species, and several experimental populations derived in different ways from a cross, were grown to investigate isozyme and character variations.

Survey of *Pox-1* polymorphism

For wild species (*O. rufipogon, O. longistaminata, O. meridionalis,* and *O. barthii*), first-generation plants of samples collected in their natural habitats were grown in short-day plots or a greenhouse in Mishima, Japan, and scored for isozymes. For cultivated species (*O. sativa* and *O. glaberrima*), strains preserved at the National Institute of Genetics were used. Five to 30 plants per population of the wild species were examined, and 1-3 plants per accession of the cultivars.

Experiments with an annual/perennial cross

An annual strain (W106 from India) and a perennial strain (W1294 from the Philippines) of *O. rufipogon* were crossed. About 100 F_3 plants were examined for 6 isozymes and various characters on an individual basis. The characters included flowering date, plant height, panicle number, panicle length, awn length, anther length (correlated with outcrossing rate; see Oka and Morishima 1967, Oka and Sano 1981), seed fertility, and reproductive allocation (harvest index). This hybrid population was divided into two groups and grown in bulk during the F_3-F_6, selecting for early- and late-flowering plants, respectively. The *Pox-1* and *Acp-1* genotypes of each individual were determined for F_2, F_3, F_4, and F_6, plants. In addition, material from an experiment carried out in Taiwan, China, by H.I. Oka was also examined for isozymes: F_5 seedlings of the above-mentioned cross were transplanted at four sites in Taiwan and left for natural propagation (Oka 1988). After 3 yr, seed was collected from surviving plants for isozyme assay.

Near-isogenic lines of *Pox-1*

Ten F_2 plants heterozygous for *Pox-1* (2A/4A) were randomly chosen from the same W106/W1294 cross and repeatedly selfed until the F_6, holding this locus heterozygous. The progenies of five F_6 heterozygotes were released for allozyme and character observations. 2A2A and 4A4A plant groups derived from a heterozygous plant thus prepared can be considered as a near-isogenic pair carrying similar genetic background except for a chromosome segment surrounding the *Pox-1* locus.

Isozyme assay

Pox-1 was particularly considered in this study; other loci are briefly mentioned only for comparison. Samples for *Pox-1* assay were prepared from mature leaves at flowering, while for other isozymes mainly plumules were used. Horizontal starch gel electrophoresis (Pai et al 1973, Second 1982) was carried out.

Results

Distribution of *Pox-1* polymorphism in cultivars and their wild relatives

At the *Pox-1* locus, two major alleles—2A (slow band) and 4A (fast band)—and one rare allele—OC—are known (Pai et al 1973). The degree of polymorphism at this locus differs markedly by species. Cultivars of *O. sativa* and *O. glaberrima* are monomorphic, carrying only allele 2A (Table 1). Populations of Asian common wild rice *O. rufipogon*, which propagates both by seed and vegetatively, are highly polymorphic.

Table 1. Allelic frequency and heterozygote frequency at the *Pox-1* locus in wild and cultivated rice species. (Means of intra-population frequencies are given for wild species.)

Species	Population (no.)	Allelic frequency			Heterozygotes (%)
		OC	2A	4A	
Oryza sativa	452[a]	0.01	0.99		0
O. glaberrima	10		1.00		0
O. rufipogon					
Perennial type	24	0.07	0.47	0.46	20.8
Intermediate type	11	0.17	0.72	0.11	10.2
Annual type	16	0.04	0.96		0.2
Weedy type	9		0.95	0.05	3.9
O. longistaminata	9		0.02	0.98	3.6
O. meridionalis	4	1.00			0
O. barthii	11		0.99	0.01	0.4

[a] Data of Fu and Pai (1979) are included.

Table 2. Partitioning of gene diversity calculated from 64 populations of *O. rufipogon.*

Locus	H_T[a]	H_S[b]	D_{ST}[c]		
			a	b	c
Pox-1	.402	.152	.151	.075	.024
Acp-1	.506	.228	.173	.006	.079

[a]Total populations. [b]Within populations. [c]Between populations. a = between all populations, b = between ecotypes (annual, intermediate, perennial, and weedy types), c = between India and Thailand.

African perennial wild rice *O. longistaminata* preserves allele 4A at high frequency. Australian wild rice *O. meridionalis* (annual) is fixed to allele OC. African annual wild rice *O. barthii* is fixed to 2A, except for a few populations probably introgressed by *O. longistaminata.*

Populations of *O. rufipogon* were classified into perennial, intermediate, and annual ecotypes based on morphological characters, although variation among populations was continuous (Morishima et al 1961). Perennial populations were highly polymorphic, while annual and weedy (grown in ricefields) populations tended to be fixed to 2A (Table 1). To examine whether such a trend is consistently found in different localities, gene diversity between populations, D_{ST}, at the *Pox-1* locus, calculated from 64 populations, was partitioned into population, locality, and ecotype components using the method of Chakraborty et al (1982). The ecotype component was larger than the locality component at *Pox-1,* contrasting with another polymorphic locus, *Acp-1,* in which the locality component was much larger than the ecotype component (Table 2). These facts show that the frequency of 2A is associated with annual habit or seed propagating ability at the species level as well as at the ecotype level over wide areas.

Cosegregation between allozyme and quantitative traits

In the F_3 of a W106 (annual)/W1294 (perennial) cross, 10 quantitative traits and 6 isozymes were examined. Character measurements were compared between two homozygous genotypes of each isozyme locus. As shown in Table 3, the genotype effect of *Pox-1* was significant on a spectrum of characters: 2A homozygotes had higher reproductive allocation, shorter plants, earlier flowering, and shorter anthers—all characteristic of an annual life history—than 4A homozygotes. This suggests that quantitative trait loci for these characters are linked with the *Pox-1* locus. The directions of all these effects were as expected from parental or ecotypic differences. Four other isozyme loci respectively showed linkage with one of the characters, with the effects of expected or unexpected directions from parental means.

Characters examined here are known to be correlated with each other among natural populations of *O. rufipogon,* resulting in ecotypic differentiation within the species (Sano and Morishima 1982). Among the F_3 lines of the W106/W1294 cross, however, most correlations disappeared, suggesting that the character correlations were gener-

Table 3. Association between characters and allozymes in F_3 plants of W106 (annual)/ W1294 (perennial). [a]

Character	Locus (homozygous allele)					
	Pox-1 (2A:4A)	*Sdh-1* (1:3)	*Acp-1* (+9:-4)	*Amp-2* (1:2)	*Est-2* (1:2)	*Pox-2* (0:4C)
Flowering time	<	ns	ns	>>	>>	ns
Culm length	<<	ns	ns	ns	ns	ns
Panicle length	ns	ns	ns	ns	ns	ns
Panicle number	ns	ns	ns	ns	ns	ns
Awn length	ns	<[b]	ns	ns	ns	ns
Anther length	<	ns	ns	ns	ns	ns
Regeneration index	ns	ns	<[b]	ns	ns	ns
Self-fertility	ns	ns	ns	ns	ns	ns
Grain weight	ns	ns	ns	ns	ns	ns
Harvest index	>	ns	ns	ns	ns	ns

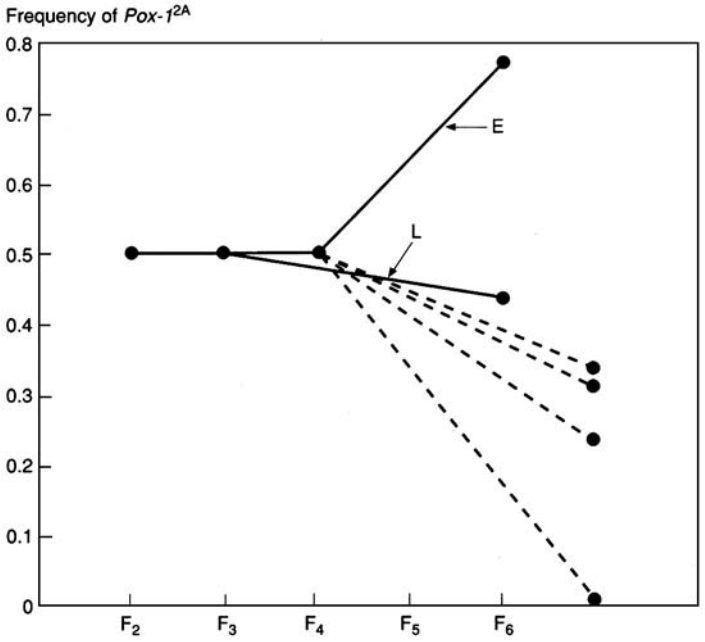
[a] Significant at the 1% (<<,>>) and 5% (<,>) levels. ns = nonsignificant. Direction of caret indicates relationship. For example, flowering time of 4A4A plants is significantly longer than that of 2A2A plants at the 5% level.
[b] Direction of association is opposite to parental type.

Table 4. Character correlations among 33 F_3 lines derived from an annual (W106)/perennial (W1294) cross (above diagonal), compared with those found among 57 strains of common wild rice from different Asian countries (below diagonal). [a]

Character	DH	CL	PN	AW	SF	GW	RG	AN	RA
Days to heading (DH)		.09	–.29	.21	–.41*	–.10	.09	.55**	–.42*
Culm length (CL)	.20		.13	.10	–.31	.11	.11	.28	–.21
Panicle no./plant (PN)	–.48**	–.28*		.07	.19	.31	.27	–.02	.49**
Awn length (AW)	–.12	–.17	.30*		–.15	.56**	.32	.25	.02
Seed fertility (SF)	–.47**	–.38**	.42**	.51**		–.05	–.23	–.58**	.65**
Single grain weight (GW)	–.35**	.17	.06	.16	.20		.17	.11	.27
Regenerating ability (RG)	.65**	.36**	–.45**	–.12	–.49**	–.14		.26	–.16
Anther length (AN)	.56**	.47**	–.41**	–.16	–.43**	.01	.62**		–.43*
Reproductive allocation (RA)	–.53**	–.32*	.39**	.17	.70**	.18	–.52**	–.61**	

[a] Significant at the 1% (**) and 5% (*) levels.

ated mostly by natural selection during differentiation (Table 4). Yet the following correlations remained in the F_3: high reproductive allocation associated with early flowering, many panicles, high seed fertility, and short anther; short anther associated with early flowering and high seed fertility; and early flowering associated with high seed fertility. This also indicated that a number of quantitative trait loci representing perennial-annual life history are partly linked.



1. Changes in frequencies of allele *Pox-1*^{2A} observed in differently selected hybrid populations derived from W l06/W1294. Solid line = selected for early (E) and late (L) flowering, dashed line = naturally grown at 4 sites in Taiwan, China.

Changes in allelic frequency in bulk populations

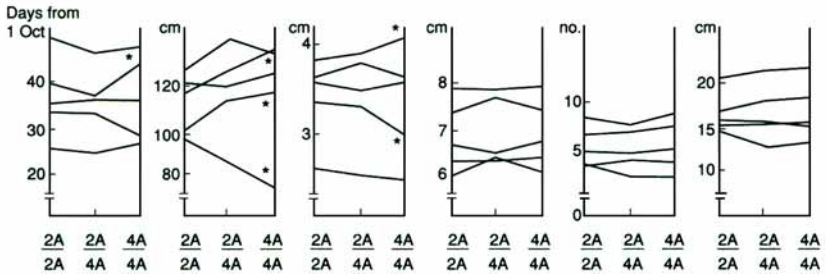
Allelic frequency at *Pox-1 and Acp-1* was monitored in two bulk populations that were originally derived from the same cross but selected for early and late flowering, respectively. The frequency of allele 2A increased in the early-selected group during the F_2-F_6: no such trend was observed in the late-selected group (Fig. 1). The allelic frequency at *Acp-1,* which was also segregating in this hybrid population, did not show any significant shift.

On the other hand, in naturally propagated populations in Taiwan, China, a decrease in 2A frequency was consistently observed at 4 sites during 3 yr. These plants were supposed to propagate mainly by vegetative means, seed propagation being unsuccessful under conditions in Taiwan (H.I. Oka, National Institute of Genetics, Japan, pers. comm.). The results of the preceding artificial and natural selection experiments indicated that 2A was selected in seed-propagated populations, particularly when selected for early flowering, while 4A was selected in vegetatively propagated populations.

Near-isogenic lines for *Pox-1*

In five sets of isogenic lines for *Pox-1,* various characters were compared among the 2A2A, 2A4A, and 4A4A plant groups (Fig. 2). No single character showed a consistent

	Heading date	Culm length	Anther length	Awn length	Panicle number	Panicle length
F_3	2A < 4A	2A << 4A	2A < 4A	2A ≒ 4A	2A ≒ 4A	2A ≒ 4A
5 isogenic pairs	ns	ns	ns	ns	ns	ns



2. Comparison of character measurements among 3 *Pox-1* genotypes (2A2A, 2A4A. and 4A4A) observed in 5 sets of near-isogenic lines. * = significant difference between 2A2A and 4A4A plant groups, ns = nonsignificant, ≒ means thecharacter measurements do not differ significantly (or are nearly equal) between 2A homozygotes and 4A homozygotes.

difference between two homozygoce groups over all five pairs. This implies that the *Pox-1* locus itself has no direct effect; nor are closely linked genes responsible in so far as the characters examined are concerned. In some pairs, however, allozyme-character associations werepreserved: between the 2A2A and 4A4A groups, flowering date was significantly different in one pair, culm length in three pairs, and anther length in two pairs. Irrespective of *Pox-1* genotype, anther length and plant height were correlated in one pair, and flowering date and panicle number were correlated in three pairs. But no correlations were found between members of the former (anther length, plant height) and those of the latter (flowering date, panicle number) groups. Judging from the rate of breakup of initial linkage blocks, the following arrangement could be presumed: anther length - plant height - *Pox-1* - flowering date - panicle number.

Discussion

Evolutionary forces influencing the conservation of multilocus associations or gametic-phase disequilibrium include selection, linkage, inversion, selfing, migration, and drift in a small population (Hedrick et al 1978). In the present study, a cluster of quantitative trait loci for flowering date, reproductive allocation, panicle number, anther length, and plant height was located on a chromosomal segment marked by the *Pox-1* locus. The chromosomal location of the *Pox-1* locus has not been determined. Other isozyme markers used in the F_3 study covered only two linkage groups (6 and 12). Therefore, the gene block detected in the present study might explain only part of natural variation. Yet, the preceding characters proved to be associated in a coadapted

manner in the detected chromosome block, as expected from ecotypic differentiation. The selectional process of clustering of coadapted genes is little known in eukaryotes. Studying a cross between two ecotypes of *Plantago lanceolata*, Wolff (1987) reported that ecologically relevant morphological genes were linked with effects expected and unexpected from parental combinations.

In the near-isogenic lines examined in the present study, the allozyme-character linkages observed in early generations largely disappeared. Selfing for six generations holding a particular locus heterozygous is expected to conserve a heterozygous segment of about 10% of the length of the chromosome on both sides of the selected locus (Hanson 1959). This means that the linkage between *Pox-1* and genes for those characters should not be strong enough to counteract the randomization force by outcrossing and recombination. There should be factors other than linkage to conserve this gene block.

In nature, an association between *Pox-1* and the perennial-annual syndrome was widely found at the species as well as the ecotype level, as mentioned. Even within a population, it was found that a seed-propagating subpopulation was fixed to 2A, while an adjacent subpopulation that propagated vegetatively was polymorphic (Morishima et al 1984). Furthermore, a similar trend of linkage between allozyme and life history traits as found in this study was also observed in other crosses of annual/perennial strains (Barbier 1990). Coadaptive interaction between genes including *Pox-1* seems to be only one plausible explanation for such an association, although whether it is because of the effect of *Pox-1* itself or some other tightly linked genes could not be detected in the present study. In the differentiation process from perennials to annuals, high seed productivity associated with earliness and selfing must have been selected for adaptation to a drier and more disturbed habitat, while, in the domestication process, rice plants must have been selected by nature and by people for high seed productivity as well as for low degree of seed shedding and seed dormancy. Such adaptive differentiation must be a gradual process to accumulate a number of coadapted genes with minor effects all over the genome. The selection of the gene block dealt with in the present study, however, could have played a key role with an appreciable effect, not only in the differentiation of annual types under natural conditions, but also in domestication. In this sense, the two processes are conceptually independent of each other, but partly interrelated in the real world.

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Notes

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Evolutionary significance of differential regulation at the *wx* locus of rice

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The waxy (*wx*) locus in rice, which specifies a major starch granule-bound protein called Wx protein, is genetically well characterized. In addition to *wx,* at least two nonwaxy alleles at the *wx* locus regulate the amount of the gene product as well as amylose content (AC). The present study was carried out to learn to what extent allelic differentiation at the *wx* locus is important for the diversity in AC observed among nonwaxy cultivars. Among the naturally occurring variants tested, AC was determined mainly by allelic changes at the *wx* locus. In addition, the amount of the gene product was affected by temperature, modifiers, and gene dosage in the same way AC was affected. These effects imply that AC is a quantitative trait in segregating populations as is often reported in rice, even though it is controlled simply by the amount of Wx protein. Allelic differentiation at the *wx* locus is discussed in relation to the evolutionary significance of gene regulation.

Amylose content (AC), a major determinant of eating quality, varies greatly among rice cultivars. The diverse AC phenotypes seem to have arisen during domestication, since the wild progenitor shows no such tendency. The waxy (*wx*) locus in rice affects the type of starch produced in the endosperm tissue. The simple inheritance contrasts markedly with the polygenic nature of AC observed among nonwaxy (or nonglutinous) cultivars. Accumulated evidence has revealed that higher organisms contain substantial genetic factors regulating gene expression. Recently, we proposed that there are two nonwaxy alleles at the *wx* locus in rice and that they regulate the quantitative level of the gene product differently, suggesting the presence of regulatory site(s) (Sano 1984). This presents a unique opportunity to examine the evolutionary change of regulatory elements that could be selected for. We present evidence here that differential expression at the *wx* locus greatly contributes to the continuous variation in AC observed in rice cultivars.

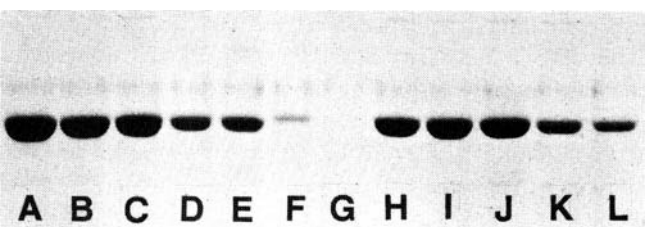
Determinants of amylose content

The *wx* locus specifies a 60-kDa major protein tightly bound to the starch granules (Sano 1984). The *wx* locus is the structural gene for the 60-kDa protein called Wx

protein, since a *wx* mutant (75-1) produced an altered form of this protein. The amount of the gene product increased in triploid endosperms through dosage effects in the same manner in which AC had been shown to increase. The *wx* locus in maize has been well characterized by biochemical and molecular techniques, and the antigenic relatedness of the Wx proteins in maize and rice (Okagaki and Wessler 1988) and their DNA sequence similarity (Hirano and Sano, unpubl. data) imply that both loci encode uridine diphosphoglucose-glucose starch glycosyl transferase. The assumption that the amount of Wx protein also causes varying degrees of AC among nonwaxy cultivars arose from the observation that two electrophoretically distinct alleles, Wx^a and Wx^b , at the *wx* locus control different amounts of the gene product as well as AC (Sano 1984). Wx^a produces about 10 times as much gene product as Wx^b, and the 2 alleles act additively in triploid endosperms. A positive correlation between the amount of Wx protein and AC (Sano et al 1985a) suggests that AC might be a good indicator of enzymatic activity of the gene product. Enzymatic activity is determined by the amount of gene product, suggesting the presence of cis-acting regulatory site(s) near or within the structural gene.

Allelic differentiation at the *wx* locus

Polyacrylamide gel analysis in extracts from starch granules readily distinguished most alleles as Wx^a or Wx^b, although some showed intermediate amounts of Wx protein and amylose, and their allelic states remain unknown (Sano et al 1985a). The quantitative differences might have been caused by a modifier that is independent of *wx* or the Wx^a and Wx^b alleles at the *wx* locus. To clarify their allelic states, alleles at the *wx* locus were introduced into a waxy line (T65*wx*, a near-isogenic line of Taichung 65 with *wx*) by successive backcrosses from 17 nonwaxy lines including different taxa. The alleles introduced into the same genetic background showed amounts of Wx protein and amylose similar to those found in the original lines (Fig. 1, Table 1). The near-isogenic lines with the alleles from cultivars that showed intermediate amounts of Wx protein and amylose also gave the same tendency. This confirmed that the alleles



1. Comparisons of starch granule-bound Wx proteins among 5 near-isogenic lines (A-E) of Taichung 65 with alien alleles at the *wx* locus, and their donor parents (H-L). A and H = W025 (*O. glaberrima*), B and I = Patpaku (indica type of *O. sativa*), C and J = W593 (*O. rufipogon*). D and K = Macan Coranglan (javanica type of *O. sativa*), E and L = Nagaewase (japonica type of *O. sativa*), F = Taichung 65 with Wx^b (japonica type of *O. sativa*), G = near-isogenic line of Taichung 65 with *wx* from Kinoshitamochi, the recurrent parent.

Table 1. Four types of nonwaxy alleles found in 17 accessions of *Oryza* spp.[a]

Type	Wx protein		Amylose content[d]	Distribution
	Relative amount[b]	2-D pattern[c]		
I	High (9.4-11.3)	A	High (25.7-29.1)	*O. sativa* indica (3), *O. rufipogon* (4), *O. longistaminata* (1)
II	High (9.7-10.6)	B	High (24.8-28.1)	*O. glaberrima* (3), *O. barthii* (1)
III	Low (0.9-1.0)	A	Low (15.2-15.8)	*O. sativa* japonica (2)
IV	Intermediate (2.2-3.6)	A	Intermediate (20.3-22.5)	*O. sativa* japonica (1), *O. sativa* javanica (2)

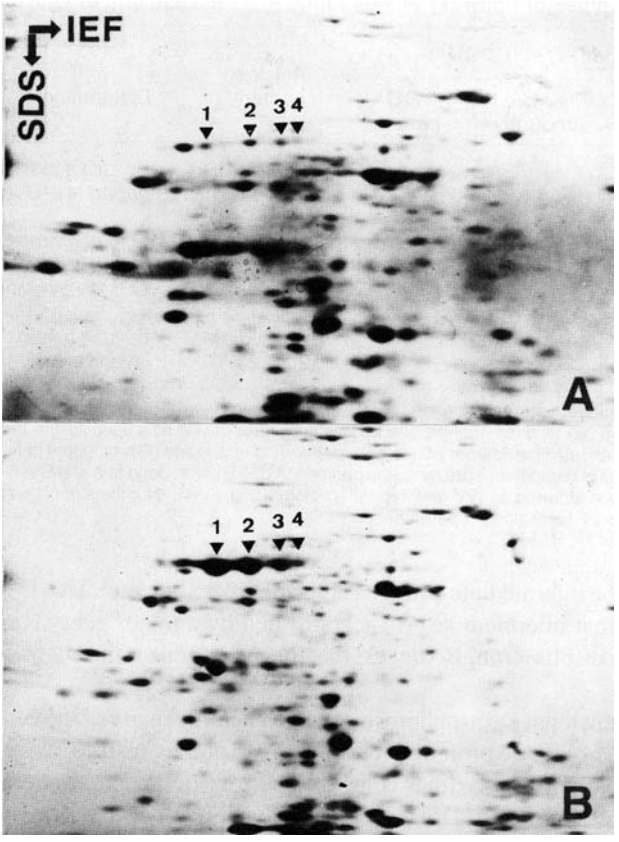
[a]Gene expressions were analyzed after the alleles at the *wx* locus were introduced into a waxy line by successive backcrosslng. T65 *wx* (a near-isogenic line of Taichung 65 with *wx*) was used as the recurrent parent The backcross generations differed from BC_3 to BC_{11} depending on the line. [b]The amount in Norin 8 was regarded as unity. Numbers in parentheses are ranges in percent. [c]A and B correspond to A and B in Figure 3, respectively, Types I and II were reported as Wx^a and Type III as Wx^b (Sano 1984). [d]Numbers in parentheses are ranges in percent. [e]Number of accessions in parentheses.

that specify the intermediate amounts are neither Wx^a nor Wx^b. This is supported by the observation that intermediate AC is controlled by a major gene (Kumar and Khush 1987). These results strongly suggest that the major gene is the allelic change at the *wx* locus itself.

Two-dimensional electrophoresis revealed heterogeneity of the gene product. Four polypeptides in soluble proteins were specific to nonwaxy lines, since those polypeptides disappeared in waxy lines (Fig. 2). The four polypeptides were detected in all nonwaxy lines examined. However, one polypeptide was not detected in the starch granule-bound proteins. In addition, the position of the missing polypeptide was different between lines with alleles from *O. glaberrima* and *O. sativa* (Fig. 3). The heterogeneity of the gene product appeared to result from modifications after transcription, since a *wx* mutant (75-1) showed a simultaneous change in those polypeptides. Based on these results, four types of alleles were recognized among nonwaxy accessions (Table 1).

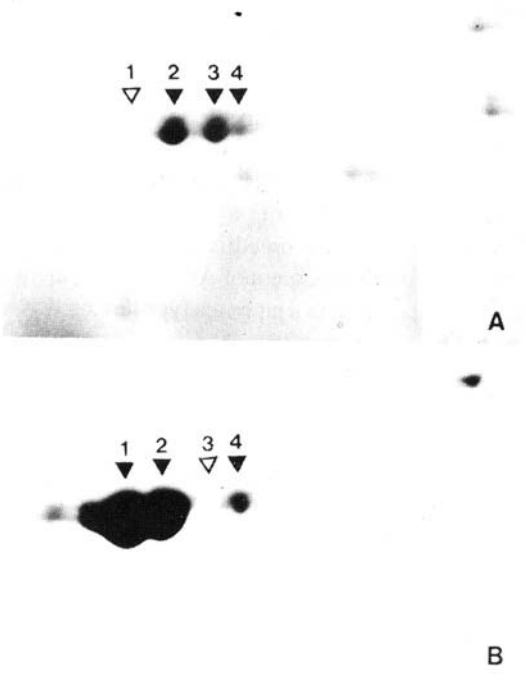
Induced mutants affecting gene expression

In naturally occurring variants it is difficult to interpret the regulatory function without the use of near-isogenic lines, because their genetic backgrounds may lead to misinterpretation, and their evolutionary origins are obscure. Comparing induced mutants of maize and rice, Amano (1981) found that leaky (or low-AC) mutants were more frequently induced in rice. Induced mutants obtained from Norin 8 were distinguished by their endosperm appearance, and their phenotypic changes were controlled by a recessive gene. Allelism tests were conducted to examine if the mutant genes were allelic to *wx*. The 12 *wx* mutant alleles were classified into 4 types according to the



2. Heterogeneity of Wx proteins (spots 1–4) revealed by 2-dimensional polyacrylamide electrophoresis in total proteins solubilized from endosperm with Tris-HCl buffer (pH 6.8). A = Taichung 65 with Wx^b, B = near-isogenic line of Taichung 65 with Wx^a from *O. glaberrima.* IEF and SDS show isoelectric focusing dimension and sodium dodecyl sulfate dimension, respectively.

amounts of Wx protein and amylose (Table 2). This contrasts with the fact that all waxy cultivars tested showed no Wx protein and almost no amylose (Sano 1984). On the other hand, none of the five low-AC mutants were allelic to wx, and all showed different segregation ratios when crossed with T65*wx.* The mutant alleles were independent of each other and had different effects on endosperm appearance. They were classified into four types according to their phenotypic differences and segregation patterns (Table 2). The low-AC mutant gene generally showed no gene dosage effect in the F_2 (Okuno et al 1983, Sano et al 1985a), suggesting that the gene product acts as a trans-acting regulatory factor. In addition, the manner of trans-action markedly differed between plants with Wx^a and those with Wx^b, since a mutant allele reduced AC only in plants with Wx^b (Kikuchi and Kinoshita 1987).



3. Starch granule-bound Wx proteins as revealed by 2-dimensional polyacrylamide electrophoresis. Of the 4 polypeptides (spots 1–4) detected in total proteins as shown in Figure 2, only 3 were found (open triangle indicates no spot). A = Taichung 65 with Wx^b, B = near-isogenic line of Taichung 65 with Wx^a from *O. glaherrima.*

Table 2. Classification of induced mutants affecting AC in rice endosperms according to gene expression at the *wx* locus.[a]

Type	Amylose content (%) (a)	Relative amount of Wx protein (b)	Ratio (a/b)	F_2 segregation in cross with T65wx	Mutants
wx mutants					
I	0.0	0.00	–	No segregation	73-1, 74-4, 74-7
II	0.3-0.6	0.61-0.77	0.01-0.004	No segregation	74-8, 75-1
III	0.0	0.19-0.56	0.00	No segregation	74-1, 75-5, 76-1, 76-2, 76-5, 74-2
IV	1.2	0.09	0.13	No segregation	74-2
Low-AC mutants					
I	7.2-7.4	0.26-0.27	0.27-0.28	9:3:4	74-5, 74-6
II	5.1	0.26	0.20	3:1	74-9
III	4.6	0.70	0.07	9:3:4	75-2
IV	3.4	0.27	0.13	9:7	76-3
Controls					
Norin 8	15.6	1.00	0.16	3:1	
T65 *wx*	0.0	0.0	–	No segregation	

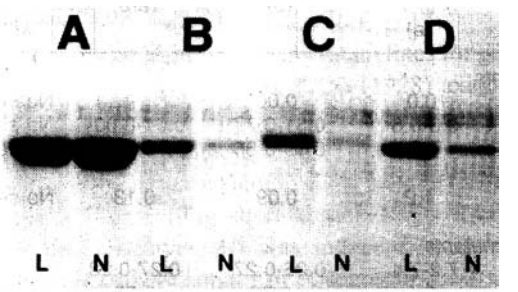
[a] The mutant gene in 76-3 is allelic to *du-2.*

Altered gene expression

As mentioned, alleles at the *wx* locus showed a dosage effect, with continuous variation of AC in segregating populations. The dosage effect affected AC in different ways according to the cross examined, which could be explained from allelic differences as well as the curvilinear regression observed between the amounts of Wx protein and amylose (Sano 1984, 1985). Another factor affecting AC is temperature. Lower temperature during grain development increases AC (Cruz et al 1989, Takeda and Sasaki 1988). Temperature insensitivity has great value for breeding in a region where low temperatures reduce grain quality because of the increment of AC. The amount of Wx protein was shown to increase with low temperature in a japonica type having Wx^b, suggesting that the AC increment is caused by an enhanced level of the gene product responding to low temperature (Sano et al 1985b). Temperature response was examined in the near-isogenic line with Wx^a and in the mutant line (75-1) with a partially inactivated form that was induced from Norin 8 with Wx^b. The line with Wx^a showed no temperature response, while in 75-1 the amount of Wx protein increased with low temperature (Fig. 4). These results strongly suggest that temperature response is also controlled by allelic changes at the *wx* locus but is independent of an inactivation of the gene product. The results also support the assumption that AC is controlled through changes in the amount of gene product, especially by allelic differentiation, even when the temperature effect is considered (Fig. 4).

Diversification in Asian cultivars

A number of accessions, including wild and cultivated rices, were examined for level of *Wx* protein in the endosperm starch. The results showed that the allelic states of the



Relative amount of Wx protein	10.5	10.6	3.3	1.0	2.7	0.9	4.0	1.0
Amylose content (%)	25.8	27.1	18.7	14.9	5.7	3.4	20.9	17.4

4. Effects of low temperature on amounts of Wx protein and amylose in 4 rice lines carrying different alleles at *wx* locus. L = 20 °C, N = 26 °C. A = near-isogenic line of Taichung 65 with Wx^a from Patpaku, B = Taichung 65 with Wx^b, C = *wx* mutant (75-1, induced from Norin 8) showing 62-kDa protein that seems partially inactivated, D = Norin 8 with Wx^b. Amount of Wx protein in Norm 8 (26 °C) was taken as unity.

near-isogenic lines with different alleles could be estimated from the amount of Wx protein, since few changes from the original lines were detected, and their alleles could be classified into three states: Wx^a, Wx^b, and the intermediate. The distribution of these alleles among rice taxa with the AA genome clearly showed that only Asian cultivars have great diversity, since African cultivars and all wild taxa have only the Wx^a allele (Sano et al 1986). In addition, the frequency of the three alleles was markedly different among three ecogeographical races: Wx^a and Wx^b were predominant in indica and japonica types, respectively, whereas the javanica type was heterogeneous. This strongly suggests that Wx^b emerged from Wx^a as a mutant with reduced level of gene product, and that the frequency of the allele increased during evolution of the japonica type.

Naturally occurring nonwaxy alleles fell into three major types—Wx^a, Wx^b, and intermediate—in terms of the amount of gene product; however, there might be intergrades within each allele, since near-isogenic lines with the same allele differed slightly in amount of gene product as well as in AC (Table 1).

Evolution at the *wx* locus

There is little evidence that regulatory changes of gene expression relate to phenotypic changes. The present results provide strong evidence that differential regulation at the *wx* locus plays a significant role in differential AC among diverse phenotypes, which affects grain quality. The *wx* locus in rice is not essential for growth, and defective mutants can survive. However, *wx* mutant alleles might be slightly deleterious, since homozygotes are rarer in introgressed wild populations than expected (Oka and Chang 1961). This suggests that the *wx* allele tends to disappear in wild populations by natural selection. Similar selection might act against the mutant allele with reduced expression, since no other taxa except *O. sativa* contain divergent phenotypes, even though *wx* alleles can occur by mutation. An attempt to find a waxy line in African rice (*O. glaberrima*) was made in vain; however, a *wx* mutant was induced with ethyl methanesulfonate treatment. The mutant showed a similar property in the starch granules (Uematsu and Yabuno 1988) and carried *wx* at the same chromosomal location as in *O. sativa* (Sano 1989), suggesting that African farmers had never selected *wx* mutants for their use regardless of their potential. Waxy *O. sativa* cultivars often have an independent origin, because intragenic recombinations occur among them. This clearly shows that farmers have selected *wx* mutants repeatedly during domestication. The frequency of intragenic recombination varied according to cross, and the maximum frequency of revertant pollen exceeded 0.5×10^{-3} (Li et al 1968). This suggests that the mutated sites responsible for the waxy phenotype are not restricted to a limited area of the DNA sequence.

The distribution of Wx^b is restricted to East Asian countries where waxy cultivars prevail. Most Japanese modem cultivars carry Wx^b. Allelic states were examined in traditional cultivars from southern Japan in addition to weedy forms from Japan and Korea (Table 3). The results suggest that fixation of Wx^b took place recently. Moreover,

Table 3. Distribution of nonwaxy alleles in Japanese rice cultivars and weedy forms.

Source	Accessions (no.)	Distribution (no.)		
		Wx^a	Wx^b	Intermediate
Modern cultivars	25	0	25	0
Traditional cultivars				
from Kyushu	17	0	6	11
from Okinawa	20	8	8	4
Weedy forms	14	9	3	2

Wx^a itself appears to have no adverse effects, since weedy forms frequently carry it. Intermediate alleles were also frequently detected in cultivars from Nepal (4 of 26) and Manipul, India (11 of 24), where no Wx^b has been detected. This suggests that the intermediate allele might have emerged directly from Wx^a. However, it is difficult to speculate whether Wx^b and the intermediate alleles have different origins. Recently, opaque endosperm with about 10% AC was found in an indica from Nepal (Heu and Kim 1989). Opaque endosperm was controlled by an allele at the *wx* locus. In the present study, *wx* mutants induced from Wx^b always showed no or only a trace of amylose. The opaque mutant might be a leaky mutant but might have a considerable amount of amylose if it has mutated from Wx^a, which produces higher levels of gene product and amylose than Wx^b.

Importance of differential regulation

New functional genes seldom emerge in the evolutionary process. Microevolution in crops proceeds with mutation, recombination (or hybridization), and selection. The diversity in gene expression at the *wx* locus observed only in Asian cultivars is regarded as the result of utilization of rare mutants by farmers; otherwise, such mutants are mostly doomed to extinction. In addition to allelic diversity, fluctuations in gene expression by temperature, modifiers, and dosage effects, if any, tend to make AC a quantitative trait, similar to most agronomically important traits. Nonfunctional *wx* alleles produce only a waxy phenotype, which almost lacks amylose. On the contrary, the regulatory changes result in various mutants with quantitatively different levels of gene expression but without losing the gene product entirely. Although the *wx* locus is not essential in rice, agronomically important traits like yield and flowering time may be controlled by essential genes whose dysfunction makes a plant nonviable. In such a case, only slightly deleterious mutants may be allowed to occur and to be maintained within a population. Mutants with modified gene expression but without the entire loss of gene product are expected to be regulatory mutants in nature. Traits like yield and flowering time may involve more than one basic gene, so regulation becomes more complex than with the *wx* locus. Such an example has already been reported in a continuous pattern of anthocyanin pigmentation, which is controlled by an allelic

series at three independent loci in addition to tissue-specific regulatory elements (Takahashi 1957). To detect major factors from polygenic traits, restriction fragment length polymorphism analysis will be used. More studies on gene regulation in quantitative traits are also needed for understanding the polygenic nature of agronomically important traits and for manipulation of the gene system.

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Cytogenetic, electrophoretic, and root studies of javanica rices

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Intercrosses among cultivars of traditional upland varieties and bulu varieties of Indonesia as well as of the dual-purpose type, the aus varieties, were made to elucidate their cytogenetic and biochemical relationships as well as similarities in their root systems. Based on pollen and spikelet fertility, the bulu and upland varieties showed slightly higher genetic affinity with each other than with the aus rices; however, the aus group bore similarities to the upland and bulu varieties based on their root characteristics. Pollen mother cells of the F_1 hybrids showed slightly lower frequencies of loose pairing at pachynema and of laggards, tri-valents, bridges, and fragments at meiosis than the parents, which also showed such chromosomal aberrations at low frequencies. Tris-HCI-soluble seed proteins indicated a more primitive state of the aus varieties than the two other groups, and the protein bands also supported a close relationship between the bulu and upland groups. The parents differed in their grouping by peroxidase isozymes. Moreover, the 13 F_1 hybrids formed 4 distinct clusters based on the combined esterase-peroxidase bands. Both bulu and upland varieties have more advanced plant characteristics than does the aus group. The javanica race can be expanded to include both bulu and upland. The upland varieties appear more diversified than the bulu varieties because of their broad geographical dispersal.

The great antiquity of the genus *Oryza* and the broad dispersal of the Asian cultigen (*Oryza sativa* L.) following its cultivation and domestication have led to enormous diversity among its cultivars. The traditional cultivars (land races) probably numbered about 100,000 before the advent of the modem varieties and the subsequent displacement of the unimproved germplasm. The major ecogeographic races (variety-groups) are indica and japonica (the latter also designated sinica), which were known in China 1800 yr ago as "hsien" (nonsticky) and "keng" (sticky) types, respectively. A third group of tall and 1ong-panicled Indonesian varieties was designated as javanica by Morinaga (1954) following the term coined by Körnicke (1885). Javanica corresponds to Group Ic of Terao and Mizushima (1944), the B plant type of Matsuo (1952), and the tropical insular group (IIa and IIb) of Oka (1958). Later Oka (1983) changed the

designation to "tropical japonica." Another variety-group comprising the aus varieties of Bangladesh and eastern India, which also show high genetic affinity with either one of the two major races, was later combined with the Indonesian cultivars as the intermediate type by Morinaga and Kuriyama (1958).

We became intrigued by the morphological similarity between the javanica cultivars and the traditional hill (upland) rices of Southeast Asia—tall stature, low tillering, rather broad leaves, stout culms, long and well-exserted panicles, large and bold grains, often awned—although they belong to distinct hydro-edaphic regimes, lowland and upland, respectively. We were also interested in the aus varieties because they share some of the drought avoidance mechanism of the hill rices in having moderately deep and thick roots (Chang et al 1986).

This paper synthesizes our biosystematic studies on the genetic relationship, karyomorphology and meiotic behavior, root characters, and electrophoretic properties of selected cultivars in the javanica race, hill rices, and aus varieties and in the F_1 hybrids among the three groups. Our aim is to elucidate their relationships in the multifaceted process of ecogenetic differentiation and diversification.

Materials and methods

Thirteen varieties selected from three ecotypes were used as the parents of a diallel cross (Table 1). The pollen fertility of the parents and F_1 hybrids was determined by staining pollen grains with I-KI. Spikelet fertility was obtained by counting the filled spikelets of 10 panicles per entry.

For cytological observations, pollen mother cell smears were prepared following a modified iron-acetocarmine technique (Engle et al 1969). Meiotic chromosomal aberrations were examined from the mid-pachytene stage through anaphase II.

Table 1. Parental materials, their origin and cultural type.

Cultivar	IRRI accession no.	Country of origin	Ecocultural type
Baok	19248	Indonesia	Bulu, lowland
Ijo Gading	17741	Indonesia	Bulu, lowland
Hawara Batu	13524	Indonesia	Bulu, lowland
Rodjolele	16544	Indonesia	Bulu, lowland
Khao Lo	12904	Laos	Hill or upland
Khao Youth	12901	Laos	Hill or upland
Kinandang Patong	23364	Philippines	Upland
Moroberekan	12048	Guinea	Upland
63-83	14725	Ivory Coast	Upland
Aus 61	28924	Bangladesh	Aus, dual purpose
Aus 3761	29571	Bangladesh	Aus, dual purpose
Black Gora	40275	India	Aus, dual purpose
Dular	32561	India and Bangladesh	Aus, dual purpose

Forty dehulled seeds from each variety were used in seed protein electrophoresis. The extraction of seed protein followed the procedure used by Siddiq et al (1972), except that whole seeds were used in this study.

Isozymes were extracted from 85-h-old germinating embryos in a 2-°C cold room. For esterases, 300 mg of embryos were ground in 1.5 ml sodium phosphate buffer (0.2 M, pH 7.0) and homogenized. For peroxidases, 400 mg of embryos with 2 ml Tris-HCl buffer (0.2 M, pH 6.0) was used. The homogenates were centrifuged at 20,000 g for 20 min at 4 °C. Two hundred microliters of the supernatant for esterases or 300 µl for peroxidases was loaded over each gel. Each sample was loaded over three gels as replications.

A modified procedure of Davis (1964) for anodic gels was adopted. Electrophoresis was performed under a constant electric current of 2–3 mA/gel tube in a 2-°C room. Bromophenol blue was used as the tracking dye. The gels were stained and fixed following the method standardized by Ng (1977) for seed proteins and esterase isozymes.

Relative mobility (R_f) was calculated by a formula modified from Weber and Osborn (1969). Similarity index and average Euclidean distance (d_{jk}) were computed by the methods of Sokal and Sneath (1963).

The root systems of 6 parents and 15 F_1 hybrids were studied using the aeroponic culture technique modified by Armenta-Soto et al (1982). Root length, thickness, and number were recorded at 45 d after sowing.

Results and discussion

Our findings on the parents and F_1 hybrids are presented under 6 headings: fertility of F_1 hybrids, karyomorphological study of 9 parents, meiotic behavior of 13 parents and 146 reciprocal F_1 hybrids, Tris-HC1-soluble seed proteins, esterase and peroxidase isozymes, and root systems under aeroponic culture.

Fertility of F_1 hybrids

The data on pollen and spikelet fertility obtained from the 13 parents and 141 reciprocal F_1 hybrids are given in Table 2. Pollen fertility values showed more stability within a variety-group because they are less subject to environmental influences than are spikelet fertility values. Pollen fertility in the bulu and upland rices averaged 96.0 and 94.9%, respectively, while their F_1 hybrids averaged 85.5%. The aus varieties averaged 93.7%, and their hybrids from bulu/aus and upland/aus crosses averaged 83.2 and 85.3%, respectively. The mean values were not significantly different among crosses, but individual hybrids ranged from 49.7% in Khao Lo/Ijo Gading to 99.9% in Moroberekan/ljo Gading. Eight crosses showed significant differences (19–48%) with their reciprocal crosses.

Mean spikelet fertilities of the three parental groups were lower than pollen fertilities: 77.9–90.1%. A wide range of spikelet fertilities was obtained in the F_1 hybrids, ranging from 9.7% in Kinandang Patong/Aus 61 to 95.3% in Aus 61/Black

Table 2. Pollen and spikelet fertility of 13 parents and 141 F_1 hybrids of bulu, upland, and aus varieties.

Parent or hybrid	Pollen fertility (%)		Spikelet fertility (%)	
	Range	Pooled mean	Range	Pooled mean
Bulu	91.5–98.5	96.0 ± 2.1	76.4–81.7	77.9 ± 7.7
Upland	86.0–98.7	94.9 ± 5.8	63.3–91.3	80.3 ± 11.1
Aus	94.3–100	93.7 ± 3.9	79.6–92.2	90.1 ± 5.4
Bulu/upland	49.7–99.9	85.5 ±17.0	28.6–92.9	70.4 ± 19.9
Bulu/aus	50.9–9.3	83.2 ±16.3	2.7–91.3	65.3 ± 2.3
Upland/aus	52.9–98.7	85.3 ±15.1	30.7–93.0	77.7 ± 13.8
Within bulus	93.3–99.5	96.0 ± 2.1	52.3–85.2	77.9 ± 7.7
Within upland	87.5–99.9	94.9 ± 5.8	46.5–92.3	80.3 ± 11.1
Within aus	84.0–98.0	93.7 ± 3.9	82.8–96.5	90.1 ± 5.4

Gora. However, the three parental array means did not show significant differences.

Thus, the three parental groups indicated a high degree of affinity between any two. Moreover, the F_1 pollen fertility readings were generally higher than those of earlier studies involving indica/japonica crosses (Demeterio et al 1965, Jennings 1966, Morinaga and Kuriyama 1958, Terao and Mizushima 1939), japonica/aus crosses (Morinaga and Kuriyama 1955), Indian indica/Indonesian indica crosses, and Indian indica/javanica crosses (Engle et al 1969). The fertility values were also slightly higher than those of the F_1 hybrids of upland/semidwarf indica crosses (Lin and Chang 1981).

Meiotic behavior of 13 parents and 146 F_1 reciprocal hybrids

The commonly observed chromosomal aberrations in the 146 F_1 reciprocal hybrids included loose pairings, univalents, chains-of-four, straggling chromosomes and laggards, bridges and fragments, and deficiency loops (Table 3). These occurred at rather low frequencies, rarely reaching 6.9% for any type in an F_1 hybrid. Low frequencies of the same kind of chromosome aberration were also observed in the parents.

No obvious correlation between the chromosomal aberrations and F_1 sterility could be found. Only in the F_1 hybrids of Aus 61/Ij0 Gading and Ijo Gading/Kinandang Patong crosses were significantly higher frequencies of loose pairing (20 and 10%, respectively) as well as high spikelet sterility (10.1 and 9.7%, respectively) observed. Fewer chromosomal aberrations were identified than in earlier indica/japonica (Demeterio et al 1965) and in Indian indica/javanica crosses (Engle et al 1969).

Tris-HCl-soluble seed proteins

The 13 parental varieties had 32 seed protein bands, varying from variety to variety as well as from group to group. The smallest number of protein bands (22–27) with the largest variation was found in the aus group. The bulu group had 26–27 bands and the upland group 26–29. This difference may suggest that the aus rices evolved earlier than the bulus and upland rices. This finding is also in general agreement with the 12–16 seed

Table 3. Chromosome behavior at meiosis in 13 parents and 146 F_1 hybrids.

Stage	Behavior [a]	Parents (%)		F_1 hybrids (%)		Cells examined (no.)
		Range	X	Range	X	
Pachynema	Normal	90.9 – 100.7	97.2	80 – 100	96.9	509 (P)
	Loose pairing	0 – 3.7	1.5	0 – 20	1.6	
	Inversion and translocation loops	0 – 3.0	0.6	0 – 4.2	0.2	3,570 (F_1)
	I (2)	0 – 3.7	0.5	0 – 6.9	0.8	
	Others	0 – 2.4	0.2	0 – 6.7	0.4	
Diplonema and diakinesis	Normal	89.8 – 100	95.7	89.9 – 100	95.9	1,529 (P)
	I (2)	0 – 4.3	1.9	0 – 5.7	1.8	
	Chain-of-4	0 – 4.8	1.0	0 – 4.5	1.1	19,813 (F_1)
	Ring-of-4	0 – 1.4	0.7	0 – 3.6	0.7	
	Others	0 – 2.6	0.8	0 – 3.2	0.5	
Metaphase I and anaphase I	Normal	90.1 – 99.5	95.2	90.1 – 100	95.3	2,608 (P)
	Laggards	0.4 – 3.3	1.7	0 – 5.0	1.8	
	I (2)	0 – 1.5	0.6	0 – 2.0	0.7	31,747 (F_1)
	I (3)	0 – 1.2	0.6	0 – 1.7	0.5	
	Bridges and fragments	0 – 2.9	1.0	0 – 2.9	0.8	
	Others	0 – 5.8	0.9	0 – 2.5	0.9	
Metaphase II and anaphase II	Normal	97.9 – 100	99.2	93.6 – 100	97.1	1,348 (P)
	Laggards	0 – 1.3	0.4	0 – 3.5	0.7	
	III	0 – 0.6	0	0 – 2.3	0.3	13,738 (F_1)
	Bridges and fragments	0 – 1.5	0.3	0 – 3.1	0.6	
	Others	0 – 0.9	0.1	0 – 2.6	0.3	

[a] Others included (a) trivalent association, deficiency loops, fragments, unequal bivalents at pachynema; (b) 13II, 1 III + 1III + 2I + 10II, 12II +nucleolar bodies at diplonema and diakinesis; (c) persistent nucleolus, late disjunction, straggling chromosomes, early division chromosomes, and 12II + fragments at metaphase I and anaphase I; (d) 2 laggards, fragments without bridges, 1 dyad with early disjunction at metaphase II and anaphase II.

protein bands in indica varieties, 14–15 in japonicas, and 15–17 in javanicas found by Siddiq et al (1972). The more advanced nature of the upland rices in relation to other ecotypes was pointed out by Chang (1976).

The mean similarity indices of seed protein profiles for the within-group and between-group comparisons are shown in Table 4. The mean index within the upland group (86.9%) was the highest and was significantly higher than the index within the aus group (75.5%). Moreover, the mean index of the bulu-upland comparison (75.5%) was significantly higher than the mean index between aus and bulu (66.8%). These comparisons suggest that there is a close genetic relationship among the three variety-groups and that the upland rices are closer to the bulu rices than to the aus rices. These indices are much higher than those among indica, japonica, and javanica races as reported by Siddiq et al (1972).

Table 4. Mean similarity indices and standard deviations (%) of electrophoretic patterns showing within- and between-group comparisons.[a]

Varietal comparison	Mean similarity indices ± SD (%)		
	Seed protein profiles	Esterase zymograms	Peroxidase zymograms
Within-group			
Aus	75.5 ± 8.7 bc	93.8 ± 4.0 a	100 ± 0.0 a
Bulu	83.3 ± 7.0 ab	85.4 ± 9.4 ab	96.4 ± 3.9 abc
Upland	86.9 ± 7.0 a	85.0 ± 7.9 ab	92.9 ± 5.8 bc
Between-group			
Aus vs bulu	66.8 ± 4.4 e	73.4 ± 4.3 c	94.7 ± 3.2 bc
Aus vs upland	71.1 ± 8.7 de	82.5 ± 9.2 b	95.7 ± 5.9 abc
Bulu vs upland	75.5 ± 5.3 cd	71.3 ± 10.0 c	92.5 ± 4.3 c

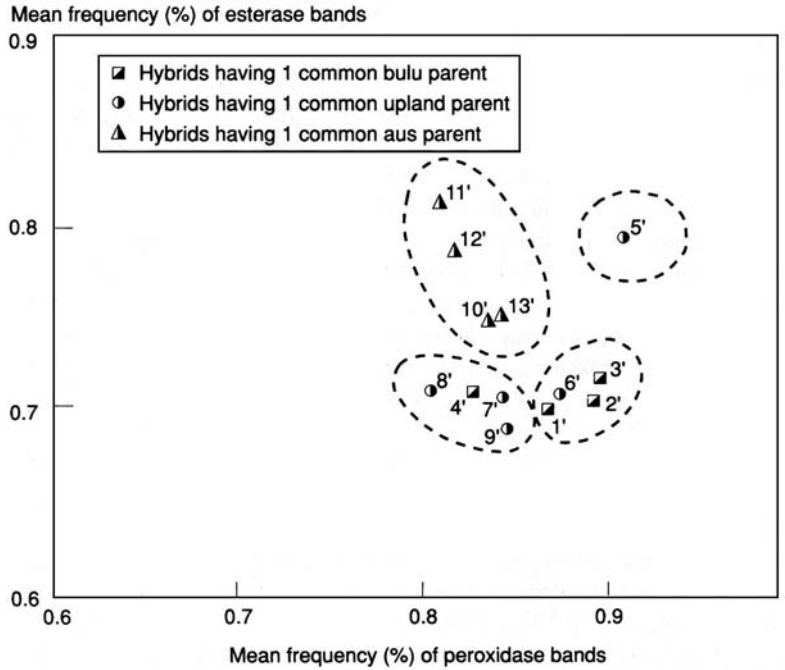
[a] In a column, mean similarity indices followed by a common letter are not significantly different at the 5% level by LSD test.

Esterase and peroxidase isozymes

Sixteen bands of esterase isozymes in the 13 varieties were identified. Eight bands (2, 3, 11, 12, 13, 14, 15, and 16) were constantly present, while the other 8 were variable. There were as many as 10 zymogram patterns in the 13 varieties. No hybrid band was detected in any of the 138 hybrids studied.

On the other hand, 12 isozymic bands of peroxidase were found in the 13 varieties, and little variation was detected. It was difficult to identify or pinpoint each varietal group by its zymogram patterns of both enzymes; authors of previous studies also failed to do so (Ng 1977, Palanichamy and Siddiq 1977, Shahi et al 1969). Nevertheless, it is likely that the three groups could be compared by the presence or absence of certain group-specific bands. For esterases, band 7 at R_f 0.39 was limited to the aus and upland varieties, whereas band 8 at R_f 0.41 was present in the bulu rices and in only one upland variety, Kinandang Patong. The bulu rices could also be separated from the aus and upland rices by the absence of peroxidase band 5.

High values of the mean similarity indices on zymogram patterns of both enzymes for within-group and between-group comparisons were observed (Table 4). Unlike with seed protein electrophoresis, the highest value was observed within the aus varieties in both cases (93.75% for esterases and 100% for peroxidases). The mean indices of the aus-upland comparison were significantly higher than the other mean indices of between-group comparisons, suggesting that the aus and upland varieties share more similar isozymic banding patterns. This is not surprising, because many workers generally place the two groups under the indica race. This finding also coincides with those of Shahi et al (1969) and Second (1982) that the cultivars of *O. sativa* could be grouped primarily into two, the indica and japonica clusters, with a range of intermediate varieties. Nakagahra (1978) classified rice varieties into five groups on the basis of esterase isozymes in the leaves, and placed the hill and mountain rices of Southeast Asia under Javanica 1 and 2. Glaszmann (1986) combined Chinese "keng," Japanese varieties, upland rices, and bulus under enzymatic group VI.



1. Distribution of the 13 hybrid populations based on mean frequencies of all isozymic bands of the 2 enzymes (esterase and peroxidase).

The isozymic bands of esterases and peroxidases found in the 13 parents were also found in their hybrids, but with different frequencies. When the distribution frequencies of all esterase or peroxidase bands in the parental arrays of 138 F_1 hybrids were averaged and arranged in a 2-way diagram (Fig. 1), 4 clusters could be identified: 1) all hybrids of the four aus varieties, 2) hybrids from 3 bulu varieties (Baok, Rodjolele, and Ijo Gading) and 1 upland variety (63-83), 3) hybrids of 1 bulu variety (Hawara Batu) and 3 upland varieties (Khao Lo, Khao Youth, and Moroberekan), and 4) hybrid populations derived from the upland variety Kinandang Patong, located in the upper right comer and away from the three other clusters. Kinandang Patong is an old cultivar from the Philippines (Bortolini 1844), morphologically different from most other upland varieties in panicle and grain features.

Root systems under aeroponic culture

Root length, thickness, and number were examined.

Root length. There was no significant difference in root length among groups (Table 5). All the F_1 hybrids produced longer roots than their parents. There was no significant difference among the F_1 hybrids within each of the three groups or among groups. However, root lengths of F_1 hybrids among groups were longer than those of the within-group F_1 hybrids.

Root thickness. The upland and aus groups had significantly thicker roots than those of the bulu varieties (Table 5). The upland group produced slightly thicker roots than the aus group. The root diameter surpassed that of most irrigated lowland varieties (0.51 mm and upward), although some varieties grown in drought-prone rainfed lowland areas also had thick roots (up to 0.88 mm), but not as thick as those of Moroberekan (1.26 mm) (Loresto et al 1983).

In between-group F_1 hybrids, the thickest roots were observed in the aus/upland group—significantly thicker than those of the F_1 hybrids of aus/bulu crosses, but not different from those of the F_1 hybrids of bulu/upland crosses. The within-group F_1 hybrids also showed that the bulu group had the thinnest roots (0.69 mm). Our earlier findings indicate that both dominant and recessive genes control root thickness in different crosses (Armenta-Soto et al 1983, Chang et al 1986).

Root number. The bulu varieties had a significantly higher root number than the upland and aus varieties (Table 5).

The root number of F_1 hybrids in crosses between groups was generally higher than that of the parents. The aus/upland F_1 hybrids gave the lowest root number, while crosses with the bulus gave significantly higher root number. Within-group crosses showed higher root numbers of the bulu group than those of the aus and upland groups. Our past studies have indicated the dominant nature of high root number (Armenta-Soto et al 1983; Chang et al 1982, 1986).

Among the three root characters in our earlier experiments, thickness had higher narrow sense heritability estimates (62%) than length (60%) and number (44%) (Armenta-Soto et al 1983), and, therefore, was a more important criterion for comparison.

Based on a matrix of correlation coefficients, Moroberekan in the upland group and Aus 61 in the aus group ($r = 0.54$*) showed similarity in both root length and number. On the other hand, similarities in root thickness between Kinandang Patong and Aus

Table 5. Mean root length, thickness, and number of 6 parents and 15 F_1 hybrids grown in aeroponic culture.

Parent or hybrid	Root length (cm)	Root thickness (mm)	Root number
Parental			
Bulu	92.8	0.58	61
Upland	86.4	0.82	25
Aus	84.7	0.80	28
Between-group			
Bulu/upland	104.1	0.84	55
Bulu/aus	1203.9	0.79	57
Upland/aus	102.8	0.90	47
Within-group			
Bulu/bulu	91.0	0.69	65
Upland/upland	95.6	0.88	35
Aus/aus	91.1	0.81	35

61 were shown (r = 0.69**), and also between Dular (an aus) and Rodjolele (a bulu) (r = 0.61**). The similarity could arise from the fact that aus and upland varieties are sown in dry soil and grown under rainfed culture.

Conclusions

A high level of genetic affinity among the three variety-groups as shown by fertility in the F_1 hybrids and by essentially normal meiotic behavior has been confirmed. It appears logical to expand the javanica race to include the bulus and the upland (hill) rices, although they differ in hydro-edaphic regime. In most cases, both types produce thicker roots than conventional irrigated lowland varieties, an advanced feature in varietal diversification. Other plant and grain characteristics such as organ size, low photoperiodicity, and weak grain dormancy of the two groups are also more advanced features (Chang 1976). Many hill rices of Southeast Asia have glabrous leaves. Another distinctive feature is their intermediate amylose content (18–25%), lying between those of the typical indica and japonica types (Chang 1988), a crucial factor overlooked by rice workers in the past.

Table 6. Ecogeographic races of *Oryza sativa:* comparison of their morphological and physiological characteristics (adapted from Chang 1988).

Indica	Sinica (or japonica)	Javanica
Broad to narrow, light green leaves	Narrow, dark green leaves	Broad, stiff, light green leaves
Long to short, slender, somewhat flat grains	Short, roundish grains	Long, broad, thick grains
Profuse tillering	Medium tillering	Low tillering
Tall to intermediate plant stature	Short to intermediate plant stature	Tall plant stature
Mostly awnless	Awnless to long-awned	Long-awned or awnless
Thin, short hairs on lemma and palea	Dense, long hairs on lemma and palea	Long hairs on lemma and palea
Easy shattering	Low shattering	Low shattering
Soft plant tissues	Hard plant tissues	Hard plant tissues
Varying sensitivity to photoperiod	Zero to low sensitivity to photoperiod	Low sensitivity to photoperiod
23–31 % amylose	10–24% amylose	20–25% amylose
Variable gelatinization temperatures (low or intermediate)	Low gelatinization temperature	Low gelatinization temperature

The aus group is also closely related to the bulu and upland groups but appears to have differentiated earlier. Whether the aus varieties, which are also called "upland' varieties by some Indian workers, are the progenitors of the typical hill rices or not could be the subject of further study. The aus group generally has intermediate to high (21–30%) amylose content.

Since variation among rice cultivars is continuous, and many cultivars have been transported by rice growers from one geographic area to another, critical studies should include an analysis of crop ecosystem in relation to varietal differentiation and diversification. The necessity of having multiple lines of evidence and an integrated research approach in interpreting evidence for the origin and dispersal of cultivated plants has been well expounded by Harlan and de Wet (1973).

The morphological and physiological characteristics of the three ecogeographic races are contrasted in Table 6.

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Notes

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Screening and analysis of wide compatibility loci in wide crosses of rice

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With indica and japonica testers to screen out wide compatibility types, a number of varieties seemed to be indicas but differed from them by showing semisterility in crosses with Ketan Nangka, a donor of the wide compatibility allele (neutral allele). Another varietal group showed good fertility with indica and japonica testers, but revealed sterility in crosses with Ketan Nangka. Thus, Ketan Nangka is suggested as a standard variety, along with the aus varieties, which show semi-sterility in crosses with indica and japonica testers but normal fertility with most aus varieties. A set of four varieties—Achar Bhog, Ketan Nangka, IR36, and a japonica type—is proposed as standard testers for hybrid sterility. F_1 hybrid sterility in rice is understood with allelic interactions at the *S-5* locus. With the identification system for *S-5,* a large number of crosses were made to test the extent to which the neutral allele at the *S-5* locus is effective. Hybrid sterility in Penuh Baru II and aus varieties, which is not explained by the testers for *S-5,* was found to be due to an additional locus rather than to a new allele. The neutral allele at the *S-5* locus can now be effectively used, but a new neutral allele indicated by Dular would also be important in rice breeding.

Hybrid sterility limits the application of wide crossing in rice breeding and lowers the productivity of hybrid rices. Its genetic basis was not understood for a long time. Recently, it was found that the F_1 sterility of hybrids between indica and japonica varieties is caused by an allelic interaction at a locus at which the indicas have $S\text{-}5^i$, the japonicas have $S\text{-}5^j$, and some javanicas have a neutral allele, $S\text{-}5^n$. The donor of $S\text{-}5^n$ is termed a wide compatibility variety (WCV). The $S\text{-}5^i/S\text{-}5^j$ genotype is sterile because of the abortion of gametes carrying the $S\text{-}5^j$ allele, but $S\text{-}5^n/S\text{-}5^i$ and $S\text{-}5^n/S\text{-}5^j$ are fertile (Ikehashi and Araki 1986). Allele $S\text{-}5^n$ has been incorporated as a wide compatibility gene into indica and japonica backgrounds to overcome sterility problems in wide cross and hybrid rice breeding (Araki et al 1988). This approach has been promising for breeding commercial hybrids between indicas and japonicas. A record of very high yield is reported in indica/japonica hybrids using the wide compatibility allele (Maruyama 1988).

Following initial successes in utilizing $S\text{-}5^n$, a number of problems emerged: the extent to which the locus is effective, the possibility of an additional locus, and systems

to identify the wide compatibility alleles. So far, the *S-5^n* allele seems to be effective for most typical indicas and japonicas. However, in the expansion of our screening of WCVs, many varieties were found for which *S-5^n* was not effective. Hybrids between some aus varieties and javanicas including WCVs show clear semisterility, while some aus varieties such as Dular show fertility when crossed with javanica WCVs as well as with indicas or japonicas. As hybrid sterility caused at the *S-5* locus can be identified with tester varieties and marker genes, it was possible to know whether the hybrid sterility between javanica and aus varieties is caused at the *S-5* locus or not. Contrasting results between the javanica WCV and Dular in their crosses with aus varieties appeared to be caused at a new locus at which Dular has another neutral allele and the javanicas and aus varieties each have interacting alleles. Genetic analyses suggested that a new locus is linked with *Rc* (red pericarp) in linkage group IV. Simultaneous use of neutral alleles at the two loci may solve the F_1 sterility problem in practically all kinds of crosses in rice.

A set of indica and japonica varieties has been used for screening WCVs. With our expanded knowledge of the types of hybrid sterility, it is now necessary to use more varieties as standards for screening WCVs. Several varieties are suggested.

Male sterility was recorded for individual plants in all the experiments, but no relationship was found between male sterility and the marker genes. Information is necessary to identify the genetic basis of male sterility; however, spikelet sterility is discussed here for simplicity. So far, pollen semisterility does not seem to lower the seed fertility of indica/japonica hybrids.

Hybrid sterility within and between varietal groups

To identify WCVs, more than 80 varieties, most of which were aus or javanicas, were crossed with indica and japonica tester varieties, and the pollen and spikelet fertility of the F_1 hybrids was examined (Ikehashi and Araki 1984). Along with the screening, a large number of crosses were made to determine compatibility types in each varietal group. The compatibility types of a given variety are here defined by hybrid sterilities shown in a set of crosses between the variety and testers. In the initial tests, several compatibility types in the javanica as well as in the aus varieties were found with the use of indica and japonica tester varieties. Additional crosses were made between the different types within each varietal group, and the F_1 hybrids were tested for fertility from 1983 to 1985. Many crosses were also made between javanica and aus varieties in the same period. All the tests were conducted at the Okinawa Branch of the Tropical Agriculture Research Center, where spikelet fertility is least affected by cold temperature due to the subtropical climate. For all the crosses, pollen and spikelet fertilities of the F_1 hybrids were determined by a standard method (Ikehashi and Araki 1984).

Compatibility types in javanicas

Following the identification of WCVs such as Ketan Nangka, Calotoc, and CPSLO-17, other compatibility types in the javanica group were found. Of 24 javanicas, 15

were classified into 1 group based on their high pollen fertility with both the indica and japonica testers, normal spikelet fertility with the japonica tester, and clear semisterility with the indica tester. These 15 varieties were designated Banten types. Six varieties showed semisterility in their crosses with indicas as well as japonicas. A representative variety, Penuh Baru II, was selected from this group. Only one variety, Padi Bujang Pendek, was identified as a WCV. The rest seemed to be exceptional.

Hybrids between types of javanicas

The six javanicas that showed semisterility in their crosses with the indica and japonica testers showed normal fertility in their cross with Ketan Nangka, a javanica WCV, and with varieties of the Banten group. It was thus concluded that, with a few exceptions, F_1 hybrids between different compatibility types in the javanica group show normal fertility.

Compatibility types in aus varieties

Forty-one aus varieties were tested; all the data are presented by Ikehashi and Araki (1987). The fertilities of some hybrids between aus varieties and indica or japonica testers are shown in Table 1. Aus 373 and Dular seemed to be widely compatible, although the pollen fertility in their crosses with IR varieties was marginal. Next to these two, five varieties including Panbira showed good fertility with the testers. A majority of 18 varieties were not classified into any definite category. Of them, five including Achar Bhog showed semisterility with indica and japonica testers. In many cases, the hybrid sterility exhibited by these varieties was marginal, leaving some possibility of reclassification.

Table 1. Spikelet fertility (%) of F_1 hybrids between testers and some aus varieties, 1984-85.[a]

Variety	Source	Spikelet fertility (%)				
		Testers		Javanicas		
		Japonicas	IR36	Ketan Nangka	Banten	Penuh Baru II
Achar Bhog	Acc. 25826	71.1	62.0	59.2	39.1	48.6
Aus 373	Acc. 29158	88.0	91.3	50.1	24.0	58.6
CH972	200011	30.5	84.8	65.3	–	50.0
D1123	Acc. 8455	87.2	70.9	57.9	64.4	–
Dular	200041	83.7	86.7	90.9	89.6	73.1
Ingra	Acc. 27552	61.4	68.3	48.8	85.5	
Kaladumai	200040	94.7	81.1	97.5	–	89.2
Kele	210013	69.4	88.2	22.6	50.0	–
Panbira	VT. 64	84.8	92.1	18.1	25.0	44.6
Prambu Vattan	200049	89.1	34.0	92.4	81.4	54.7
Satika	210003	81.8	54.4	60.1		59.4

[a] Details in Ikehashi and Araki (1987).

Hybrids between aus varieties

Because there were different compatibility types in the aus varieties—some were like indicas and others like japonicas—many varieties from different types were chosen and crossed with each other to test the fertility of the F_1 hybrids. The fertility of such hybrids was normal regardless of the compatibility type of the parent variety. Exceptionally low fertility was found only in the crosses of Prambu Vattan, which seemed to be a japonica in various aspects.

Crosses between aus varieties and javanica WCVs

Some aus varieties were crossed with WCVs, and the fertility of the F_1 hybrids was examined. The fertilities of some hybrids between aus varieties and Ketan Nangka are shown in Table 1. Many aus varieties showed hybrid sterility in their crosses with Ketan Nangka. Two japonica-like varieties—Kaladumai and Prambu Vattan—and Dular showed normal fertility in the cross with Ketan Nangka (Table 1).

To determine whether semisterility is a common fact in F_1 hybrids between WCVs and aus varieties, additional aus varieties were crossed with other WCVs, viz., Calotoc and CPSLO, and with other javanicas. With some exceptions, the F_1s between WCVs and aus varieties showed semisterility. Earlier, it was indicated that the Penuh Baru group of javanicas and some aus varieties such as Achar Bhog were similar in their semisterility both with indica and japonica testers; however, F_1 hybrids between Penuh Baru II and some aus varieties such as Achar Bhog showed clear semisterility (Table 1).

Tests of South Indian varieties

Varieties from South India or Sri Lanka were tested together with additional varieties in the aus group (Table 2). Karalath, Pusur, and Eat Samba showed good fertility in their cross with the indica or japonica testers, but not so with Ketan Nangka. Only

Table 2. Spikelet fertility (%) of F_1 hybrids between testers and aus or South Indian varieties, 1985.

Tester	Spikelet fertility (%)					
	Aus varieties			South Indian varieties		
	Karalath	Pusur	Surjamukhi	Triveni	Eat Samba	Dahanala
Japonicas	91.7	85.1	96.2[a]	38.7[a]	90.7[a]	45.0[a]
IR36	77.2	91.1	41.4[a]	85.7[a]	87.5[a]	74.3[a]
Ketan Nangka	58.8	47.2	91.2[a]	37.9	49.9[a]	67.6[a]
Panbira	91.6	95.0	–	–	–	–
Achar Bhog	–	–	83.1[a]	98.6	–	–

[a]Used as pollinator.

Surjamukhi showed high fertility with Ketan Nangka. Triveni and Dahanala were definitely not classified into indicas or japonicas. These varieties from South India or Sri Lanka seemed to be similar to most aus varieties in that they showed semisterility when crossed with Ketan Nangka.

Tests of varieties from Bhutan, China, and Korea

Crosses between some testers and Asian varieties gave the results shown in Table 3. Two improved Korean lines and Nanjing 11 seemed to be typical indica types. Three native varieties from China were similar to indicas but differed in their lower fertility with Ketan Nangka. They were similar to Triveni and Dahanala. The Bhutan varieties Jyakuchem and Kuchem showed good fertility both with indica and japonica testers, suggesting their wide compatibility. Jyakuchem was found to possess $S\text{-}5^n$.

Standard varieties for identifying compatibility types

The compatibility tests revealed a number of varieties in China and India that can be identified as indicas with the use of the indica-japonica testers. But they differ from indica testers such as IR36 and IR50 in their low compatibility with Ketan Nangka. Examples of such varieties are CH972, Triveni, Dahanala, Pe-Bi-Hun, and Tao-Jen-Chiao. Another type shows good fertility with both indica and japonica testers but significantly lower fertility in crosses with Ketan Nangka. To this type belong Aus 373, Panbira, Pusur, and Eat Samba. Ketan Nangka can thus be considered a standard variety. Whether or not a hybrid between a given variety and Ketan Nangka shows semisterility can be a criterion for classifying compatibility types. As most aus varieties show good fertility in crosses with each other, aus variety Achar Bhog was also selected as a standard variety. This variety shows semisterility in its cross with indica and japonica testers, so that any variety showing good fertility when crossed with it may be a kind of aus. Thus, a set of four varieties—Achar Bhog, Ketan Nangka, IR36, and japonica variety Taichung 65 or Akihikari—would be useful to identify compatibility types.

Table 3. Spikelet fertility (%) of F_1 hybrids between testers and some Asian varieties showing atypical performance.

Tester	Spikelet fertility (%)							
	Korea (1984)		China (1984)				Bhutan (1985)	
	Milyang 23	Suweon 258	Pi-bi-hun	Tuan-ku-chao	Tao-jen-chiao	Nanjing 11	Jyaku-chem	Kuchem
Japonicas	38.5	44.8	41.7	37.9	12.5	48.5	90.2	89.7
IR36	85.5	86.5	89.2	94.9	82.1	94.3	90.5	—
Ketan Nangka	96.8	92.8	44.8	—	44.8	94.8	—	—
Achar Bhog	64.6	87.8	74.2	83.3	57.8	89.7	84.1	71.4
Aus 373	—	93.6	86.5	98.4	89.5	93.6	—	—

Genetic analysis of hybrid sterility between varietal groups

A large number of crosses were made to test compatibility types of varieties, and Penuh Baru II was found to show clear semisterility in its cross with indica as well as japonica testers, suggesting that the tester system for *S-5* is not applicable to this variety. Also, many aus varieties showed clear semisterility with javanica WCVs such as Ketan Nangka and Calotoc. Some aus varieties were crossed for genetic analysis.

New locus suggested by a javanica variety

Among the compatibility types in the javanica group, Penuh Baru II was found to produce semisterile F_1 hybrids when crossed with japonica and indica testers, while producing fertile F_1s in its cross with Ketan Nangka. In the three-variety cross Ketan Nangka/Penuh Baru II//IR50, close linkages were found between spikelet fertility and the *wx* or *C* gene from Ketan Nangka (Table 4). In the complementary cross, Ketan Nangka/IR36//Penuh Baru II, the same linkage relationship was shown (Table 4). Therefore, Ketan Nangka's $S\text{-}5^n$ allele, which is closely linked with the *wx* and *C* genes, must be allelic to the sterility-causing allele in the F_1 between the indicas and Penuh Baru II. Since the F_1 hybrids from Penuh Baru II and indicas are semisterile, the allele possessed by Penuh Baru II must be different from $S\text{-}5^i$. On the other hand, the sterility of the F_1 of Penuh Baru II and japonica variety Akihikari was not affected by the locus near *C* and *wx* in the related cross of Ketan Nangka/Akihikari//Penuh Baru II (Table 4). The compatibility relation among indica, javanica, and japonica varieties can thus be ascribed to an allelic interaction at the *S-5* locus. However, the sterility of the F_1s of

Table 4. Distribution among 8 fertility classes (0–30 to 91–100%) of spikelet fertility in 3-variety crosses with Ketan Nangka and Penuh Baru II.

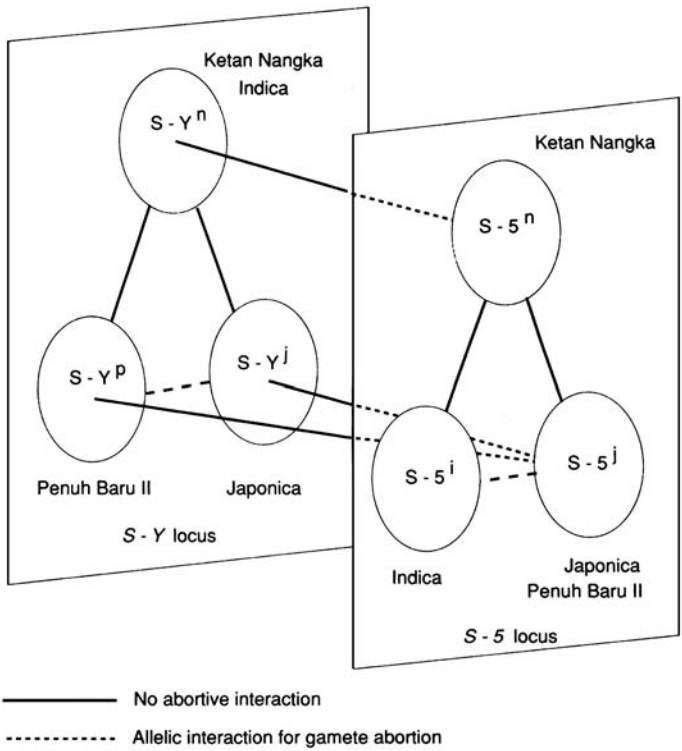
Marker	Plants (no.) in each fertility class								Total (no.)	Mean (%)
	0-30	31-40	41-50	51-60	61-70	71-80	81-90	91-100		
				Ketan Nangka/Penuh Baru II/IR50 (1984)						
C/C^+			1			7	16	3	27	82.1
C^+/C^+	1	2	7	14			1		25	50.9
wx/+			2	3		6	13	1	25	76.5
+/+	1	2	6	11		1	4	2	27	58.5
				Ketan Nangkal/IR36//Penuh Baru II (1985)						
C/C^+				2	1		12	42	57	91.7
C^+/C^+		3	26	21	6	2			58	52.1
wx/+			2	9	2	1	9	29	52	83.3
+/+		3	24	14	5	1	3	13	63	61.4
				Ketan Nangka/Akihikari//Penuh Baru II (1985)						
C/C^+	3		1	3	14	15	23	4	63	73.7
C^+/C^+			5	2	9	11	15	6	48	74.2
wx/+	3		3	3	15	16	21	5	66	73.4
+/+			3	2	8	10	17	5	45	76.2

Penuh Baru II and the japonicas must be caused at a locus other than *S-5*, where Ketan Nangka and indicas may have a neutral allele, since the sterility of the two varieties is caused only at the *S-5* locus (Fig. 1).

Detection of $S\text{-}5^n$ in wide compatible aus varieties

It has been indicated that aus varieties include various compatibility types in terms of F_1 sterility with indica and japonica testers, and that the types of compatibility are likely to differ from those of other groups. Therefore, the same method that applied to the analysis of WCVs was attempted for hybrids between aus varieties and the other types.

Aus 373 seemed to be a WCV and was tested in a three-variety cross using indica and japonica testers (Table 5). Spikelet fertility in the cross Aus 373/IR50//Akihikari was related to that of the genotype of C/C^+, suggesting that an allelic interaction between an allele from IR50 and another from Akihikari was responsible for spikelet sterility. Therefore, allelic interaction at the *S-5* locus was indicated. Similarly, Dular and Pusur were found to show normal fertility in their crosses with indica and japonica



1. Loci for hybrid sterility in Penuh Baru II. In its cross with IR36, Penuh Baru II reveals sterility due to allelic interaction at the *S-5* locus. Its sterility in crosses with japonicas is due to another locus, where both Ketan Nangka and IR36 have a neutral allele.

Table 5. Detection of wide compatibility allele near C locus by differentiating spikelet sterility in crosses to indica and japonica testers.

Marker genes	Plants (no.) in each fertility class									Total (no.)	Mean (%)	*t*-test[a]
	0-20	21-30	31-40	41-50	51-60	61-70	71-80	81-90	91-100			
Aus 373/IR50//Akihikari (1983)												
C/C^+		1	3	8	6	9	4		2	33	57.5	**
C^+/C^+	1	2	7	5	3	7	1			26	47.5	
Pusur/IR36//Akihikari (1985)												
C/C^+		1	2	1	5	4		22	12	46	80.9	**
C^+/C^+	5	4	10	12	11	3		1		46	51.4	
Akihikari/Dular//Tae baekb (1988)												
C/C^+	1	6	4	6	8	12	30	25	7	99	68.7	**
C^+/C^+	4	4	7	13	15	18	18	5		84	57.0	
alk/ +	2	2	2	7	6	10	30	25	7	91	71.4	**
+/+	3	8	8	12	17	20	18	4		90	55.4	

** = significant at the 1% level. Wigh-yielding indica variety from Korea.

testers. Three-variety crosses with the two varieties indicated the existence of an $S\text{-}5^n$ allele (Table 5). But Pusur is different from Dular in respect to its semisterility when crossed with Ketan Nangka.

Locus suggested by javanica WCVs and aus varieties

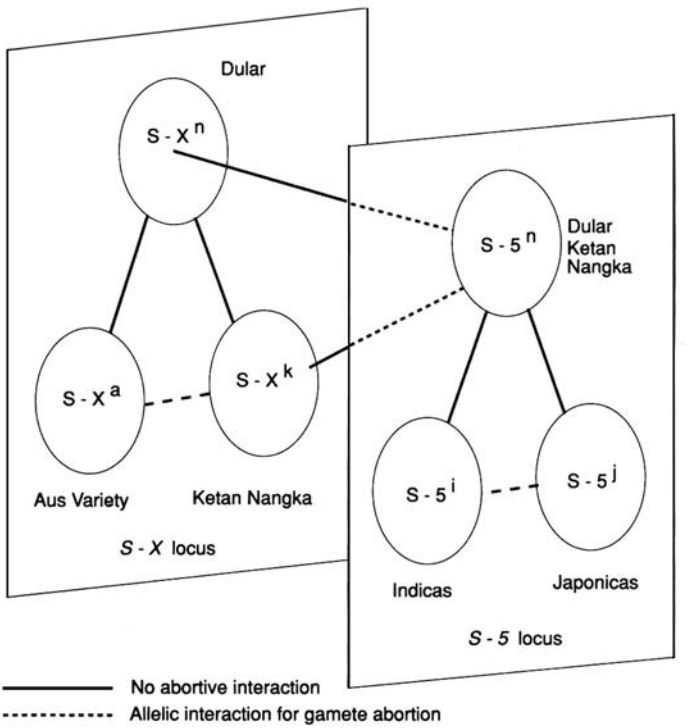
Many crosses between aus varieties and indica or japonica varieties were tested, but no clear relationship was found between fertility level and marker genotype. Allelic interactions can probably not be detected with the limited number of marker genotypes. After such tests, a different kind of three-variety cross was made, in which one aus variety and two WCVs were crossed to detect allelic interaction between the aus varieties and the javanica WCVs. An aus variety with red pericarp, Ingra, was used in Ingra/Ketan Nangka//CPSLO 17, where the cross between Ketan Nangka and CPSLO 17 did not show hybrid sterility and the sterility could be due only to Ingra and CPSLO 17. In this cross, the level of spikelet fertility was related to the *Rc* locus in linkage group IV (Table 6). Therefore, the pronounced hybrid sterility between javanica WCVs and aus varieties may be caused at this locus near *Rc.* To analyze the new locus, several crosses were tested. But in similar crosses using aus varieties with red pericarp, such as Kele and Chakila, the effect of the locus near *Rc* was not found (Table 6). It is likely that the locus near *Rc* can function in only some aus varieties.

Dular and Ketan Nangka have the same neutral allele at the *S-5* locus but differ in their cross with aus varieties. Dular also has a neutral allele at the newly suggested locus, where Ketan Nangka shows allelic interaction with some aus varieties (Fig. 2).

Table 6. Three-variety crosses with aus, javanica, or japonica varieties.

Marker genes	Plants (no.) in each fertility class									Total (no.)	Mean (%)	*t*-test[a]
	0-20	21-30	31-40	41-50	51-60	61-70	71-80	81-90	91-100			
					Ingra/Ketan nangka//CPSLO 17							
+/+			2	3	10	3	5	12	11	46	73.8	
wx/ +			2	9	9	5	1	14	12	52	71.8	
Rc/*Rc*$^+$			3	11	14	5	2	2	1	38	55.9	
Rc$^+$/*Rc*$^+$			1	1	5	3	3	19	28	60	83.7	**
					Tatsumimochi/Chakila//Banten (1989)							
Rc/*Rc*$^+$		1	1	1	2	6	12	23	22	68	82.2	
Rc$^+$/*Rc*$^+$			1	0	7	13	4	17	21	63	79.0	
					Tatsumimochi/Kele//Banten (1989)							
Rc/*Rc*$^+$	1	0	1	4	5	6	8	11	20	56	81.1	
Rc$^+$/*Rc*$^+$			2	3	4	7	7	20	26	69	77.9	

[a] ** = significant at the 1% level.



2. Loci for hybrid sterility between aus varieties and javanica WCVs. Dular and Ketan Nangka have neutral alleles at the S-5 locus. But Dular has another neutral allele at a locus near *Rc,* where an aus variety and javanica WCVs have two interacting alleles for gamete abortion.

Discussion

The system of F_1 hybrid sterility in rice is now better understood in the light of allelic interactions at a locus. The basic structure of allelic interactions can be shown as a triangular relationship between three alleles, i.e., one neutral and two interacting alleles. Gametes possessing one of the interacting alleles are eliminated in heterozygotes of such alleles, while in heterozygotes of a neutral allele and another allele no gamete abortion occurs.

The identification system for *S-5* is well constructed, with standard testers from indica and japonica types as well as such marker genes as *C* and *alk* in linkage group I. Thus, the neutral allele known as the wide compatibility gene has been used in breeding hybrid varieties.

Since this gene mechanism has been understood, a large number of crosses have been made to test the extent to which the neutral allele is effective. Then two varietal groups were found for which the identification system for *S-5* is not adequate. First, an exception was demonstrated by Penuh Baru II, which showed clear semisterility in its crosses with indica as well as with japonica testers, suggesting that the testers for *S-5* are not applicable. Second, many aus varieties showed clear semisterility in their crosses with javanica WCVs such as Ketan Nangka and Calotoc.

The limitation of the initial identification system for alleles at the *S-5* locus implies that there are more alleles at the *S-5* locus or that additional loci function independently of the *S-5.* One of the clues for determining the genetic basis was obtained by marker genes. Because any allelic action at the *S-5* locus can be definitely traced by such markers as *C* or *alk,* a sterility reaction without any relation to the markers may be due to another locus.

Hybrid sterility in Penuh Baru II and aus varieties, which is not explained by the standard system for *S-5* alleles, is found to be caused at an additional locus rather than by a new allele. In the case of Penuh Baru II, a new locus is suggested, where the indica tester and Ketan Nangka have a neutral allele, while japonicas and Penuh Baru II have interacting alleles. In the hybrid sterility between WCVs and some aus varieties, they are assumed to possess interacting alleles, with Dular possessing a neutral allele at this locus. Dular showed an exceptionally good compatibility with indica, javanica, and japonica varieties.

Although the neutral allele at the *S-5* locus is effectively incorporated into indica and japonica varieties, the use of a new neutral allele indicated by Dular would be important to breeding work on the Indian Subcontinent. Further progress in the study of hybrid sterility will depend on the availability of marker genes. The use of isozymes provides a partial solution. But biochemical markers may be more useful. Standardization of tester varieties is also necessary. Such a system would be useful in classifying varieties, inasmuch as such terms as indicas and japonicas are as confusing in studies of hybrid sterility as in other research areas.

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How was rice differentiated into indica and japonica?

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The evolutionary dynamics of the indica-japonica differentiation was studied from the viewpoint of population genetics. Indica and japonica are distinguished by genes and characters associated with each other nonrandomly. In indica/japonica hybrid progenies, the same direction of gene and character association found among the cultivars was generally observed. This means a trend toward the restriction of recombination among several independent loci. Accordingly, intermediate types between indica and japonica are relatively infrequent, even if natural hybridization occurs frequently between them. Indicas and japonicas are isolated by the restriction of recombination in the hybrids.

Two subspecies or ecogeographic races of common rice, indica and japonica, are represented by genes and characters associated nonrandomly among many cultivars (gene and character association). Oka (1958) defined the indica and japonica types as two varietal groups having associations of genes or phenotypes in contrasting states of phenol reaction (*Ph/ph*), apiculus hair length, $KClO_3$ susceptibility, and tolerance for cold and drought. Glaszmann (1987) reported that two major varietal groups represented by associations of alleles at 15 isozyme loci largely corresponded to the indica and japonica types defined by Oka (1958).

Yet the causal factor of the indica-japonica differentiation among rice cultivars remains unknown. To elucidate the factors causing nonrandom association of genes and characters that result in the indica-japonica differentiation among cultivars, the pattern of their associations was studied in hybrid populations. Here I describe patterns of association in 12 genes and characters among many cultivars, and also in F_2 and F_5 populations derived from an indica/japonica cross, and discuss the factors causing such nonrandom associations.

Materials and methods

Two hundred cultivars and 4 single seed descent populations derived from an indica/japonica cross were tested to examine 12 characters and genes.

Plant materials

A sample of 200 native cultivars collected from various localities in Asia was used to represent varietal variation occurring in nature. The cultivars were classified into indica and japonica by the method described here.

Hybrid populations used in this study were derived from the cross Acc. 419 (indica)/ Acc. 504 (japonica). Both parents were included in the varietal sample. Acc. 419 was developed by pureline selection in India. Acc. 504 is the Taiwanese cultivar Taichung 65 (T65), from a cross between two Japanese native cultivars. Acc. 419 is a typical indica, and T65 a typical japonica. They have different alleles at a number of loci, and different phenotypes.

The F_2 population consisted of 200 individuals. The F_3 and F_4 populations were raised by the single seed descent method in which seed for the next generation is prepared as a bulk of a single seed from each plant of the previous generation. The F_3 and F_4 populations consisted of 188 and 172 plants, respectively. The F_5 population was raised by bulking 2 seeds from each F_4 individual, and 300 plants were randomly chosen for analysis.

Genes and characters examined

All F_2 and F_5 plants were examined on a single-plant basis for phenol reaction (*Ph/ph,* chromosome 4), susceptibility to $KClO_3$ at the two- or three-leaf stage (genes unknown), and apiculus hair length (in millimeters) to classify them as indica or japonica. The same characters were also recorded in the 200 cultivars. Measurement methods for these characters are described by Sato et al (1986). For quantitative numerical evaluation of the indica-japonica differentiation, a discriminant score (Z) was calculated for each cultivar by combining three characters as follows:

$$Z = Ph + 1.313K - 0.82Hr - 1.251$$

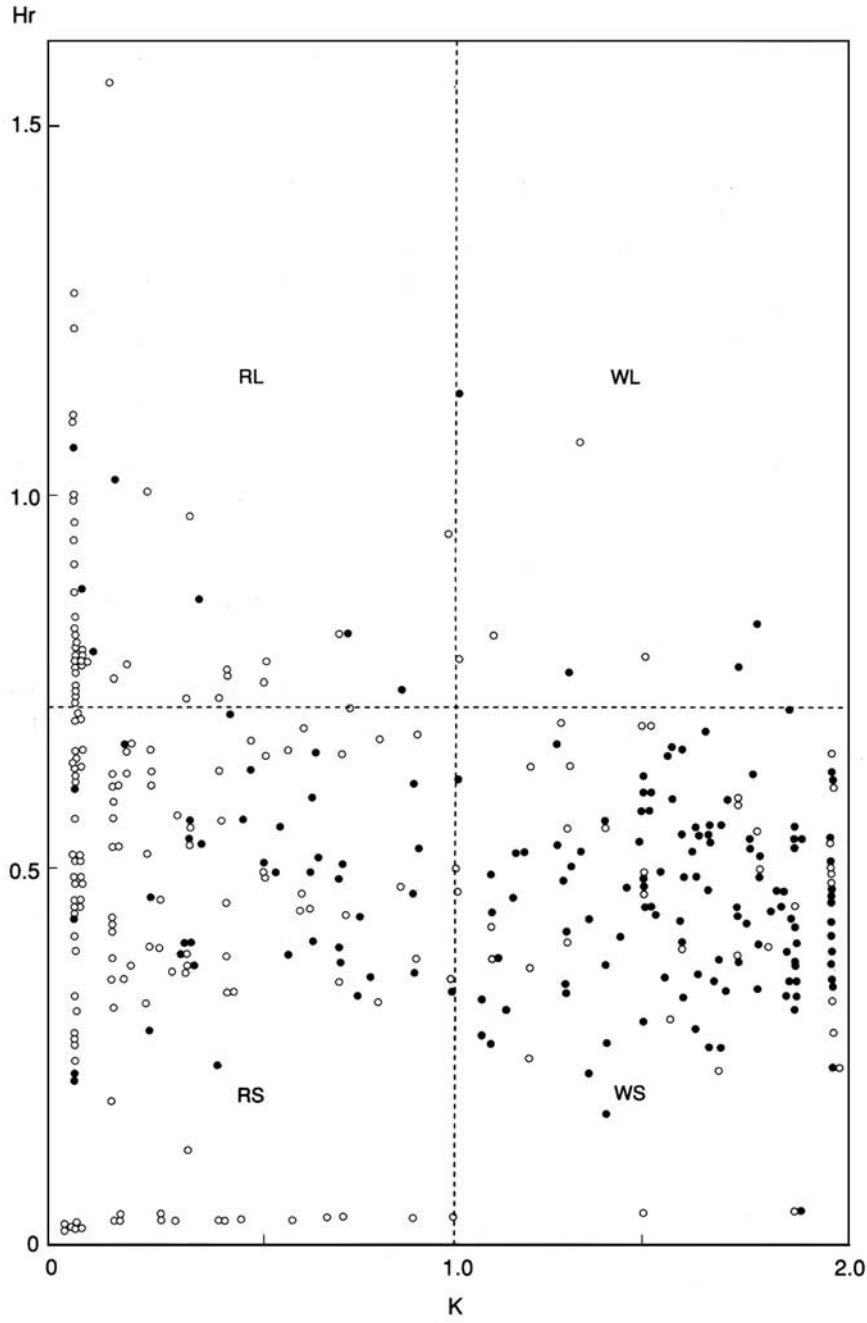
where Ph, K, and Hr indicate phenol reaction, $KClO_3$ susceptibility, and apiculus hair length, respectively. Ph is 1 if positive or 0 if negative. K varies from 0.0 (most resistant) to 2.0 (most susceptible). Hr value is given in millimeters.

The hybrids and cultivars were also examined for pericarp color (*Rc/rc,* chromosome 7), apiculus color (*C/c,* chromosome 6), hull color (black or straw, complementary action of *Ph, Bh-a,* and *Bh-b*), and awn (gene[s] unknown), which segregated in the present cross. Furthermore, they were examined for five enzyme-encoding loci that also segregated in the hybrid populations: *Est-2* (chromosome 6), *Pgi-2* (chromosome 6), *Amp-2* (chromosome 8), *Cat-1* (chromosome 6, but independent of *Est-2* and *Pgi-2*), *and Acp-1* (chromosome 12). Detailed descriptions of the methods of isozyme assay are given in Ishikawa et al (1987).

Results

Character and gene associations in the varietal sample

Correlations among Ph, K, and Hr among the cultivars are indicated in Figure 1. K values showed continuous variation, but seemed to be divided into susceptible (W) and



1. Relations among phenol reaction, $KClO_3$ susceptibility, and apiculus hair length. R = resistant, W = susceptible to $KClO_3$, L = long, S = short apiculus hair length, o = negative phenol reaction, ● = positive.

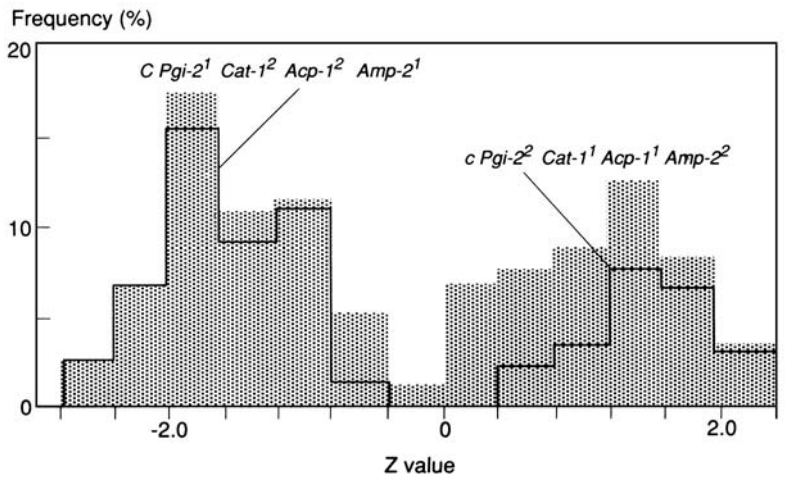
resistant (R) types. Hr values showed continuous and unimodal variation. However, this character is controlled by the *Aph* locus; *Aph* carriers mostly have apiculus hairs longer than 0.7 mm, while *aph* carriers have ones shorter than 0.7 mm (Sato 1985). Cultivars used here were divided into long (L) and short (S) hair types, taking 0.7 mm as the dividing line.

K and Hr showed a negative correlation. Cultivars of WL type were less frequent than WS, RL, or RS types. Cultivars of RL type frequently had the ph allele, while those of WS type had the *Ph* allele. Thus, the cultivars tended to be divided into *Ph* WS and *ph* RL types. These two types correspond to indica and japonica.

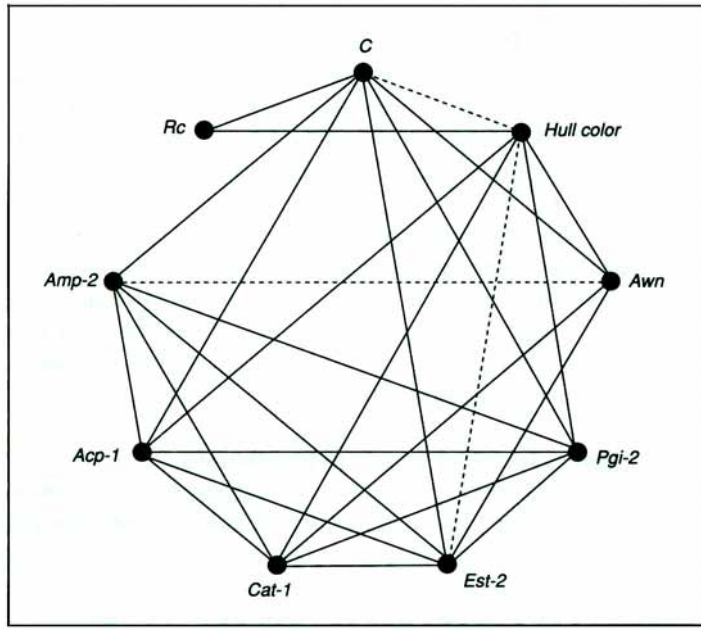
The distribution pattern of Z values is shown in Figure 2. Typical indicas had positive Z values, and typical japonicas had negative Z values. The frequency of Z values for all cultivars showed a continuous but bimodal distribution, indicating that the cultivars used here showed a fair tendency to differentiate into indica and japonica groups.

The pattern of association among nine genes and characters other than the three discriminant characters is illustrated in Figure 3. Solid and dotted lines indicate nonrandom associations significant at the 1% and 5% levels, respectively, which are based on correlation coefficients (between quantitative characters), chi-square values (between qualitative characters), or *t* values (between qualitative and quantitative characters). Of the 36 possible combinations of genes and characters, 25 (69.4%) showed nonrandom association.

Of these nine genes and characters, five genes (other than pericarp color, hull color, awn, and *Est-2*) were used for varietal classification. The cultivars were classified by allelic association at these five loci. Fourteen of 32 possible combinations (25=32, Table 1) were found. Many cultivars were classified into a few representative



2. Frequency distribution of Z values showing indica-japonica variation among 200 cultivars.



3. Association of 9 genes and characters among 200 cultivars. Significance at the 1% (solid lines) and 5% (dotted lines) levels.

Table 1. Classification of 200 cultivars into 32 genotypes based on nonrandom association among 5 loci.

Allele at					Cultivars	Coefficient of
C	*Pgi-2*	*Cat-1*	*Acp-1*	*Amp-2*	(no.)	estrangement[a]
C	1	2	2	1	61	0
C	1	2	2	1	27	1
C	1	1	2	1	2	1
C	1	2	2	2	1	2
C	1	1	2	2	2	2
C	2	1	1	1	2	3
C	1	1	1	1	1	3
C	1	1	1	2	3	3
C	2	1	1	1	2	4
C	1	1	1	2	4	4
C	2	1	2	2	5	4
C	1	2	1	2	11	4
C	1	1	1	2	16	4
C	2	1	1	2	63	5
		Total			200	

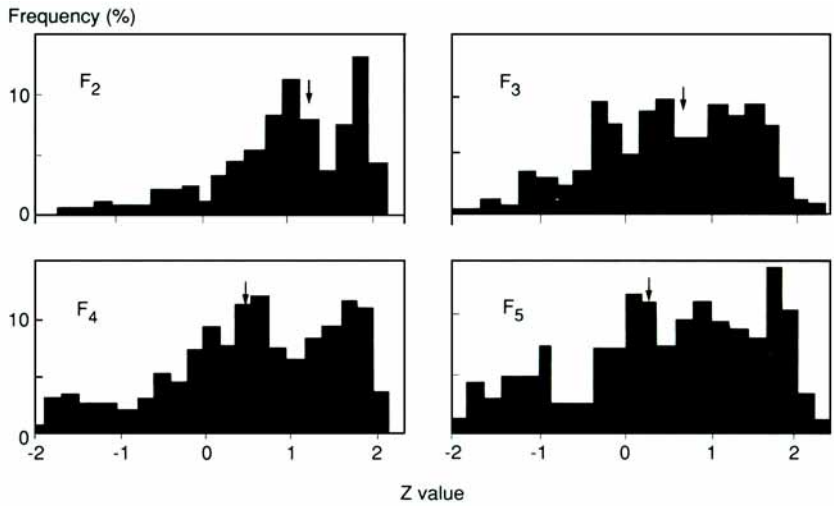
[a] Number of loci having alleles different from *C Pgi-2* ^{1} *Cat-1* ^{2} *Acp-1* ^{2} and *Amp-2* ^{1} type.

genotypes, such as *C Pgi-2*^{1} *Cat-1*^{2} *Acp-1*^{2} *Amp-2*^{1}, *c Pgi-2*^{1} *Cat-1*^{2} *Acp-1*^{2} *Amp-2*^{1}, and *c Pgi-2*^{2} *Cat-1*^{1} *Acp-1*^{1} *Amp-2*^{2}. The observed frequency was different from the expected frequency calculated from random association of the genes. Genotype *c Pgi-2*^{2} *Cat-1*^{1} *Acp-1*^{1} *Amp-2*^{2} was the most frequent. Its reverse genotype, *C Pgi-2*^{1} *Cat-1*^{2} *Acp-1*^{2} *Amp-2*^{1} and a similar one, *c Pgi-2*^{1} *Cat-1*^{2} *Acp-1*^{2} *Amp-2*^{1}, were also frequent. This indicates that the cultivars used tended to be differentiated into *c Pgi-2*^{2} *Cat-1*^{1} *Acp-1*^{1} *Amp-2*^{2} and *C Pgi-2*^{1} *Cat-1*^{2} *Acp-1*^{2} *Amp-2*^{1} types.

The Z values of the cultivars belonging to these three genotypes are indicated in Figure 2. All cultivars belonging to *c Pgi-2*^{2} *Cat-1*^{1} *Acp-1*^{1} *Amp-2*^{2} had positive Z values, while those belonging to *C Pgi-2*^{1} *Cat-1*^{2} *Acp-1*^{2} *Amp-2*^{1} and *c Pgi-2*^{1} *Cat-1*^{2} *Acp-1*^{2} *Amp-2*^{1} had negative Z values. This means that the two most frequent genotypes, *C Pgi-2*^{1} *Cat-1*^{2} *Acp-1*^{2} *Amp-2*^{1} and *c Pgi-2*^{2} *Cat-1*^{2} *Acp-1*^{1} *Amp-2*^{2}, correspond to indica and japonica, respectively.

Character and gene association in hybrid populations

The frequency distributions of Z values in the hybrid populations are shown in Figure 4. In the F_2, the Z values showed a continuous and unimodal distribution, indicating that indica-japonica differentiation did not occur. The mean of Z (1.27) was much higher than zero, the value separating indicas and japonicas. This may be because the alleles carried by indicas are largely dominant over japonica alleles. The mean of Z shifted toward the negative from the F_2 to the F_5 because more segregants showed negative values. The standard deviation of the Z value was 0.97 in the F_2 and became greater with each generation, reaching 1.28 in the F_5—still lower than that among the cultivars (1.66). However, in the F_5, the range of variation was as wide as that in the cultivars.



4. Z values in hybrid populations. Standard deviations in F_2, F_3, F_4, and F_5 populations were 0.97, 1.06, 1.16, and 1.28, respectively. Arrows indicate means.

The pattern of association among the nine genes and characters in the F_2 and F_5 populations is shown in Figure 5. Of 36 possible combinations, 4 in the F_2 and 6 in the F_5 showed nonrandom associations. The associations found in the F_2 are probably due to linkage (e.g., *c*, *Est-2*^{1}, and *Pgi-2*^{1}).

In the F_5 population, three associations that did not appear in the F_2 were recovered. They are the associations between black hull and *Rc* (red pericarp), between *Rc* and *Amp-2*^{2}, and between *Est-2*^{1} and *Acp-1*^{1}. In the associations between black hull and *Rc*, and between *Est-2*^{1} and *Acp-1*^{1}, parental combinations of alleles were more frequent than their recombinant types.

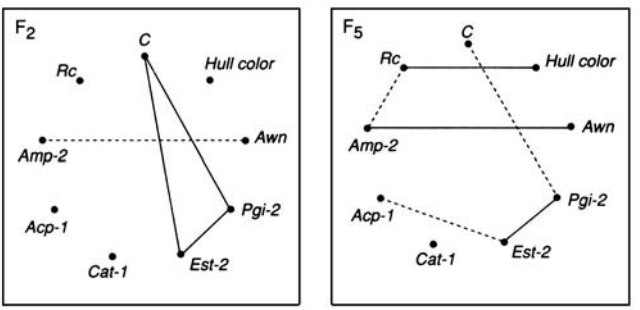
Discussion

Indicas and japonicas differ in genes and characters that are associated nonrandomly, as indicated by many authors (Glaszmann 1987, Oka 1958, Sato et al 1986), who concluded that no single gene representative of indica-japonica differentiation can be pointed out. The genetic mechanisms responsible for such nonrandom association among cultivars should be studied to elucidate factors causing indica-japonica differentiation.

The bimodality of Z values and the nonrandom association among nine genes and characters recovered in the F_5 suggest that these gene and character associations could be constructed against randomizing forces. Nonrandom associations between genes and characters are not completely understood, even though considerable outcrossing occurs. This may indicate that indicas and japonicas are genetically distant, because recombinant or intermediate types decreased in the hybrids.

Nonrandom association between alleles is caused by various evolutionary forces such as gametic selection, zygotic selection, random drift, linkage, and nonrandom mating in higher plants (Hedrick et al 1978). Artificial selection also plays an important role in cultivated species.

In the F_2 of the indica-japonica hybrid population, Z values showed continuous variation. Moreover, the associations observed among the cultivars largely disappeared. These facts indicate that the gene and character associations found among



5. Association among 9 genes and characters in F_2 and F_5 populations.

cultivars are caused largely by natural or artificial selection. Random drift may not be a causal factor of the indica-japonica differentiation, because it has been repeatedly reported in Asian and African cultivars (e.g., De Kochko 1987).

Associations found in the F_2 population are likely to be caused by linkage (e.g., *C, Est-2,* and *Pgi-2*). However, three associations found in the F_5 but absent in the F_2 could not be explained by linkage. In fact, in those three associations, the relevant loci are known to be carried by different chromosomes (*Acp-1* and *Est-2, Rc* and *Amp-2,* and *Rc* and *Bh-a, Bh-b*). In addition, *Ph* and *Rc,* which are located on different chromosomes, are also nonrandomly associated in the F_5. Since the F_5 population was raised by the single seed descent method, the effect of zygotic selection must have been eliminated. Thus, these associations are considered to be caused by gametic selection.

Gametic selection is caused by various mechanisms. Hybrid sterility, in which pollen having particular genes becomes abortive, is frequently observed in indica/japonica crosses (Ikehashi and Araki 1986; Oka 1953, 1974; Oka and Doida 1962; Yokoo 1984) and results in distorted segregation in the hybrids. Distorted segregation also occurs in distant crosses by certation, which bring about differential fertilization among normal pollen grains (e.g., Nakagahra 1972). However, it has been proven from a computer simulation study that hybrid sterility and certation do not cause the nonrandom associations found in hybrid populations (Nomura et al 1991). Perhaps the gene and character associations found in the F_5 were due to gametic selection caused by the differential fertilizing abilities of gametes with different genotypes. This trend of gametic selection may act as an internal mechanism for indica-japonica differentiation.

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Genetic diversity and intervarietal relationships in rice (*Oryza sativa* L.) in Africa

J.-L. Pham

Twelve African cultivars of *Oryza sativa* were sampled to examine the biological significance of varietal classification based on isozyme studies. Analyses of F_1 hybrid sterility and of F_2 segregations showed good correspondence between biochemical markers and observed reproductive barriers. Genetic markers for some vegetative and reproductive traits were identified. Such linkages could be involved in the relationships between classifications based on various criteria.

The variability in Africa of the Asian cultivated rice species *Oryza sativa* has been described and analyzed with regard to isozymic polymorphism (Ghesquière and Second 1983; de Kochko 1987, 1988) as well as agromorphological traits (Jacquot and Arnaud 1979, Miezan and Ghesquière 1986). These studies have shown that the genetic diversity of *O. sativa* in Africa is similar to that in Asia and that it is organized in two main groups corresponding to the indica and japonica subspecies.

The indica-japonica distinction is partly based on the existence of reproductive barriers among Asian cultivars of *O. sativa* (Oka 1988). It is thus important to know if the indica-japonica distinction, maintained in Africa by enzymatic diversity, also reflects differences in the genetic pool, as in Asia.

Materials and methods

Observations were made at the agromorphological, fertility, and biochemical levels.

Plant material

Twelve African traditional cultivars that represent part of the isozyme variation within the species on that continent (ORSTOM collection, Table 1) were chosen for their enzymatic polymorphism. Three of them possess alleles that are, in Africa, characteristic of the wild species *O. longistaminata.*

Table 1. Varieties used in crossing design.

Variety	Origin	Culture type	Enzymatic structure	Phenol reaction
ES70-6	Tanzania	Phreatic	Japonica	−
YS138-3	Guinea	Pluvial	Japonica	−
YS252-1	Guinea	Phreatic, pluvial	Japonica	−
YS45-1	Guinea	Pluvial	Japonica	−
BS117	Guinea-Bissau	Phreatic	Indica	+
BS125	Guinea-Bissau	?	Indica	+
ES44	Tanzania	Irrigated	Indica	+
ES79	Tanzania	Pluvial	Indica	+
SS404	Senegal	Irrigated	Indica	+
YS309	Guinea	Phreatic	Introgressed japonica[a]	+
BS20	Guinea-Bissau	Pluvial	Introgressed indica[a]	−
2LS102	Mali	Irrigated	Introgressed indica[a]	+

[a] See text.

F_1 fertility

A 12 × 11 crossing design was used. Variety 2LS102 was used as the female parent. Four plants of each genotype were studied. Their panicles were bagged at heading to avoid allopollination. Three panicles per plant were counted. Seed fertility was defined as number of grains over total number of spikelets. The data were treated by correspondence analysis and hierarchical ascending classification (HAC).

F_2 segregation

Numerous F_2 progenies were studied for segregation of isozyme markers by enzyme electrophoresis as described by Second (1982) and de Kochko (1987). The terminology used for isozyme loci and the correspondence with previous notations are given in Pham et al (1990).

The conformity of F_2 segregation to Mendelian proportions and the sources of distortions were tested by chi-square analysis. In segregation involving a null allele, where heterozygous genotypes were indistinguishable, the allelic frequencies p and q were estimated based on the frequency of the double-recessive homozygote genotype (assuming an F_2 distribution according to p^2:2pq:q^2).

Identification of markers for quantitative traits

Both agromorphological traits and genetic markers (isozyme and phenol reaction loci) were followed in the F_2 of the cross ES70-6/SS404 (321 plants). The methods and experimental design used to study the relationships between marker loci and quantitative traits were as described by Pham (1990). Following Tanksley et al (1982), a significant difference for a trait between F_2 genotypic classes of a marker locus was interpreted as the existence of linkage between the marker locus and at least one of the quantitative trait loci of the studied trait.

Table 2. Seed fertility of F_1 hybrids and parental lines.[a]

Female	Seed fertility (%) with indicated male parent											
	ES 70-6	YS 138-3	YS 252-1	YS 45-1	BS 117	BS 125	ES 44	ES 79	SS 404	BS 20	YS 309	2LS 102
ES70-6	**87**	83	82	*70*	12	16	*39*	*58*	35	60	*26*	–
YS138-3	84	**76**	87	84	*32*	*34*	29	48	34	87	29	–
YS252-1	67	85	**88**	84	*67*	*70*	*73*	47	*81*	80	70	–
YS45-1	74	–	89	**88**	*73*	*51*	*67*	*83*	65	79	16	–
BS117	0	*0*	*0.5*	*43*	**87**	75	80	79	81	*40*	0.2	–
BS125	*7*	*9*	*41*	*17*	88	**84**	*f64*	74	66	*53*	2	–
ES44	*85*	11	*38*	*21*	90	*44*	**75**	87	86	63	20	–
ES79	*0*	34	*0.5*	9	84	74	78	**73**	85	–	23	–
SS404	43	50	*60*	74	89	81	90	90	**77**	64	9	–
BS20	57	–	79	83	*64*	*85*	–	57	60	**90**	72	–
YS309	33	30	*43*	28	18	12	54	17	19	74	**72**	–
2LS102	44	15	*27*	29	71	78	68	65	65	92	51	**81**

[a]Parental lines in boldface, Significant differences between reciprocal crosses are indicated by italics. (-) = missing data.

F_1 hybrids

No genotype effect was detected in F_1 seed from crosses, germination rate (71%), or losses after sowing (<3%).

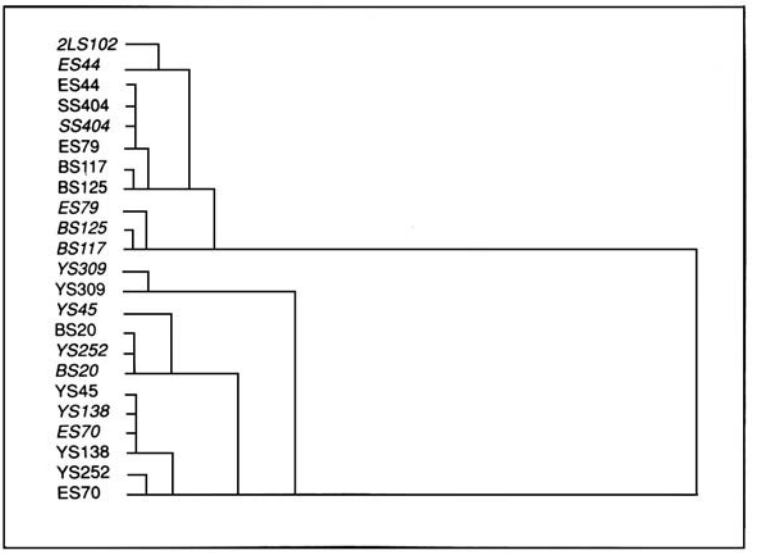
Table 2 shows the seed fertility of F_1 hybrids and parental lines, which covered a uniform range (0–90%). The sample of varieties presents various situations, showing that a diversity of relationships between genotypes corresponds to the diversity of the genotypes.

Differences occurred between reciprocal crosses for about one-third of the observed combinations (Table 2).

Classification of varieties by F_1 fertility

The data were subjected to correspondence analysis after joining Table 2 and its transposition (although the information given by the crosses with 2LS102 is thus lost, this genotype is less discriminating). The varieties were then classified by HAC using as variables the factorial coordinates on axes 1 to 4 (Fig. 1). The plane defined by the first two axes of the correspondence analysis in Table 2 allows a quick visualization of the results (Fig. 2).

Two main clusters appear: 1) the japonica (as classed by their enzymatic genotype) varieties ES70-6, YS138-3, YS45-1, YS252-1, and YS309 and the sole indica BS20; and 2) the indica varieties SS404, ES44, ES79, BS117, BS125, and 2LS102. For all varieties, classification into either of these two groups is the same for both male and female parents.



1. Hierarchical ascendant classification of 12 cultivars from fertility of their F_1 hybrids. Roman type = cultivar as male parent; italics = cultivar as female parent.

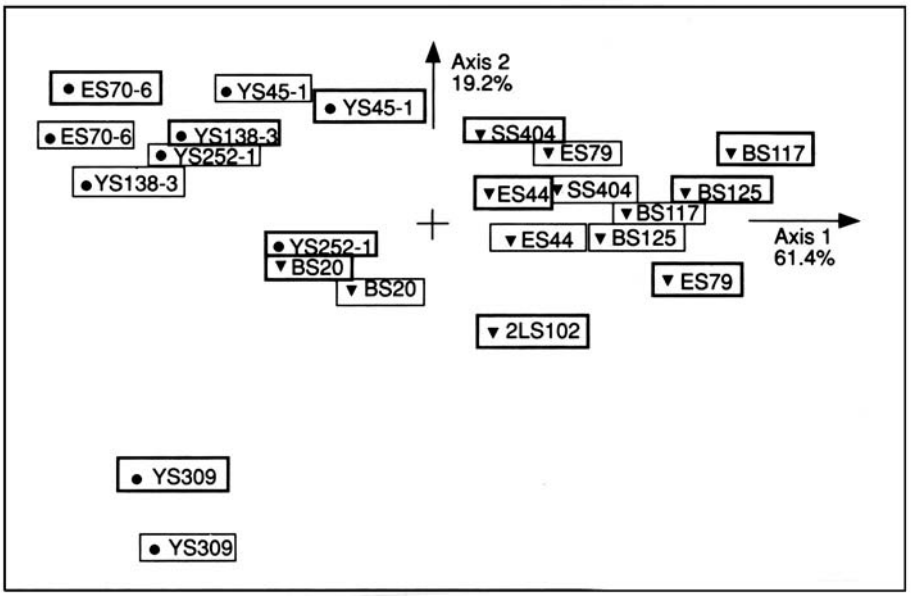
The japonica cluster + BS20 includes three unequal groups:

- The first consists of typical japonica varieties, which generate fertile within-japonica hybrids and sterile hybrids when crossed with indicas. They are ES70-6 and YS138-3 (male and female), and YS45-1 and YS252-1 (male).
- BS20 (male and female) and YS252-1 (female) are close, and YS45-1 (female) belongs to this group. These varieties could be called wide compatible (Ikehashi and Haraki 1984), but only BS20 was wide compatible in both reciprocal crosses.
- YS309 is alone. This genotype generates mostly sterile or semisterile hybrids and could be called a narrow compatibility variety.

The differences between male and female behavior play a clear part in the classification of indica varieties (Fig. 1,2). BS125, ES44, SS404, and BS117 form a group that is notably homogeneous in male behavior but is more scattered in female behavior, where two subgroups appear. The first female subgroup consists of varieties (ES79, BS125, and BS117) showing extreme reactions with japonicas, especially with ES70-6. The second consists of ES44, SS404, and 2LS102, which generate more intermediate reactions. These results suggest the existence of cytoplasmic variability in indica cultivars.

Usefulness of biochemical markers

Is there any correspondence between the information from F_1 analyses and that from biochemical markers?



2. Twelve cultivars plotted in the plane defied by axes 1 and 2 of correspondence analysis of F_1 fertility table. Heavy frame = female parent, light frame = male parent, ● = japonica cultivar, ▼ = indica cultivar.

Enzymatic polymorphism. The classification into two main groups based on F_1 fertility corresponds to that based upon enzymatic criteria. The only exception is BS20, which is grouped with japonica varieties, though enzymatically it is classed as an indica.

Furthermore, the enzymatic classification of japonica varieties (de Kochko 1988) separates YS45-1 and YS252-1 from YS138-3 and ES70-6. This separation is also apparent in fertility analysis. This correspondence is all the more interesting because YS45-1 and YS252-1 have "hybrid" genotypes (Second 1982) resulting from reciprocal introgressions between indica and japonica "ancestral" genotypes. These intermediate genotypes present a trend toward wide compatibility (as the female parent only). Our observations agree with those of Clément and Poisson (1986), who describe the wide compatibility of the japonica varieties of the G3 group (Jacquot and Arnaud 1979); however, reciprocal crosses were not studied by Clément and Poisson (1986).

On the other hand, no correspondence is found in indica varieties between enzymatic and fertility classification. This lower efficiency of enzymatic analysis confirms the possible importance of cytoplasmic variability for classifying these cultivars.

Cytoplasmic variability. Polymorphism of chloroplast DNA (restriction fragment length polymorphism) of the parental lines (except ES70-6) was studied by Z.H. Shang (pers. comm.) at ORSTOM, Montpellier, following the method described by Dally and Second (1989). Using the restriction endonuclease *Eco*RI, the indica varieties showed

three restriction patterns, while all the japonicas showed a common pattern. This difference in variability cannot be discussed because of the small size of the sample.

Although there is no overlapping between groups of indica varieties obtained from fertility analysis and the observed restriction patterns, these results show the convergence of different methods to demonstrate the cytoplasmic diversity of indica varieties. This conclusion is supported by an analysis of agromorphological traits (data not shown), which shows numerous differences between reciprocal crosses. Cytoplasmic variability must be considered in germplasm management and in breeding programs, especially those using multi-origin populations.

F_2 progenies

Thirty-two F_2 progenies were studied for marker loci segregation, including isozyme loci and phenol reaction locus.

Analysis of marker loci segregation

Table 3 shows the conformity of F_2 segregations to Mendelian proportions for each progeny and chromosome. All the abnormal segregations are analyzed in Table 4.

Crosses within subspecies. Two progenies of japonica/japonica crosses and three of indica/indica crosses were examined. Five chromosomes were marked. All segregations were normal.

Intersubspecific crosses. Of 15 F_2 progenies studied, 8 showed at least 1 distorted segregation. Among the seven marked chromosomes, six carried loci that are subject to distortion. The most common chromosomes with abnormal segregations were chromosome 6 (loci *Est-2* and *Pgi-2*) and chromosome 12 (*Sdh-1* and *Acp-1*). For the other loci, distortions seemed to be relevant to particular cases. Most of the abnormal segregations (Table 4) showed unequal allelic frequencies. Random assortment of gametes was generally observed. Thus, as noted by Pham et al (1990) for other progenies, deviations from Mendelian ratios result from gametic selection rather than from zygotic selection.

All distortions did not have the same range (Table 4). The F_2 progenies BS125/ES70-6, ES70-6/ES79, and YS138-3/ES79 presented extreme distortions at locus *Est-2* (chromosome 6), since only one recessive homozygote plant was observed instead of the 70 theoretical plants. In the F_2 of ES70-6/SS404, the ratio of allelic frequencies was nearly 1:3. Similar distortions were observed at locus *Cat-1* (chromosome 6) in the F_2 of BS125/ES70-6 and on chromosome 12 (locus *Sdh-1 and Acp-1*) in the F_2 of ES70-6/SS404. Other distortions presented an allelic frequency ratio of about 2:3. All allelic excesses were in favor of the indica allele.

Crosses involving BS20 and YS309. Of 13 F_2 progenies observed, 6 showed at least one skewed segregation. Seven of the nine marked chromosomes carried loci subject to distortions. Chromosome 6 was the most susceptible, but the allelic frequency did not exceed 60:40.

Table 3. Conformity of marker loci segregations for each F_2 progeny and marked chromosome.[a]

Cross	Chromosome and locus (loci)											Chromosomes (no.) with distorted loci	Marked chromosomes (no.)
	1 *Est-5 Got-1*	2 *Amp-1*	3 *Pgi-1*	4 *Ph*	6 *Pgi-2 Est-2*	6 *Cat-1*	7 *Est-9*	11 *Adh-1 Pgd-1*	12 *Acp-1 Sdh-1*	? *Est-1*	? *Got-3*		
Japonica/japonica													
ES70-6/YS45-1					0	0		0				0	2
YS252-1/YS45-1						0		0				0	2
Indica/indica													
BS125/SS404			0		0			0	0	0		0	5
ES79/BS117			0		0			0	0			0	4
SS404/BS125			0		0			0	0	0		0	5
Indica/japonica or japonica/indica													
BS117/YS138-3	0		0	0						0		0	4
BS125/ES70-6				0	+	+	0		+	0		2	5
BS125/YS45-1					0				0	0		0	3
ES70-6/ES79	+				+	+	+	0		+		4	5
ES70-6/SS404				+	+	0	0	0	+	0		3	6
ES79/YS45-1	0				0	0						0	2
SS404/ES70-6					+				+	0		2	3
SS404/YS252-1					+				+			2	2
SS404/YS45-1					0					0		0	2
YS138-3/ES79	0				+	0	0	0		0		1	5
YS252-1/ES79	0				0	0				0		0	2
YS252-1/SS404			0		0					0		0	3
YS45-1/ES44			+		0					0		1	3
YS45-1/ES79	0				0	0				0		0	3
YS45-1/SS404			+		0					0		1	3
With introgressed varieties													
BS117/BS20					0				0			0	2
BS20/SS404	0			0	+		0	0	+			2	6
BS20/YS309	0	0		+	+			+	0		+	4	7
BS20/YS45-1	+			0	0				0			1	4
ES70-6/BS20	0				0	0		0	0	0		0	5
ES70-6/YS309		0		0	+	0		+	0	+	0	3	7
SS404/BS20					0				0			0	2
YS138-3/BS20	0				+			0	0	+		2	5
YS138-3/YS309		0			0							0	2
YS309/BS125					+				+			2	2
YS309/SS404					0				+			0	2
YS45-1NS309		0								0	0	0	3

[a] 0 = conformity, + = distortion.

Table 4. Distorted F_2 segregations.

Chromosome	Cross (P_1/P_2)	F_2 plants (no.)	Locus	Allelic frequency		χ^2 test[a]	
				P_1	P_2	Homogeneity of allelic frequency	F_2 distribution (p^2:2pq:q^2)
1	ES70-6/ES79	120	*Est5*	.42	.58		
1	BS20/YS45-1	258	*Got-1*	.56	.44	6.52*	0.93 ns
3	YS45-1/ES44	52	*Pgi-1*	.38	.62	2.77 ns	6.83**
4	BS20/YS309	79	*Ph*	.61	.39		
4	ES70-6/SS404	281	*Ph*	.44	.56		
6	BS125/ES70-6	48	*Cat-1*	.75	.25	24.00***	0.59 ns
6	ES70-6/ES79	115	*Cat-1*	.40	.60	8.42**	0.10 ns
6	BS125/ES70-6	65	*Est-2*	1.00	.00		
6	BS20/SS404	100	*Est-2*	.40	.60	8.00**	2.78 ns
6	ES70-6/ES79	120	*Est-2*	.00	1.00		
6	ES70-6/SS404	398	*Est-2*	.24	.76		
6	ES70-6/YS309	243	*Est-2*	.44	.56		
6	SS404-ES70-6	60	*Est-2*	.78	.22		
6	SS404/YS252-1	80	*Est-2*	.59	.41	4.90*	0.41 ns
6	YS138-3/ES79	96	*Est-2*	.10	.90		
6	YS309/BS125	80	*Est-2*	.47	.53	0.63 ns	6.27*
6	BS20/YS309	80	*Pgi-2*	.34	.66	15.63***	1.48 ns
6	ES70-6/SS404	398	*Pgi-2*	.40	.60	32.16***	2.47ns
6	SS404/ES70-6	97	*Pgi-2*	.58	.42	4.64*	3.25 ns
6	YS138-3/BS20	100	*Pgi-2*	.52	.48	0.32 ns	5.86*
7	ES70-6/ES79	117	*Est-8*	.40	.60	9.85**	0.92 ns
11	BS20/YS309	76	*Adh-1*	.38	.63	9.50**	1.28 ns
11	ES70-6/YS309	309	*Pgd-1*	.62	.38	37.39***	0.07 ns
12	ES70-6/SS404	394	*Acp-1*	.36	.64	62.54***	0.38 ns
12	BS125ES70-6	64	*Sdh-1*	.61	.39	6.13*	0.02 ns
12	BS20/SS404	100	*Sdh-1*	.39	.61	9.68**	5.92*
12	ES70-6/SS404	387	*Sdh-1*	.36	.64	64.83***	0.73 ns
12	SS404/ES70-6	99	*Sdh-1*	.65	.35	16.99***	0.36 ns
12	SS404/YS252-1	80	*Sdh-1*	.69	.31	22.50***	0.18 ns
12	YS309/SS404	59	*Sdh-1*	.19	.81	43.93***	3.46 ns
12	YS309/BS125	80	*Sdh-1*	.30	.70	25.60***	2.90 ns
12	YS45-1/SS404	52	*Sdh-1*	.48	.52	0.15 ns	4.99*
?	BS20/YS309	79	*Got-3*	.58	.42	5.94*	0.16 ns
?	BS125/ES70-6	60	*Pox-3*	.59	.41	4.03*	2.58 ns
?	ES70-6/ES79	46	*Pox-4*	.33	.67	11.13**	0.01 ns
?	ES70-6/YS309	243	*Pox-4*	.48	.52	1.00 ns	6.13*
?	YS138-3/BS20	60	*Est-1*	.61	.39		

[a]ns = nonsignificant; significance at the 5% (*), 1% (**), and 0.1% (***) levels.

The case of chromosome 6

The region of chromosome 6 marked by loci *Pgi-2* and *Est-2* is apparently very susceptible to distortions. Several researchers have noted distortions on loci *wx* and *C,* which are located on the same chromosome (see Nakagahra et al 1974). Figure 3 shows the intervarietal relationships of these loci. Some results were added involving parent 108 used by Oka (1958) as an indica tester. The results support the classification obtained from studying F_1 hybrids. Japonicas ES70-6 and YS138-3 are opposed to indica varieties. The wide compatibility of YS252-1, YS45-1, and BS20 was confirmed, although a light reciprocal effect was observed with SS404. The uniqueness of YS309 was also confirmed.

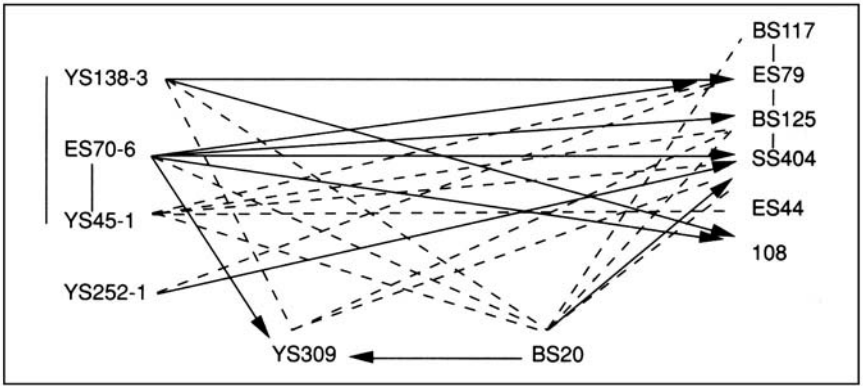
The rate of distortion in the F_2 was not proportional to F_1 sterility. One explanation is that the genetic systems involved in hybrid sterility are not all located near loci *Est-2* and *Pgi-2,* and that other mechanisms are involved in distortions (Nakagahra et al 1974).

Relationships between agromorphological traits and genetic markers

We will discuss here some of the results obtained from studying the japonica/indica F_2 progeny of ES70-6/SS404 (Pham 1990).

Table 5 presents the significant effects obtained for three loci that were used for classifying cultivated rice: *Pgi-2* (Second 1982), *Acp-1* (Inouye and Hagiwara 1980, Shahi et al 1969) and *Ph* (Oka 1958). Our detection of quantitative trait loci indicates that these loci have different allelic states in ES70-6 and SS404. Polymorphism among quantitative trait loci seems therefore to correspond to enzymatic polymorphism.

Assuming that these results could be extended to other varieties, the linkage of these markers with traits that were often considered useful in classifying cultivated rice (tillering, shape of the grain, size of the flag leaf, length of the panicle) could partially



3. Intervarietal relationships of loci *Est-2* and *Pgi-2* on chromosome 3. Broken lines indicate normal F_2 segregations; solid lines correspond to skewed F_2 segregations. Arrows show parent whose allele is in excess.

explain the correspondence between the classifications obtained using enzymatic and morphological criteria.

Locus *Pgi-2* appears to be a marker common to morphological and reproductive traits, since linkage was also demonstrated with fertility. This result agrees with the genetic map of rice, which locates numerous genetic sterility systems on chromosome 6. The existence of reproductive barriers contributing to the isolation of indica and japonica subspecies could therefore be correlated with the preservation of a morphological identity.

Conclusion

The genetic structure of the sample of African varieties may be clearly revealed by studying the diversity of biochemical markers. The indica-japonica distinction revealed by isozyme studies corresponds to a distinction based on reproductive barriers like hybrid sterility and abnormal transmission of genetic information in F_2 progeny. Although our study was limited by the small number of varieties, the results favor using biochemical markers for evaluating rice germplasm collections, since the resulting classifications have biological significance.

Table 5. F_2 progeny of ES70-6/SS404. Tests for difference between genotypes for 3 marker loci with respect to some quantitative traits.[a]

Trait	Locus										
	Acp-1				*Pgi-2*				*Ph*		
	ES/ES	ES/SS	SS/SS	Test	ES/ES	ES/SS	SS/SS	Test	ES/ES	?/SS	Test
No. of tillers at 50 d after sowing				ns	4.72	5.70	6.11	***			ns
Heading date (no. of days from sowing)				ns	99.0	92.8	94.5	***			ns
Plant height (cm)	107	100	104	**	110	102	102	**			ns
Length-width ratio of flag leaf	24.8	26.1	27.2	**				ns	26.0	26.5	*
No. of primary branches of panicle	12.2	11.3	1.7	*	11.6	11.3	11.9	*	12.7	11.2	***
Length-width ratio of grain	2.76	2.88	2.93	**				ns			ns
Seed fertility (%)				ns	31.2	24.4	34.0	**			ns

[a] 321 plants were studied. ES/ES = homozygous for ES70-6 allele, SS/SS = homozygous for SS404 allele, ES/SS = heterozygous, ?/SS = ES/SS and SS/SS are indistinguishable. ns = nonsignificant, * = significant at the 5% level, ** = 1%, *** = 0.1%.

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Traditional highland rices originating from intersubspecific recombination in Madagascar

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Genetic divergence among traditional rices from Madagascar was investigated on the basis of 39 morphophysiological traits and 19 isozyme genes. Comparison with Asian and African rices revealed the existence of new varietal types that do not fit the existing classification schemes. These types are mainly lowland cultivars grown in the high plateau region at altitudes ranging from 1,000 to 1,500 m. Based on morphophysiology, they are intermediate between indica and tropical japonica types for most traits, although they are the tallest. Isozyme data show a limited global gene diversity and a marked bipolar structure similar to the classical indica-japonica structure with, however, a peculiar predominance of allele 2 at locus *Amp-1,* forming multilocus types that are rare or absent in Asia. Classical associations between some isozymes and some morphological traits are almost nonexistent. The introduction of rices from Asia to Madagascar was thus probably accompanied by a strong founder effect and was followed by intensive intersubspecific recombination. Adaptation to new ecological niches took place without pronounced disruption of subspecific complexes of coadapted genes.

The indica-japonica differentiation is the main feature of varietal diversity in Asian cultivated rice (see Oka 1988 for a review). Such a pattern most probably arose from multiple domestications and the associated founder effects. Post-domestication varietal migrations were extensive, and the two types are now distributed over most Asian regions. There remains evidence of ecological specialization, leading indica varieties to be grown mainly under tropical lowland conditions, and japonica varieties mainly under temperate conditions and tropical upland conditions. In some environments, such as tropical highlands, both types are sympatric and are thus exposed to intersubspecific introgressions. An isozymic survey of Asian traditional varieties (Glaszmann 1987, 1988) suggested that few indica-japonica intermediates exist, and that most intermediate-like varieties are more probably consequent to the contribution of local wild rices rather than to intervarietal recombination.

The introduction of Asian rice to other continents has enabled the study of various aspects of rice evolution. Continental Africa has been extensively studied, and the genetic structure of local cultivated rice is considered the result of new opportunities for intra- and interspecific introgressions (Bezançon and Second 1984, Ghesquière 1988, Kochko 1987b). Madagascar offers a simpler situation. Because of geographic isolation due to insularity, ancient rice introductions were limited in number. Historical and linguistic hints (Boiteau 1977, Dez 1965, Domenichini-Ramiaramanana 1988, Domenichini-Ramiaramanana and Domenichini 1983) suggest two main origins: from Indonesia with the Protomalagasy people, and from the Indian subcontinent. Recent studies showed that Madagascar exhibits a large proportion of varieties showing particular morphophysiological and biochemical character associations, beside the typical indica and japonica varieties (Ahmadi et al 1988, Kochko 1988, Rabary et al 1989).

In this paper, we review the peculiarities of the rices from Madagascar. We compare them with the Asian morphological types, and with the Asian and African isozymic types. Both sources of information lead to deductions about the evolutionary past of cultivated rice in Madagascar that are relevant to the interpretation of cultivated rice diversity in other parts of the world.

Materials and methods

Morphophysiological and isozymic evaluations were carried out on 179 and 182 varieties, respectively, from the Madagascar national collection maintained by the Centre National de la Recherche Appliquée au Développement Rural (FOFIFA) and from recent field collections by FOFIFA and the International Board for Plant Genetic Resources; 145 varieties were common to the 2 samples.

The morphophysiological evaluation was performed at Alaotra Lake, Madagascar, and involved 24 quantitative and 15 qualitative characters. The procedures followed were those described by Jacquot and Arnaud (1979). The data were subjected to a factor analysis of correspondences (FAC) (Benzecri 1973) and “nuées dynamiques” (Diday 1971) after transformation of the quantitative data into qualitative data.

The isozyme study involved 10 enzymes encoded by 19 polymorphic genes as described by Glaszmann et al (1988), namely catalase (CAT), esterase (EST), aminopeptidase (AMP), acid phosphatase (ACP), shikimate dehydrogenase (SDH), alcohol dehydrogenase (ADH), isocitrate dehydrogenase (ICD), phosphogluconate dehydrogenase (PGD), phosphoglucose isomerase (PGI), and glutamate oxaloacetate transaminase (GOT).

Results

Both morphophysiological and isozymic evaluations identified varietal types specific to Madagascar, besides types commonly found in Asia.

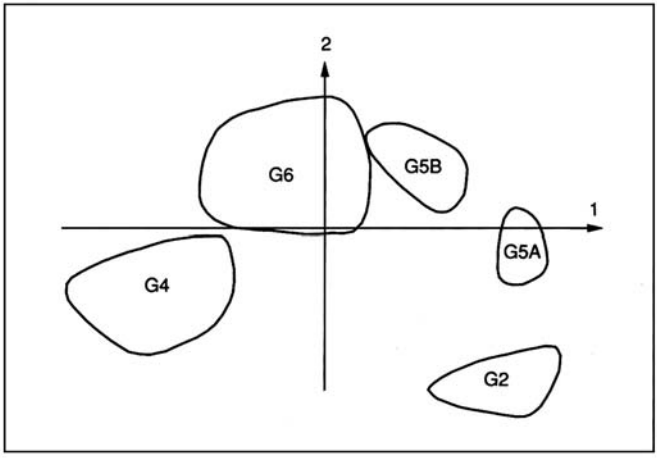
Morphophysiological variability

The FAC of the morphophysiological variability identified four groups, named G2, G4, G5, and G6 (Ahmadi et al 1988) for the sake of homology with a previous study (Jacquot and Arnaud 1979). The distribution of these groups on plane 1, 2 of the FAC is shown in Figure 1. Axis 1 positively attracts the varieties with high tillering capacity, thin organs, and short panicles with few branches. Axis 2 positively attracts the varieties with high stature; intermediate tillering; and big, heavy grains with a positive phenol reaction. The main traits of the morphological groups defined in Asia (Matsuo 1952, as reported by Angladette 1966) and in Madagascar are presented in Table 1.

The indica type in Asia, with high stature, high tillering capacity, thin organs, and fine and light grains with a positive phenol reaction, is represented in Madagascar by group G5, and is associated with lowland culture (with standing water) in regions having low altitude. In Madagascar, this group could be subdivided into two subgroups, G5A and G5B;G5A varieties display higher tiller numbers and thinner organs.

The temperate japonica type in Asia, with short stature, short panicles with little branching, low grain shattering, and bold grains with negative phenol reaction, is represented in Madagascar by group G2. Since these are modem varieties introduced from Asia less than a century ago, they are not considered here.

The tropical japonica type (often referred to as javanica) in Asia, with high stature, few tillers, long panicles with profuse branching, low grain shattering, and heavy, thick grains with negative phenol reaction, is widely represented in Madagascar by group G4. This type of rice has been grown under slash-and-bum culture in low-altitude forest areas on the eastern coast for more than 1,500 yr (Labatut and Raharinarivonirina 1969).



1. Distribution of 4 groups of varieties in plane 1, 2 of the factor analysis of correspondences among 39 morphophysiological characters of rice in Madagascar (after Ahmadi et al 1988).

Table 1. Main features of morphological groups defined in Asia (general description, Angladette 1966) and in Madagascar (average and range, Ahmadi et al 1988).[a]

Character	Varietal group						
	Temperate japonica		Tropical japonica		Indica		Atypical
	Asia	Madagascar (G2)	Asia	Madagascar (G4)	Asia	Madagascar (G5A/G5B)	Madagascar (G6)
Length of 1st leaf under flag leaf(cm)	Short	30.9 (24.6–36.2)	Long	46.3 (39.4–51.9)	Long	32.8 (23.0–40.1)	39.1 (29.8–57.7)
Width of 1st leaf 1st leaf underflag leaf(cm)	Narrow	10.3 (8.7–12.3)	Wide	16.0 (10.7–18.1)	Narrow	10.6 (8.0–13.8)	12.4 (9.7–17.3)
Tillering	Inter-mediate	14.8 (9.5–18.1)	Low	8.7 (5.5–16.6)	High	14.6 10.9–23.2)	11.3 (8.2–19.7)
Plant height (cm)	Short	85 (74–101)	Tall	120 (97–137)	Tall	111 (73–135)	122 (104–145)
Culm diameter (mm)	Inter-mediate	4.3 (3.6–4.8)	Thick	5.3 (3.8–6.5)	Inter-mediate	4.3 (3.3–5.6)	5.0 (3.3–6.0)
Panicle length (cm)	Short	16.8 (15.2–19.5)	Long	24.4 (18.8–29.3)	Inter-mediate	20.7 17.2–23.2)	22.1 (17.7–26.6)
Shattering (%)	Little	10.6 (4.7–24.6)	Inter-mediate	7.9 (1.7–17.9)	Much	17.4 (4.5-32.8)	12.5 (5.1–32.0)
Panicle secondary branches	Few	25.5 (22.1–30.5)	Many	44.0 (28.0–66.4)	Inter-mediate	31.5 21.5–39.5)	35.9 (23.3–50.4)
Grain length (L, mm)	Short	8.5 (7.9–9.3)	Inter-mediate	9.4 (7.1–11.3)	Long	10.0 (7.8-11.8)	9.6 (7.6–12.1)
Grain width (W, mm)	Wide	3.5 (2.8–4.1)	Wide	3.6 (3.1–4.2)	Thin	2.9 (2.5–3.4)	3.3 (3.0–3.8)
Grain shape (L/W)	Bold	2.4 (2.1–2.7)	Big	2.6 (2.1–3.6)	Fine	3.4 (2.7–4.2)	2.8 (2.7–3.8)
100-grain weight (g)	Heavy	2.7 (2.0–4.9)	Heavy	3.0 (2.2–4.4)	Light	2.7 (2.2–3.5)	3.0 (2.0–3.8)
Phenol reaction	Negative	–	Negative		Positive	+	+/-

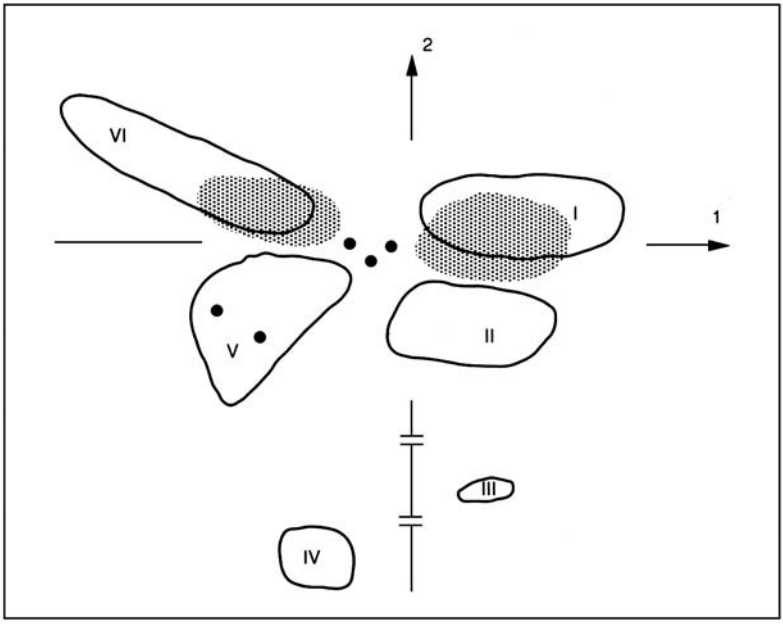
[a] Figures in parentheses are ranges.

The group specific for Madagascar, namely group G6, encompasses varieties grown under lowland conditions, having the highest stature, and whose other characters are intermediate between those of the tropical japonica group (G4) and the indica group (G5). Group G6 is numerically important in Madagascar and has no equivalent in Asia.

Thus, the morphophysiological variability of rice is particular in Madagascar: beside varietal groups G2, G4, and G5, which fully correspond to the temperate japonica, tropical japonica, and indica Asian types, respectively, an additional group, G6, stands out with atypical character combinations. Its members, particularly those with the vernacular designations “Rojo” and “Latsika,” are grown mostly at altitudes higher than 1,000 m and have a high level of cold tolerance (Rasolofo et al 1986).

Isozymic variability

The FAC of the isozymic variability identified two main clusters close to the indica and japonica groups (groups I and VI of Glaszmann 1987; Fig. 2). Two representatives of



2. Distribution of varieties from Madagascar in plane 1, 2 of the factor analysis of correspondences of isozyme data at 15 loci. Delimitations of the 6 varietal groups found in Asia are shown. Among the varieties from Madagascar, 129 cluster in the shaded area close to group I, 48 cluster in the shaded area close to group VI, 2 fall into group V, and 3 are intermediate (from Rabary et al 1989).

group V, otherwise characteristic of the Indian subcontinent, were also found. The main features of the genetic diversity in the main enzymatic groups in Asia (Glaszmann 1987, 1988), in Africa (Kochko 1987a), and in Madagascar (Rabary et al 1989) are given in Table 2.

Among the 19 loci surveyed, all polymorphic in Asia and Africa, only 13 exhibit some variation in Madagascar, and they display 30 alleles vs 46 in Asia and 33 in Africa. The missing alleles are those rare in Asia. Thus, for most polymorphic loci, the allele most frequent in Asia further gained importance in Africa and Madagascar. This tendency is reversed in Madagascar for several loci. The reversal is particularly pronounced for *Amp-1,* where allele 2, rare in Asia (13%) and in Africa (10%), has become frequent (61%).

When the indica and japonica groups are compared with their Asian counterparts, the loss of alleles is less pronounced than when the overall allele frequencies are considered, because the alleles specific for the minor Asian groups are no longer taken into account. However, some of the reversals noted above become stronger.

For the indica group, Madagascar is characterized by a reversal of allele frequencies at loci *Amp-1, Est-2,* and *Sdh-1* as compared with Africa, and at those loci and *Pgi-2* as compared with Asia. The most significant peculiarity is the high frequency of allele 2 at locus *Amp-1,* which is very rare in Asia and much less frequent in Africa.

Table 2. Genetic diversity of the main enzymatic groups in Asia (Glaszmann 1988), in Africa (including Madagascar, Kochko 1987b), and in Madagascar (Rabary et al 1989); allele frequencies; and diversity indices (H) (Nei 1975).[a]

Locus	Allele	Allele frequency (%)												
		Whole sample			Indica group					Japonica group				
							Madagascar					Madagascar		
		Asia (n= 1688)	Africa (n= 688)	Mada-gascar (n=181)	Asia (n=900)	Africa (n=359)	Indica (n=129)	I (n=32)	I* (n=97)	Asia (n= 451)	Africa (n= 329)	Japonica (n=47)	J (n=41)	J* (n=6)
Cat-1	1	71	83	97	100	100	99	97	100	4	65	81	93	–
	2	29	17	3	tr	–	1	3	–	96	35	19	7	100
	3	–	–	–	–	–	–	–	–	tr	–	–	–	–
	H	0.41	0.28	0.05	0	0	0.02	0.05	0	0.08	0.46	0.31	0.13	0
Sdh-1	1	37	30	13	43	58	18	50	7	1	–	–	–	–
	2	62	67	87	54	40	82	50	93	99	100	100	100	100
	3	1	3	–	2	2	–	–	–	–	–	–	–	–
	4	1	–	–	1	–	–	–	–	–	–	–	–	–
	H	0.48	0.46	0.22	0.52	0.50	0.30	0.50	0.13	0.02	0	0	0	0
Pgi-1	1	49	39	66	87	76	90	100	87	tr	–	2	2	–
	2	51	61	34	13	24	10	–	13	100	100	98	98	100
	H	0.50	0.48	0.44	0.23	0.36	0.18	0	0.23	tr	0	0.04	0.04	0
Pgi-2	1	60	92	98	47	84	98	97	98	100	100	100	100	100
	2	29	7	1	53	14	2	3	2	–	–	–	–	–
	3	8	tr	–	–	1	–	–	–	–	–	–	–	–
	4	3	tr	1	–	1	–	–	–	–	–	–	–	–
	H	0.55	0.15	0.04	0.50	0.27	0.04	0.06	0.04	0	0	0	0	0
Est-1	0	9	32	12	3	–	7	12	6	25	67	25	29	–
	1	91	68	88	97	100	93	88	94	75	33	75	71	100
	H	0.17	0.44	0.21	0.06	0	0.13	0.21	0.11	0.38	0.44	0.38	0.41	0
Est-2	0	36	36	64	13	21	59	19	72	76	53	75	74	100
	1	42	38	22	47	30	24	31	22	24	47	23	24	–
	2	22	26	12	40	49	17	50	6	–	–	2	2	–
	H	0.65	0.66	0.52	0.60	0.63	0.57	0.62	0.43	0.37	0.50	0.36	0.39	0

continued

Table 2 continued.

Locus	Allele	Allele frequency (%)												
		Whole sample			Indica group					Japonica group				
							Madagascar					Madagascar		
		Asia (n= 1688)	Africa (n= 688)	Mada-gascar (n= 181)	Asia (n=900)	Africa (n=359)	Indica (n=129)	I (n=32)	I* (n=97)	Asia (n= 451)	Africa (n= 329)	Japonica (n=47)	J (n=41)	J* (n=6)
Est-5	0	tr	3	–	tr	6		–	–	–	–	–	–	–
	1	99	97	100	100	94	100	100	100	100	100	100	100	100
	2	1	–	–	–	–		–	–	–	–	–	–	–
	H	0.02	0.06	0	tr	0.11	0	0	0	0	0	0	0	0
Est-9	1	40	43	62	64	43	62	81	87	tr	–	–	–	–
	2	60	57	38	34	57	38	19	13	100	100	100	100	100
	H	0.48	0.49	0.47	0.45	0.29	0.47	0.23	0.28	tr	0	0	0	0
Cat-1	1	71	83	97	100	100	99	97	100	4	65	81	93	–
Amp-1	1	78	88	39	93	78	25	100	–	90	100	88	100	–
	2	13	10	61	tr	19	75	–	100	–		12		100
	3	4	2	–		3	–	–	–	10	tr	–	–	–
	4	5	–	–	6	–	–	–	–	tr	–	–	–	–
	5	tr	–	–	1	–	–	–	–	–	–	–	–	–
	H	0.37	0.22	0.47	0.13	0.35	0.38	0	0	0.18	tr	0.21	0	0
Amp-2	1	39	P	30	1	P	2	–	3	99	P	100	100	100
	2	61	P	70	99	P	98	100	97	tr	–	–	–	–
	3	tr	–	–	–	–	–		–	tr	–	–	–	–
	4	tr	–	–	–	–	–	–	–	tr	–	–	–	–
	H	0.48	–	0.42	0.02	–	0.04	0	0.06	0.02		0	0	0

continued

Table 2 continued.

Locus	Allele	Allele frequency (%)												
		Whole sample			Indica group					Japonica group				
							Madagascar					Madagascar		
		Asia (n= 1688)	Africa (n= 688)	Mada-gascar (n = 181)	Asia (n=900)	Africa (n=359)	Indica (n=129)	I (n=32)	I* (n=97)	Asia (n= 451)	Africa (n= 329)	Japonica (n=47)	J (n=41)	J* (n=6)
Amp-3	0	4	P	1	–	–	–	–	–	–	–	–	–	–
	1	48	P	71	51	P	73	63	76	73	P	67	62	100
	2	43	P	28	47	P	27	37	24	27	P	33	38	
	3	1	–	–	2	–	–	–	–	–	–	–	–	–
	4	3	–	–	–	–	–	–	–	–	–	–	–	–
	5	tr	–	–	tr	–	–	–	–	–	–	–	–	–
	6	1	–	–	–	–	–	–	–	–	–	–	–	–
	H	0.58	–	0.41	0.52	–	0.39	0.47	0.36	0.39	–	0.44	0.47	0
Acp-1	1	62	52	71	98	99	96	91	98	1	–	– 2	–	17
	2	67	48	29	2	1	4	9	2	99	100	98	100	83
	3	1	–	–	–	–	–	–	–	–	–	–	–	–
	H	0.48	0.50	0.42	0.04	0.02	0.08	0.16	0.04	0.02	0	0.04	0	0.28
Adh-1	0	tr	–	–	–	–	–	–	–	tr	–	–	–	–
	1	95	P	99	99	P	99	100	99	88	P	100	100	100
	2	4	P	–	–	–	–	–	–	12	–	–	–	–
	3	1	–	1	1	–	1	–	1	tr	–	–	–	–
	H	0.09	–	0.02	0.02	–	0.02	–	0.02	0.21	–	0	0	0
Pgd-7	1	67	64	76	61	59	68	84	63	96	70	85	83	100
	2	6	9	21	15	17	32	16	37	–	–	–	–	–
	3	27	27	3	24	24	–	–	–	4	30	15	17	–
	H	0.47	0.51	0.42	0.55	0.56	0.44	0.27	0.47	0.08	0.42	0.26	0.28	0
All loci		0.62	0.38	0.29	0.28	0.30	0.23	0.20	0.15	0.10	0.16	0.05	0.12	0.03

[a]tr = traces, 0.5 > tr > 0; P = present, >0, but undetermined. * = presence of allele 2 at locus *Amp-1*.

For the japonica group, Madagascar differs from Africa by the reversal of allele frequencies at locus *Est-1,* and from Asia by the reversal at locus *Cat-1.* For this group, too, the frequency of allele 2 at *locus Amp-1* is significant, whereas this allele is absent from the japonica group in Asia and Africa.

On the basis of the allele at locus *Amp-1,* Rabary et al (1989) tentatively distinguished subgroups I and I* in the indica group, and J and J* in the japonica group, * indicating the presence of allele 2. The subgroups I and J display only minor differences from their African and Asian counterparts. The same is true for J*, besides the difference at locus *Amp-1.* For I*, more significant differences account for all the peculiarities of the indica group in Madagascar.

Thus, as morphophysiological variability does, isozyme variability of rice in Madagascar identifies specific types beside the usual indica and japonica types observed in Asia.

Congruence between the two classification schemes

The distribution of the 144 varieties analyzed for both morphology and isozymes on plane 1, 2 of the FAC of the morphological data is shown in Figure 3. The isozyme groups and the culture type—upland vs lowland—are distinguished, and two classes of elevation are considered.

Enzymatic group I contains all lowland varieties, distributed mostly in morphological group G5, but a few falling into group G6. All are grown at elevations lower than 1,000 m.

Varieties of enzymatic group I* are scattered in all morphological groups at all altitudes with, however, higher concentration in group G6 and at elevations above 1,000m. Among the 78 I* varieties, 3 are grown under upland conditions.

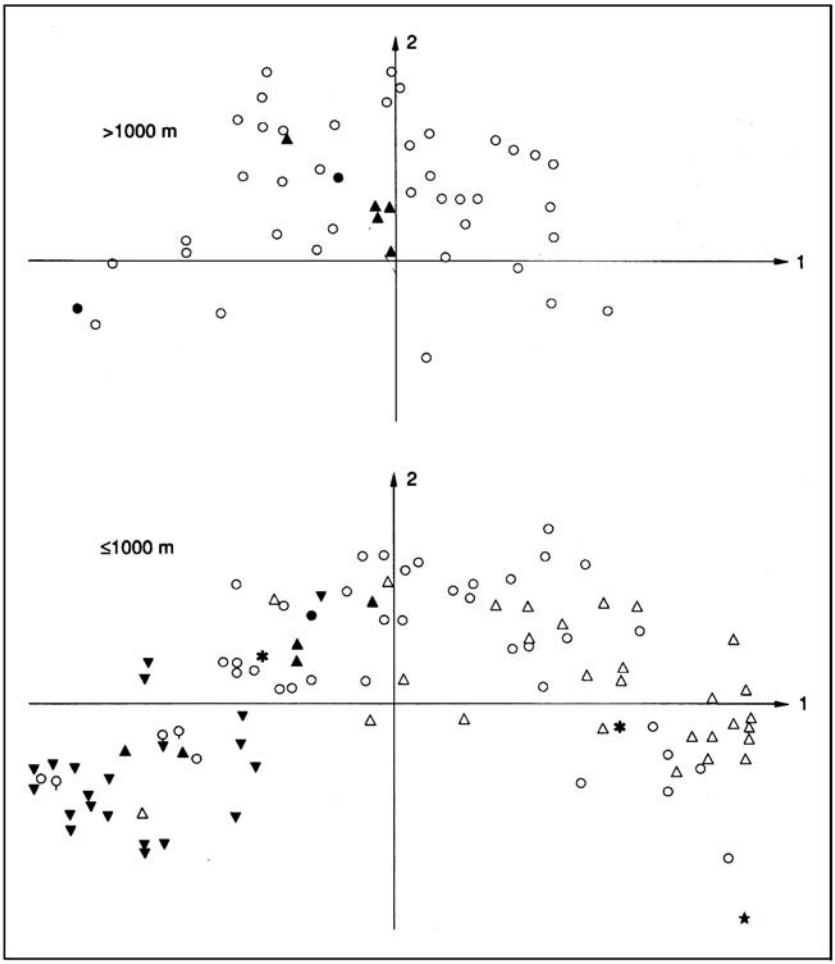
Varieties of enzymatic groups J and J* cluster around morphological groups G4 and G6. In G4, most are upland varieties (tropical japonica) grown at low elevations, whereas in G6, all are lowland varieties belonging to the vernacular families "Vary Lava" and "Latsika." The former are well known for their very long and wide grains; the latter are grown only above 1,500 m.

These are the main noteworthy outputs of this comparison:

- About 75% of the morphologically atypical varieties (G6) are also atypical regarding isozymes.
- A higher frequency of atypical varieties is observed above 1,000 m.
- The cold-tolerant lowland japonica varieties do not morphologically resemble the temperate Asian japonica varieties.
- Some upland varieties possess both the typical morphology of tropical japonica (G4) and basically indica isozymes (I*).

Discussion

The genetic diversity of rice in Madagascar is characterized by the presence both of varietal groups similar to the indica and japonica groups from Asia and Africa and of



3. Varieties from Madagascar scattered in plane 1, 2 of the factor analysis of correspondences among 39 morphophysiological characters (see Figure 1) and differentiated according to enzymatic group and culture type for 2 classes of elevation. Δ = I, lowland; ○ = I*, lowland; ⊖ = I*, upland; ▲ = J, lowland; ▼ = J, upland, ● = J*, lowland; ★ =group V, lowland; * = intermediate, lowland (Rabary et al 1989).

groups specific to the island, where morphophysiological and isozymic variations are not fully congruent.

Comparison of isozyme allele frequencies among Asia, Africa, and Madagascar reveals that the introduction of rice to Africa and Madagascar was accompanied by the loss of minor Asian alleles. But in Madagascar, a few exceptions are noted, such as allele 2 at locus *Amp-1*, which is restricted to the Indian subcontinent in Asia and is predominant in Madagascar.

Thus, rice introduction in Madagascar caused genetic drift linked to founder effects. Allele *Amp-1*^{2} in Asia is found mainly among varieties that differ from indica and japonica types in several other specific alleles; in a survey of 1,688 Asian varieties (Glaszmann 1987,1988), only 2 indicas displayed this allele: one from southern India and one from Sri Lanka. In Madagascar, this allele is found in an array of genotypes, all very rare or absent in Asia. Its introgression from local wild rices can be excluded, for only *Oryza longistaminata* is present on the island, and it does not possess this allele (Ghesquière 1988, Second 1985). Therefore, these genotypes must have arisen from local genetic mixing within *O. sativa.* Moreover, since this allele is found in both indica and japonica isozymic backgrounds, mixing has involved both intrasubspecific and intersubspecific recombinations. The higher frequency of japonica-prone alleles *Est-2*^{0} and *Sdh-1*^{2} in group I* than in group I confirms that I* varieties probably arose from intersubspecific recombination. The situation is different in Africa, where the frequency of *Amp-1*^{2} is higher than in Asia only among indicas, with a low frequency of alleles *Est-2*^{0}and *Sdh-1*^{2}.

Evidence drawn from the isozyme data alone is reinforced by the morphological evidence of many intermediate forms. The environmental conditions of high plateaus in Madagascar certainly exerted particular selection pressures that favored certain recombinant forms, as shown by the high frequency of these forms at high elevations and their cold tolerance (Rasolofo et al 1986).

The data are conclusive, thanks to the peculiarities of Madagascar, where varietal migrations and gene introgression from wild rices did not complicate the pattern of variation. Some firm conclusions are relevant to other, less favorable situations. In particular, it is striking that intersubspecific recombination has left so few intermediates as far as isozymes are concerned. This is to be compared with the tendency of parental gene combinations to increase among the progenies of indica/japonica crosses (see Oka 1988 for a review). Those genes that kept their initial assortment are certainly tightly linked to components of coadapted gene complexes involved in the maintenance of indica-japonica differentiation. These are *Pgi-1* on chromosome 3, *Cat-1* on chromosome 6, *Est-9* on chromosome 7, *Amp-2* on chromosome 8, and *Acp-1* on chromosome 12 (Wu et al 1988), using the new chromosome nomenclature. Therefore, other regions in Asia or Africa where these genes form multilocus associations should not be considered without indica-japonica gene introgression.

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Prospective use of *Oryza longistaminata* for rice breeding

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Morphological types, fertility, and outcrossing rates were studied in a population of 10 interspecific backcross progenies (*O. longistaminata/ O. sativa// O. sativa)* left under open pollination conditions. By segregation analysis at eight electrophoretic loci, single-locus and multilocus estimates of the outcrossing rates were calculated. In the first generation, 75% of the seeds came from outcrossing; this rate decreased to 35% in the second generation, following pollen fertility restoration. Outcrossing rates appeared primarily related to plant sterility and secondarily to stigma length and exsertion. At the morphological level, an important diversity of plant types was observed in the first generation, but plants were characterized by various wild traits. The second generation spontaneously evolved toward a more cultivated type, and transgressive segregants were observed for different morphological traits. Allelic segregations at the F_1 level were normal, but the second generation exhibited highly significant distortions. A loss of alleles coming from the wild species was observed for 5 of the 8 loci and for all 10 families.

Oryza longistaminata is a wild species of rice that grows widely throughout intertropical Africa. It covers a large range of ecological sites, from flooded plains to temporary ponds, and propagates itself by developing vigorous rhizomes (Ghesquiere 1985). This species is allogamous, with a self-incompatibility system, and shows the extreme maximum values of stigma and anther length and number of pollen grains within the Sativa species group (Oka and Morishima 1967).

This species shows significant diversity at the isozyme level (Ghesquiere 1988) and appears to be among the most distant species from *O. sativa* within the Sativa group (Second 1985). *O. longistaminata* has not intervened during the domestication of *O. sativa,* nor in the latter's diversification on the African continent since its introduction there, because of the strong reproductive barrier that isolates the former from all other species. This barrier is due to the action of two complementary lethal genes that cause abortion of the embryo (Chu and Oka 1970a, Ghesquiere 1988). In spite of this barrier, hybrid plants may be obtained, either by artificial crossing or, rarely, in seed sets collected from wild plants along the borders of ricefields.

The spontaneous hybrids were called Obake plants by Chu and Oka (1970b). They are characterized by different features shared with artificial hybrids: absence of rhizomes, high tillering habit, photoperiod insensitivity, low male fertility, and variable female fertility. When pollen fertility is not limiting, their inbred offspring are highly depressed. The genetic structure of these plants has been extensively studied by Ghesquiere (1988), who showed their possible origins from F_1 seeds to alternate backcrosses of *O. sativa* and *O. longistaminata.*

The use of *O. longistaminata* in rice breeding has been envisaged for the introgression of some of its allogamous characteristics into *O. sativa* to produce hybrid varieties (Taillebois and Guimaraes 1987). *O. longistaminata* may also be useful for the introgression of special disease resistance genes. This study considered the wild species as a source of diversity in general, and as a source of allogamy to favor spontaneous intermating in a hybrid population with a wide genetic base. This experiment was conducted to show that it was possible to avoid the classical reduction of variability of the backcross technique. The morphological diversity of a population composed of backcross progenies between an Obake plant and 10 varieties of cultivated rice was described for two generations. Outcrossing rates were estimated and related to the fertility of the population. Intergenomic recombination was studied at the isozyme level. The possibility of rapidly restoring a cultivated and fertile type, and the efficiency of selection were estimated in a third trial.

Materials and methods

The maternal parent of all progenies was an Obake plant obtained from a seed collected in North Cameroons. Its isozyme pattern was similar to that of a hybrid between *O. longistaminata* and an indica variety of *O. sativa.* This plant was male sterile. It had been pollinated by 10 upland rice varieties well adapted to West African conditions. The 10 progenies (300 plants each) were observed in field trials during 2 generations. To enhance natural intermating, the completely randomized trials were left under open pollination conditions for two generations. Because the first (G_1) generation showed significant variation in plant fertility, two trials were carried out in the second (G_2) generation. In one (the single seed descent [SSD] method), one seed of each G_1 plant was cultivated; in the other (the bulk method), all the seeds of each family were mixed, and a sample of 300 plants/family was cultivated. A third trial (G_3), composed of 20 self-pollinated progenies of G_2 plants chosen for their fertility, was studied. Each progeny was composed of 60 plants. To compare the hybrids with their cultivated parents in the G_1 and G_3, the parental varieties were studied, but they did not participate in the pollination. Measurements from single plants were taken to describe morphological characters including plant height, tillering, flowering date, panicle architecture (length, number of primary and secondary branches), pigmentation, awn development, and seed shedding. Fertility was described by pollen fertility and total number of seeds produced per plant. In the G_2, the stigma and anther length and the rate of exserted stigma were measured on plants whose progenies were used for estimation of outcrossing rates.

Electrophoretic analyses, based on the technique described by Second and Trouslot (1980), were performed on samples of each generation. Seven enzymatic systems were studied, corresponding to nine polymorphic loci: *Amp-1, Cat-1, Est-2, Est-5, Est-9, Enp-1, Pgd-1, Pgi-1,* and *Sdh-1*. Distinguishing the parental species origin of the alleles was possible for five loci. Estimates of outcrossing rates in the G_1 were calculated from the genotypic frequencies observed in the G_2 populations, and estimates of outcrossing rates in the G_2 were calculated from the studies of open-pollinated progenies of G_2 single-plant arrays. Sixty-four progenies of six plants were analyzed. Two maximum likelihood methods were used: one gives an estimate for each locus (method derived from Brown and Allard 1970) and the other, based on the simultaneous analysis of genotypes at different loci, gives a multilocus estimate (Shaw et al 1981). The maternal genotypic frequencies were known in both cases, but pollen pool allele frequencies were estimated on the same samples as outcrossing rates. Heterogeneity of the single-locus estimates was tested by chi-square analysis.

Results

The evolution of the population is first presented according to its reproductive behavior and morphological traits. The extent of intergenomic recombination as shown at the isozyme level is then presented. Finally, the implications of these results for the use of *O. longistuminata* for plant breeding and genetic studies are discussed.

Reproductive behavior of interspecific progenies

The mean pollen fertility was very low in both generations: 18% in the G_1 and 37% in the G_2. Nevertheless, there was high variability between plants. In each generation, a few fertile plants composed the efficient pollen pool. In the G_1, 5% of the plants had a pollen fertility above 60%; in the G_2, 20% of the plants had a fertility slightly higher. This low rate of pollinators is a source of drift. Nevertheless, the distributions of pollen fertility among families were not statistically different. Seed production was also very low: the mean was 20 seeds/plant in the G_1 and 47 in the G_2. In both trials, 20% of the plants produced no seed and therefore did not contribute to the next generation. Although the SSD and bulk methods differed in seed contribution of G_1 plants to the G_2, the distribution of fertility of the plants in these two methods exhibited no difference. The early selection, at low intensity, in the bulk method was inefficient in increasing the fertility of the progeny.

In the third trial, selection of the most fertile plants in the G_2 was efficient, and strong correlations were found between the pollen and seed fertility of G_2 selected plants and the mean fertility of their offspring (0.68 and 0.75, respectively); yet large variability was found in each progeny. These results show that it is possible to rapidly restore the fertility of hybrid-derived plants.

The evolution of apparent outcrossing rates in the G_1 and G_2 is presented in Table 1. Significant differences between loci estimates were found in each generation.

Estimation of outcrossing rates is usually applied to natural populations with homogeneous fertility. Differences between genotypic frequencies at the adult stage

Table 1. Mean outcrossing rates (m) and standard deviation (SD) in G_1 (estimates based on genotypic frequencies observed in 2 G_2 experiments) and G_2 (estimates based on genotypic frequencies of 64 single G_2 plant progeny arrays). Estimates for each locus, means over loci, and multilocus estimates.

Estimate from locus	G_1				G_2	
	G_2:SSD m	(n=371) SD	G_2:bulk m	(n=304) SD	Progenies m	(n=435) SD
Est-5	1.165	(0.154)	0.564	(0.200)	0.492	(0.122)
Enp-1	1.124	(0.109)	1.040	(0.118)	0.519	(0.141)
Amp-1	0.886	(0.098)	0.662	(0.157)	0.340	(0.159)
Cat-1	0.472	(0.102)	0.184	(0.120)	0.582	(0.121)
Sdh-1	0.563	(0.087)	0.551	(0.102)	0.240	(0.082)
Pgd-1	0.891	(0.088)	0.579	(0.077)	0.031	(0.097)
Mean over loci	0.82		0.63		0.35	
Mean, multilocus	0.74		0.54		0.35	

and pollen frequencies might be a source of deviation in estimates, the existence of selective factors usually being the cause of variations between estimates provided by different loci (Kesseli and Jain 1985).

Here the artificial hybrid population was submitted to different selective pressures. Despite certain aberrant values, the mean estimates look coherent. A comparison of estimates provided by the two methods confirms this result, because the multilocus estimate is supposedly less affected by variations between pollination and estimation of genotypic frequencies (Shaw et al 1981). Actually, at least 75% of the plants observed in the G_2 came from a detectable outcrossing event in the G_1. In the G_2, the rate was reduced and involved 35% of the plants.

A relationship between inbreeding rate and pollen fertility was found at the level of the individual: in the G_2, plants whose pollen fertility was higher than 40% were preferentially inbred. This relationship might also be extended to the succession of generations. Lower values of the outcrossing rate estimates provided by the bulk experiment, compared with the SSD experiment, reflect the importance of inbreeding in the origin of bulk plants. In the G_3 progenies, outcrossing rates were estimated by comparing seed production with obligate inbreeding and with open pollination. At this level, the mean rate was 15%. The outcrossing rates were related to the stigma length of plants (r = 0.39) and to their exsertion rates, but were independent of anther length. This result agrees with studies of floral characteristics influencing outcrossing in *O. sativa* (Xu and Shen 1988).

Morphological changes along generations

In the G_1 and G_2, considerable diversity was found for all the morphological traits (Table 2). However, G_1 plants were characterized by very high and continuous tillering, partly due to high general vegetative vigor and to the ability to emit new tillers from

Table 2. Means (m) and standard deviations (SD) of one of the 10 parental varieties (P_6) and its progeny, in cross with an Obake plant, in G_1 and G_2 (SSD).

Character	P_6 (n=10)		G_1 (n=62)		G_2 (SSD)	
	m	SD	m	SD	m	SD
Panicle length (cm)	25.5	1.05	26.8	3.71	23.8	4.70
Primary branches (no.)	15.5	0.93	11.4	2.44	9.9	3.74
Secondary branches (no.)	34.0	4.71	31.5	10.35	29.8	21.72
Panicle density[a]	2.19	0.27	2.76	0.67	2.36	1.25
Earliness (no. of days)	94.2	3.62	102.7	7.15	110.9	12.39
Tillering[b]	24.1	9.30	37.1	18.00	24.7	15.20
Height (cm)	92.5	7.16	87.7	13.16	75.9	15.41

[a]The ratio of secondary to primary branches. [b]Number of panicles produced in 4 wk after the beginning of flowering.

buds located on aerial nodes. This capacity, observed in every family, allowed the overlapping of flowering periods of plants with different growth durations. Panicles were usually longer but with fewer branches than those of the cultivated parents, although the ratio of secondary to primary branches appeared higher in G_1 plants than in the cultivated parental varieties. *O. longistaminata* plants and the mother Obake plant, observed in irrigated conditions, showed long panicles with few secondary branches. Other wild traits characterized hybrid progenies: they were frequently pigmented (60% of G_1 plants showed collar, spikelet, or stigma pigmentation) and long awned, and, where measurement was possible, shedding was high in almost every plant. Rhizomes never developed; this trait appeared to have been totally eliminated since the first hybridization.

G_2 plants evolved toward a more cultivated type. A reduction in the rates of pigmented plants and awned spikelets was observed with lower tillering habit and reduced perenniality. The genetic load of *O. longistaminata,* a wild and allogamous species, was expressed through weakness and many panicle or spikelet abnormalities. At the same time, an increase of total variance was found for every trait, while the between-family variances decreased (Table 3). Interspecific hybridization does not seem to induce high hereditary modification of quantitative traits. Analysis of the regressions between the cultivated parents of the population and their progenies in the G_1 and G_2 shows intermediate values for the majority of characters, all significantly different from zero, except for height (Table 3). Higher coefficients were observed in regressions between G_2 plants and their inbred progenies, indicating the expected efficiency of selection within these progenies. Though the choice of G_3 mother plants had been based only on the fertility of the hybrids, comparison of the G_3 progenies with four cultivated rice varieties showed the existence of transgressive segregants for all traits studied, particularly for early developmental characteristics and panicle architecture. In the G_1 and G_2, the majority of the plants were arrested, and seed shedding was important. Segregation for these traits was analyzed in the G_3 progenies in relation to the parental phenotypes. These two characters showed complex segregation and

Table 3. Comparison of 1) ratios of between-family variance (Vb) to total variance (Vt) for the 10 parental varieties (Pi), and the corresponding families in G_1 and G_2 (based on 10 families of 62 individuals), and 2) coefficients of parent-offspring regressions for different generation associations.[a]

Character	Vb/Vt			Regression coefficients		
	Pi	G_1	G_2	Pi/G_1	Pi/G_2	G_2/G_3
Panicle length	0.611	0.157	0.052	0.732	0.533	0.674
Primary branches	0.797	0.176	0.010	0.378	0.2220	0.765
Secondary branches	0.580	0.099	0.023	0.301	0.344	0.462
Earliness	0.762	0.271	0.064	0.337	0.502	0.679
Tillering	0.836	0.123	0.045	0.262	0.129	0.117
Height	0.334	0.095	0.009	0.018	0	0.330

[a]For parent to G_1 (Pi/G_1) and G_2 (Pi/G_2), the regressions are based on 10 families of 62 individuals; for G_2 to G_3 (G_2/G_3), the regressions are based on 20 progenies of 30 individuals.

appeared independent of each other. Some fertile and nonshedding offspring were observed. Segregation of seed shedding is independent of plant fertility. These results are consistent with those of Morishima (1985), who suggested that the "domestication" traits had been acquired by the accumulation of genes spread throughout the genome.

Intergenomic recombination

Linkage studies were carried out on 2 G_1 and 10 G_3 progenies. Except for *Enp-1* and *Cat-1,* which were found tightly linked (with 0.04 and 0.13% recombinants in the 2 progenies studied), all the other markers were independent. This result is consistent with previous studies of isozyme location (Pham et al 1990). Knowing the parental genotypes, allelic frequencies were compared with expected ones (Table 4). The gametic segregation of the Obake plant, tested through G_1 genotypes, appeared significantly skewed for only one (*Amp-1*) of the seven loci studied, although others also showed slight losses of the alleles coming from the wild species; *Sdh-1* was the only exception. In G_2 SSD, significant losses of wild alleles were found for five loci (*Amp-1, Est-9, Enp-1, Pgd-1, Sdh-1*). The importance of the loss varied between families and between loci. Differences between bulk and SSD frequencies were a consequence of differences in the contribution of G_1 plants to the next segregation. In bulk, losses were of the same or had increased intensity for all families for loci *Amp-1, Enp-1, Pgd-1,* and *Pgi-1*; they differed from one family to another for *Sdh-1* and *Est-9.* Study of open-pollinated progenies of G_2 plants (used mainly to estimate outcrossing rates) allowed us to follow this evolution. Losses seemed to become stable for loci *Pgi-1* and *Enp-1* but still increased for *Est-5, Amp-1,* and *Sdh-1.*

Segregation was also studied in inbred G_3 progenies, which presented different parental genotypes. Among 43 segregation patterns studied, 11 showed significant distortions, but their intensity and direction varied between loci and progenies. *Amp-1* was the only locus to show high loss of the wild allele in both progenies where it segregated. *Est-5* and *Cat-1* were studied in five progenies; they showed loss of the wild

Table 4. Evolution of the frequencies of the alleles coming from *O. longistaminata* in the backcross population over generations.[a]

Locus	G_1	G_2-SSD	G_2-bulk	G_3-op
Est-5	0.200**	0.140**	0.090***	0.095***
Enp-1	0.230	0.163**	0.135***	0.135***
Amp-1	0.200**	0.140**	0.140**	0.125***
Sdh-1	0.270	0.195*	0.230	0.140**
Pgi-1	0.209	0.186*	0.090***	0.095***

[a]Level of significance of the test of comparison between observed and expected frequency = 0.25. Estimates are based on the genotypes of 170 plants of the G_1, 371 and 304 of the G_2 observed in SSD (G_2-SSD) or bulk (G_2-bulk), and 435 open-pollinated offspring of G_2 plants (G_3-op).

allele in one, an excess in another, and no distortion in three others. *Est-2, Pgi-1,* and *Sdh-1* studied in four, six, and five progenies, respectively, showed no distortion. Contrary to expectations from the comparison of allelic frequencies in SSD and bulk, a systematic analysis of the relationship between isozyme genotype and fertility showed no significance. Losses of wild alleles could be attributed not only to differences in fertility between genotypes but also to other sources of deviations, linked or not to the genetic load of the wild species, such as losses at germination, weakness, or selective assortment of gametes.

Discussion and conclusions

This experiment showed that it is possible to obtain spontaneous intermating in a wide experimental population despite a high level of sterility. The mixing of two distant species under two mating systems led to highly sterile plants; few fertile plants composed the efficient pollen pool in each generation. Long and well-exserted stigmas, contributed by the wild species, tended to enhance outcrossing rates. Perenniality, expressed by continuous tillering, also favored intermating between plants of different families. Because of this important outcrossing rate in the G_1, variability remained high in the G_2. However, the parallel evolution of pollen fertility and inbreeding showed the limits of the intermating system. Nevertheless, introgression of the long stigma in a male sterile variety should lead to increased outcrossing rates necessary for high production of hybrid seed.

Spontaneous evolution of the population toward a more cultivated type was found at the morphological and isozyme level, and different origins were proposed for that evolution. Evidence for genetic exchanges between cultivated rices and *O. longistaminata* was suggested at the isozyme level, but because of its reproductive barrier, this species did not play a major role in the diversification of cultivated rices in Africa. Nevertheless, a very high morphological diversity could be expected in the offspring of such crosses. With distant phenotypes and complementary growth habits, introgressive hybridization may be useful for rice breeding. Though plants in the first generation were not attractive from a plant breeding point of view, it was possible to restore

fertility and to eliminate unfavorable traits in a few generations. Interesting transgressive segregants were observed in G_3 progenies. Without major variation in the heritability of quantitative traits, selection among and within the progenies might be efficient. Even if introgressive lines derived from this cross are not directly exploitable as cultivated varieties, they might be an interesting source of new diversity for rice breeding.

New technologies, such as restriction fragment length polymorphism (RFLP) marker-assisted selection, may facilitate the elimination of unfavorable wild traits. Shedding and awning are governed by a few genes spread throughout the genome, which should be easily located by RFLP studies. The detection and location of quantitative trait loci involved in other morphological traits that distinguish rice species might also help greatly in selecting hybrid derivatives. In an interspecific population showing a strong linkage disequilibrium, selection by target markers should be easy. An RFLP map of the rice genome is already available (McCouch et al 1988) and has been transferred to an interspecific backcross population involving *O. longistaminata* (Tanksley et al 1991), which will allow testing such a hypothesis.

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Discussion

Session 1: Varietal differentiation and evolution

Comment—*Juliano:* Our studies at IRRI have shown that the main difference between the Wx^a and Wx^b alleles is the higher amount of the Wx gene product (protein)in WX^b—about three times that in Wx^a at the same amylose content—suggesting higher specific activity of granule-bound starch synthase in Wx^b. Our recent measurements confirm comparable starch synthase activities in Wx^a and Wx^b alleles of developing grains grown at IRRI, as well as similar amylose contents.

Question—*Wang Xiangmin:* Do your data on distribution of the waxy locus (loci) support the opinion that japonica rices originated after indica rices?

Answer—*Sano:* It is difficult to speculate about the evolution of organisms from the results obtained at a single locus. Regarding the *Wx* locus, I am rather interested in the evolution at a single locus. We can say that the original state is Wx^a and the other allelic changes were derived as regulatory mutants from Wx^a.

Q—*Fan Yun-liu: Wx* gene expression is temperature-dependent. You mentioned low temperature. Could you tell how low?

A—*Sano:* Low and high temperatures were 20 and 26 °C during grain development.

Q—*Fan Yun-liu:* Have you identified the *cis* elements involved in temperature regulation of the *Wx* gene?

A—*Sano:* Near-isogenic lines show a differential response to temperature, indicating that the introduced segment must carry an element responsible for it. It is not certain that it acts in *cis* or in *trans.*

Q—*Chaudhuri:* Can you recognize any gene near the waxy locus and do you think it may be a modifier of the waxy gene? Second, do you think that the gene is responsible for differential gene expression of Wx^a and Wx^b?

A—*Sano:* There are many low-amylose mutants that reduce the amount of *Wx* protein. Mostly, they act in *trans* because they are independent of *Wx.*

Wx^a and Wx^b act in *cis.* Our recent northern hybridization work clearly shows transcriptional control. We highly suspect that a *cis*-acting element must be involved in this case rather than other modifiers.

Q—*Wang Xiangmin:* Are javanica rices more closely related to japonica or to indica rice?

A—*Chang:* Because of the great divergence among *Oryza sativa* cultivars, it is difficult to make a generalized answer. Dr. Morinaga has used, appropriately in my view, the term "intermediate." Depending on the particular cultivar of the two other ecogeographic races used, the F_1 hybrids could vary from highly fertile to highly sterile. Even indica/indica crosses have produced highly sterile progenies and aberrant segregation and recombination.

Q— *Yuan:* The cause of sterility between indica/japonica crosses is the abortion of female gametes. Are there some problems in male gamete development?

A— *Ikehashi:* Since the beginning of this work we have compiled data on pollen sterility (male gamete abortion), but we have not yet found any locus for it. We know that wide compatible varieties also have an n-allele for pollen sterility in indica/japonica crosses. Pollen sterility in wide crosses affects yield under cool conditions. We have done some work to clarify the genetic marker for this.

Q— *Oka:* The use of the term "compatibility" to show F_1 fertility is not adequate, because compatibility means the ability to co-exist. Would "affinity" as used by Morinaga be better?

A— *Ikehashi:* I have no objection. But the term "wide compatibility" is commonly used by rice breeders. Breeders seem to understand what is meant by wide compatibility.

C— *Second:* Looking at the indica/japonica differentiation from an evolutionary perspective, there are two main models:

- differentiation occurred during the process of domestication (as you seem to assume), and
- differentiation pre-existed in the wild ancestors, and characters selected during domestication were superimposed.

I support the second model based on many molecular markers scattered on the chromosomes and also on chloroplast DNA in both cultivars and wild species. From this second model's standpoint, your presentation could be called "How does the indica/japonica differentiation remain in hybrid derivatives?" I do not see any reason to prefer one or the other model from your data.

C— *Sato:* The question, "Are indicas and japonicas derived from a common ancestor?" seems not to be so meaningful, because all creatures ultimately derive from a common ancestor. So, this question could be rephrased as, "Which occurred earlier: domestication or indica-japonica differentiation?" I think indica-japonica differentiation might have occurred prior to domestication, because a similar trend of association has been observed among wild rice populations.

Q— *Shao Quian:* Do you have some idea about the different ancestors of japonicas and indicas?

A— *Sato:* Recent archeological findings in East China suggest that japonicas arose there independently from indicas. However, I feel the data are insufficient.

Q— *Chaudhuri:* Don't you think that among your identifiable evolutionary forces "preferential mating" and "gametic selection" are identical?

A— *Sato:* I use "preferential mating" to mean the premating selection of pollen grains by various causes. Gametic selection refers to post-mating, i.e., during pollen tube elongation, etc.

Q—*Chang*: The earliest remains of rice found in Shanghai (c. 6500 yr old) and Cheking (c. 7000 yr old) were of the Keng (sinica) type. More recently, Hsien (indica) grains and Jeng types, also 7000 years old, were found side by side at another site (Liu-jai-jaio) in Chikiang. (These are the oldest carbon-dated archeological findings to date.) How can we explain this situation, because the indica type is generally considered to be of southern origin and the Keng a temperate zone derivative?

A and **Q—*Sato***: Recent archeological finds in China are very interesting. I agree that East China is one of the places where rice cultivation first began, but I cannot agree that the excavated rice seeds were all japonica, because grain shape is not a good discriminating character. I think we need more data to be able to say whether indica or japonica arose first. My point can be posed as a question: "What factor(s) caused the separation of indicas from japonicas?"

C—*Glaszmann*: In response to Dr. Chang on the ancient geographic distribution of indicas and japonicas based on excavated grains, the main character used to classify excavated grains has been grain shape. If we look at present-day varieties, there are many japonicas with rather thin grains, and many indicas with wide grains. Oka and Morishima estimate that classifying varieties by grain length/width ratio is difficult, having a 40% chance of error. Thus the putative classification of excavated grains may be misleading.

Q—*Zhang Qifa*: How many markers are used in constructing a discriminant function, and what is the criterion to define indica and japonica?

A—*Sato*: I used three markers, but recently we have used an additional five isozyme loci. The discriminant function was used. Indica and japonica cultivars show positive and negative values of this function, respectively.

C—*Oka*: In addition to the introduction of rice from Indonesia and India to Madagascar and East Africa, respectively, some varieties could have been introduced by the Portuguese from Europe, some 500 years ago.

C—*Glaszmann*: Of 24 upland rices from Madagascar that were studied, 21 were japonicas and 3 were indicas with the *Amp-1*^{2} allele (i.e., indicative of indica-japonica recombination).

C—*Chang*: In response to Dr. Glaszmann's remark (that javanica is a part of japonica, the former being tropical and the latter being from the temperate zone), I would like to reiterate the need to examine complex issues from different perspectives: biosystematic, ecological, anthropological, and agrodevelopmental. In our paper, we emphasized how multiple lines of evidence may reveal more of the enormous genetic diversity in the Asian cultigen after wide dissemination, cultivation, and selection under diverse environments. No single approach can provide a definitive answer to the complex process under which a tropically based semiaquatic plant evolved into a dryland ecotype and others intermediate between wetland and dryland types.

Q— *Sato:* Intermediate types between indica and japonica have been found in lowland cultivars, but in Asia those intermediate types are found mainly in upland fields. What do you think about this?

A— *Glaszmann:* Intermediate types are found where both indica and japonica types are sympatric. One such environment is the upland ricefield in the tropics, where most varieties are japonicas but where a few indicas can also grow. Another one is the lowland field at a high elevation in the tropics. In the extreme conditions in Madagascar (1800 m), japonica types are found (Latsika varieties). At slightly lower altitude are found most intermediates.

Q— *Vaughan: O. longistaminata* is the most difficult *Oryza* to conserve ex situ. Since *O. longistaminata* is genetically diverse, can you recommend ex situ techniques to conserve this diversity to make it readily available to rice scientists now and in the future?

A— *Ghesquiere:* Individual plants can be conserved through vegetative propagation (rhizomes or cuttings). Seed multiplication of natural populations should be done in small populations of 25–50 plants with good water supply and fertilizer to get sufficient seed set. Another way is multiplication of artificial populations by bulking individuals from different accessions to increase seed set.

C— *Zhang Qifa:* There might be an alternative conclusion to natural selection as the cause of the observed association between isozyme loci and traits. The closeness of the linkage mating system and the parental associations that went into a cross may have major impacts on associations in later generations. The individual and joint effects of these three aspects should be investigated before reaching the conclusion of natural selection.

C— *Morishima:* I would like to consider that the genes responsible for these characters are linked, forming an "adaptive gene block." This gene block tends to be broken as the generations proceed in a hybrid population. This is why I consider that a nonrandom association consistently found in nature cannot be explained only by selection.

Q— *Zhang Qifa:* What is the linkage relationship between *Pox-1* and the traits investigated? The mating system used should also have a major impact on the association. How many generations of selfing were done in the preparation of isogenic lines?

A— *Morishima:* The recombination value was not estimated. But judging from the fact that six generations of selfing theoretically retain 10% of the chromosomes in a heterozygous state at one side of the particular locus, the linkage observed in the early generations is not so tight. Since wild rices are partly allogamous, nonrandom association must have been broken by outbreeding and recombination. We did six generations of selfing.

Q— *Vaughan:* Typical annual and perennial AA wild *Oryza* species can be clearly distinguished in the field by the characters you presented. However, could a similar set of morphological characters be provided to help distinguish the intermediate from weedy ecotypes in the field?

A—*Morishima*: Since most of the weedy types have an intermediate perennial-annual habit, it is difficult to distinguish weedy types and wild-intermediate types by morphology. Our "weedy type" consists of naturally growing rice plants in or adjacent to a ricefield.

Q—*Virmani*: Is there a relationship between the polymorphism of the *Pox-1* locus and the presence or absence of genes for apomixis in wild rices?

A—*Morishima*: If you use "apomixis" in the broad sense (asexual propagation), *Pox-1* polymorphism is associated with variation in the extent of apomixis. Judging from my data, *Pox-1* is linked to at least one of the loci responsible for asexual propagation in nature. But it seems that many factors influence the relative proportion of sexual vs asexual propagation of natural populations of wild rice.

SESSION 2

Genetic Markers, Linkage Groups, and Aneuploids

Analysis of genes for stigma coloration in rice

H.I. Oka

The anthocyanic pigmentation of the rice apiculus is controlled by three complementary genes—*C, A,* and *P*—which serve as the basic coloration genes. The genetic control of stigma color is more complex. To shed more light on genes for stigma coloration, F_1 and F_2 data for 196 varietal crosses were investigated to explain the whole pattern of segregation by assuming certain Mendelian genes. In ordinary cases, the stigma is colored only in plants having *C, A,* and *P.* Two independent genes, *Ps-2* and *Ps-3,* take part in stigma coloration, *Ps-2* being relatively frequent in indicas and *Ps-3* in japonicas. For *Ps-3,* an inhibitor, *I-Ps-3,* was found, which seems to have two loci according to variety. In addition, two complementary inhibitors are assumed to be present in some of the varieties. A Japanese upland variety, Gaisen-mochi, having a colorless apiculus and colored stigma, has *Ps-1,* which expresses stigma color even when *P* is absent (recessive). An inhibitor for this gene, *I-Ps-1,* needs *P* to function. Four genes—*P, Ps-3, Ph,* and *Ps-1*—are located in linkage group II in that order. Their recombination values were estimated.

Most rice varieties and derivatives from varietal crosses express anthocyanin color in the stigma only when the apiculus is colored. The coloration of the stem node and some other plant parts also shows such a relation, and the genes for apiculus color can be considered as basic for anthocyanin formation (Takahashi 1964). Apiculus color at heading ranges from pink to dark purple, for which several isoalleles have been described, but when only the presence or absence of color is considered, it is controlled simply by three complementary genes: *C* (chromogen production), *A* (activation), and *P* (spreading pigment). These three genes are located in linkage groups I, III, and II, respectively (cf. Kinoshita 1984, pp. 29–30). Accordingly, all varieties with colored apiculus have the genotype CAP, while colorless varieties can have varying genotypes (Oka 1989a). Setty and Misro (1973) reported that the *P* gene has different loci according to variety. In the material used in this study, however, all F_2 plants derived from crosses between apiculus-colored parents had a colored apiculus, and the different loci of gene *P* were not confirmed.

In contrast to apiculus color, the genetic control of stigma color is more complex, as reported in this paper, but space is too limited to describe the procedures for identifying genes and determining parental genotypes for all crosses observed.

Materials and methods

Forty-one strains were used as parents, including 21 indicas and 19 japonicas (*Oryza sativa*), and an Indian wild annual type of *O. rufipogon* (Table 1). The F_1 and F_2 plants of 196 crosses were observed for apiculus and stigma color at heading, pollen and seed fertilities, phenol reaction, and some other traits. In 20 cases, reciprocal crosses were made and the F_2 populations were observed more than once, even though no significant differences were detected between reciprocal crosses and replications. In each cross, about 80–300 F_2 plants were grown and observed in Taichung, Taiwan, China, with the assistance of several students of Chung Hsing University.

Purple to dark red (P) and red to pink (R) color tones were distinguished, but it was difficult to classify them into several grades to identify multiple alleles at the *C* and *A* loci. This was largely because distant crosses were tested under varying conditions during the winter and summer cropping seasons.

In distant crosses, F_2 ratios are often distorted (Oka 1989b). Yet, the underlying genes could be deduced in many cases from observed segregation patterns. To estimate recombination values from distorted ratios, the methods described by Oka (1989c) were employed. To confirm the parental genotypes presumed from the F_2 data, F_3 lines from selected F_2 plants were examined in 001/325, 108/532, and 5 other crosses, and some colorless F_3 lines were intercrossed.

Results

The results of the experiments are presented in two sections: one discussing ordinary varieties in which stigma coloration occurs only in plants with a colored apiculus, and the other discussing a special variety, Gaisen-mochi (532), and its crosses, in which plants with colorless apiculus and colored stigma occur.

Crosses between ordinary varieties

In the F_2 populations of crosses between ordinary varieties, three types of segregants are found: + + (apiculus and stigma both colored), + - (apiculus colored, stigma colorless), and - - (apiculus and stigma both colorless). No - + (apiculus colorless, stigma colored) type occurs. The parental genotype for apiculus coloration (the combination of *C, A,* and *P*) can be judged by the F_1 phenotypes from crosses with known genotypes and by observation of F_2 plants when necessary (Oka 1989a). The F_2 ratios for stigma color observed among segregants with colored apiculus (+ + and + - types) are summarized in Table 2, excluding those with variety 532.

Different segregation patterns were found; representative ones are briefly described in Table 3.

Table 1. Varieties used as parents and their apiculus and stigma color.[a]

Code	Origin	Vernacular name	Color	
			Apiculus	Stigma
		Indicas		
001	Vietnam	70[a] som cau	–	–
022	Vietnam	II dauh	–	–
060	Vietnam	RTS23	–	–
101	Taiwan	U-kuh-tsing-you	–	–
108[b]	Taiwan	Peh-ku	–	–
124	Taiwan	Shuang-chiang	–	–
143	Taiwan	Lui-tou-tzu	–	–
160	Taiwan	Hong-ka-chiu	P	P
414[b]	India	PTB10	–	–
421	India	PTB8	P	–
435	India	Pachchai perumal	P	P
451	India	Surjamkhi	P	P
612	Sulawesi	Padi bali	–	–
619	Sulawesi	Padi hotjong	P	–
706	North China	He-nan-tsao	P	P
717	China	Nan-chang-wan	–	–
719	China	Chin-sen	–	–
724	South China	Kunming tsieh-huan	–	–
727	South China	Chin-tsao	–	–
761	Hainan	Siao-chung-kuh	–	–
1091	South China	Lui-kung sen	P	P
		Japonicas		
219	Philippines	Garumbalay	P	–
221	Philippines	lnakupa	P	P
236	Philippines	Olag ayau	–	–
242	Philippines	Malagkit pirurutong	–	–
318	Indonesia	Boegi inda	P	P
325	Indonesia	Kaniranga	P	P
T65[b]	Taiwan	Taichung 65	–	–
501	Japan	Urasan	–	–
521[b]	Japan	Kisshin	–	–
532	Japan	Gaisen-mochi	–	P
535	Japan	Hirayama	R	P
545[b]	Japan	Shinriki	–	–
552	Japan	Aikoku	R	–
563[b]	Japan	Kinoshita-mochi	P	R
571	Japan	Mansaku	–	–
647[b]	Sulawesi	Padi ase-banda	–	–
701	North China	Tatung-tsailai	P	P
703	North China	Tamao tao	–	–
871	Taiwan	Nabeshi	–	–
		O. rufipogon		
W106[b]	India	Cuttack annual type	P	P

[a] P = purple to dark red, R = red to pink, (–) = colorless. [b] Used as maternal parent and intercrossed.

Table 2. Pooled F_2 ratios for stigma color (++:+–) among segregants with colored apiculus and genotypes presumed for respective strains.[a]

P_2	P_1							Genotype of P_2 strain	
	001	545	647	219	521	T65	W106	Common genes[b]	Extra[c]
Group A[d]	0	3:1	51:13	3:1	0	3:1	1:1	*c A P Ps-2 ps-3 I-3*	
O22	0				0	0:1		*c A P ps-2 ps-3 I-3*	
Group B[e]	1:0	51:13	1:0	13:3	51:13	15:1	1:0	*C A P Ps-2 Ps-3 i-3*	
421	49:15		1:3	1:15	3:61	3:13	(61:3)	*C A P ps-2 ps-3 I-3'*	
619	49:15	3:61	1:3		(3:61)			*C A P ps-2 ps-3 I-3'*	
Group C[f]	1:0	51:13	1:0	13:3	51:13	15:1	1:0	*C A P Ps-2 Ps-3 i-3*	
545	3:1	–	0	0:1	0			*C a P ps-2 ps-3 I-3*	*(I-a)*
871	3:1			0:1				*C a P ps-2 ps-3 I-3*	
647	51:13	0	–		0	0	1:0[i]	*C a P ps-2 Ps-3 i-3*	*(I-a)*
219	3:1	0:1	7:57[g]	–	0:1	21:1235		*C A P ps-2 Ps-3 I-3*	*(I-b)*
242	0		7:57[g]	1:15				*c A P ps-2 Ps-3 I-3'*	*(I-b)*
325	1:0	3:1	15:1	51:13	3:1			*C A P Ps-2 ps-3 i-3*	
501	0		21:43[h]			0		*c A P ps-2 ps-3 i-3*	*(I-b)*
521	0	0	0	0:1	–	0		*c a P ps-2 ps-3 I-3*	
571	0		0	3:13	0	0	(63:1)	*c a P ps-2 ps-3 i-3*	
236	3:1					0:1	(63:1)	*C A p ps-2 ps-3 i-3*	
703	1:0	0	0		0	0	1:0	*C a p Ps-2 ? ?*	
535	1:0	201:55[j]	15:1					C^{Br} *A P Ps-2 Ps-3 i-3*	
552	3:1					0:1		C^{Br} *A P ps-2 ps-3 i-3*	
T65	3:1	0				–	(63:1)	C^{Br} *a P ps-2 Ps-3 i-3*	*(I-a)*
W106							–	*C A P Ps-2 Ps-3 i-3*	*(Ps-1)*

[a]Ratios in parentheses = assumed without confirmation. [b]*I-3/i-3 = I-Ps-3/i-ps-3, I-3' = I-Ps-3'* supposedly having a locus different from that of *I-Ps-3. I-a* and *I-b* = complementary inhibitors for *Ps-3,* corresponding to *I-Ps-a* and *I-Ps-b* in Kinoshita (1984, p. 30). [c]Strains with no "extra" gene are expected to have *i-a, i-b,* and *ps-i.* [d]Group A = 001, 060, 101, 108, 124, 143, 414, 612, 717, 719, 724, 727, and 761. [e]Group B = 160, 435, 451, 706, and 1090. [f]Group C = 221, 318, and 701. [g]7:57 = (1:3) (7:9). (1:3) comes from cosegregation for *I-3/i-3* (given in b). (7:9) comes from complementary inhibitors *I-a/i-a* and *I-b/i-b* (given in b). [h]21:43 = (3:1) (7:9). (3:1) comes from *Ps-3/ps-3.* (7:9) is expected from *I-a* and *I-b* as mentioned above. [j]21:235 = (3:13) (7:9). (3:13) comes from interaction between *Ps-3/ps-3* (3:1) and *I-3/i-3* (1:3). (7:9) is expected from *I-a* and *I-b* as mentioned above. [j]201:55 = (3:1) (9:7) (1:3). (3:1) comes from *Ps-2/ps-2.* (9:7) is due to C/C^{Br} and *Ps-3/ps-3,* where plants with *C* and *Ps-3* are expected to show stigma coloration. (1:3) is expected from *I-3/i-3* as mentioned above (b and *g*).

Table 3. Representative segregation patterns, F_2 ratios, and genes for stigma color.

Case	P_1	P_2	F_1	F_2 ratio ++:+–	Example	Genes for stigma color
0	––	––	––	"0"	Many	(No apiculus-colored segregants)
1	––	++	++	1:0	108/160	Same gene in both parents
2	––	––	++	3:1	108/545	Segregation for a coloration gene
3	––	+–	+–	1:3	647/421	Segregation for an inhibitor
4	+–	––	+–	3:13	219/571	A coloration gene and an inhibitor
5	––	++	++	13:3	219/221	A color gene and an inhibitor for another color gene
6	––	––	++	51:13	108/647	Two color genes and an inhibitor
7	+–	––	+–	0:1	219/521	Same inhibitor in both parents

Underlying genes in these different cases are presumed as follows:

- Crosses between colorless indicas produced no colored hybrids, because both parents have *c A P* (group A; Table 3, case 0). In their crosses with colored indicas (group B) or colored japonicas (group C), the F_1 and all F_2 plants with colored apiculus had colored stigmas (+ +:+ – = 1:0; Table 2; Table 3, case 1). In this case, both parents are thought to have the same gene for stigma coloration, assigned the symbol *Ps-2* to agree with the description in Kinoshita (1984, p. 30).
- When varieties of group A (*c A P Ps-2*) were crossed with variety 545 (*C a P),* the F_1 was colored (+ +) and the F_2 plants with colored apiculus segregated into 3 + +:1 + – type (Table 3, case 2). Variety 871 showed the same pattern. These varieties seem to have *ps-2.* Variety 647, whose F_2 ratios in crosses with group A varieties were near 3:1, may also be considered to have *ps-2.*
- When 647 was crossed with varieties of groups B and C, all F_2 plants with colored apiculus had colored stigmas (1:0; Table 2; Table 3, case 1). This cannot be explained without assuming another gene for stigma coloration, *Ps-3,* present in both parents (to agree with Kinoshita 1984, p. 30), since 647 would have *ps-2.* Then the crosses of 647 (*ps-2 Ps-3*) with group A varieties (*Ps-2 ps-3*) are expected to show a 15:1 ratio in the F_2. The occurrence of nearly 3:l ratios in those crosses suggests that group A varieties have an inhibitor for *Ps-3, I-Ps-3* (abbreviated *I-3*); in view of the 1:0 pattern in crosses with groups B and C (*Ps-3 Ps-2*), 647 cannot have *I-3.* Accordingly, the nearly 3:1 ratio observed in crosses between 647 and group A varieties may be considered to be 51:13 (Table 2; Table 3, case 6) resulting from 3:1 for *Ps-2* and 3:13 for *Ps-3* and *I-3.* The observed ratio of 374:105 (pooled for 4 crosses whose ratios were homogeneous) agrees with this expectation ($c^2 = 0.8$, $P > 0.5$).
- A colored variety 325, showing a 15:1 F_2 ratio in its cross with 647 and the 1:0 pattern with group A varieties, is considered to have *Ps-2 ps-3 i-3.* Variety 545 is thought to have *ps-2 ps-3 I-3,* because its crosses with 325 and group A varieties gave a 3:1 ratio, and its crosses with varieties of groups B and C gave a nearly 3:1 ratio (51:13; Table 2; Table 3, case 6). The observed F_2 ratios in crosses of 545 with groups B and C—468:132 (4 crosses pooled, homogeneous)—agree well with 51:13 ($c^2 = 1.2$, $P > 0.25$).
- When variety 219 was crossed with group A varieties, the F_1 was colored (+ +) and the F_2 showed a 3 + +:1 + – ratio (568:145, $c^2 = 1.2$, $P > 0.25$; 4 homogeneous crosses pooled). This suggests that 219 has *ps-2 Ps-3 I-3.* The F_1 and F_2 plants from 219/545 and 219/871 showed no stigma color, because they had *I-3* in common (Table 3, case 7).
- Varieties 242 and 421 showed nearly 1:15 F_2 ratios for stigma color in their crosses with 219, suggesting that they have an inhibitor for *Ps-3* at a locus different from that in 219, which is tentatively designated as *I-Ps-3′* (abbreviated *I-3′*). Variety 619, which showed a segregation pattern similar to that of 421, is also assumed to have *I-3′.* When 1:15 is combined with 3:1, a 49:15 ratio is expected, although it is not distinguishable from 3:1 (e.g., 421/group A, Table 2).

- F_2 ratios lying between 1:3 and 1:15, and between 1:3 and 1:1 were found in some crosses. These may be regarded as resulting from segregation distortion, but some of these crosses showed high F_1 fertilities. Hsieh (1960, 1961) pointed out the presence of a set of complementary inhibitors (I^{ps} and $I^{ps}2$; redesignated *I-Ps-a* and *I-Ps-b* in Kinoshita (1984, p. 30); *I-Ps-3-a* and *I-Ps-3-b* or *I-a* and *I-b* in this paper), based on the finding that T65, which seemed to have no ordinary inhibitor, showed in its crosses with some colored-stigma strains (7111 and two others) colorless F_1 plants, and nearly 1:2 F_2 ratios. Such complementary inhibitors will bring about a 7:9 ratio in the F_2 and will give rise to 21:42, 7:57, and other ratios when combined with 3:1,1:3, and others. Although the data from this study give no conclusive evidence for their existence, this assumption facilitates explanation of some of the F_2 ratios observed. Tentatively, it is assumed that varieties T65, 545, and 647 have *I-a;* and that 219, 242, and 501 have *I-b* (Table 2). Then, for instance, the F_2 ratio found in 647/219 (22:226) can be regarded as 7:57 (c^2 = 1.1, P>0.25), and that observed in 647/501 (66:159) may be regarded as 21:43 (c^2 = 1.2, P>0.25). Probably, if such complementary inhibitors exist, they would be distributed more commonly, although their detection is not always feasible.
- When a colorless japonica, 521, having *c a P,* was crossed with varieties of groups B and C, the F_2 ratios (++:+–) observed were 89:5, 86:7, 59:5, etc. Its cross with 219 produced no colored-stigma F_2 plants (0: 1; Table 3, case 7), while its cross with 421 (*CAP Ps-2 Ps-3 I-3'*) gave a nearly 1:15 (3:61) ratio. Variety 521 is then assumed to have *ps-2 ps-3 I-3,* like 545, and the above ratios with groups B and C are assumed to be 51:13. The observed ratio in 521/451 (86:17) agrees with 51:13 (c^2 = 0.9, P>0.25), but others do not. This is considered due to segregation distortion, since the crosses with 521 showed low F_1 pollen fertilities (20–50%). A similar colorless japonica, 571, was assumed to have *ps-2 ps-3 i-3,* because its F_2 with 219 gave a 3:13 ratio (13:95. c^2 = 3.2, P>0.05). Another colorless japonica, 703, having *C a p,* showed the 1:0 pattern in its F_2s with group A varieties, suggesting that it has *Ps-2.* However. its alleles at loci *Ps-3* and *I-3* remain unknown. Through similar exercises in symbolic logic, the genotypes for stigma coloration of several varieties were presumed, e.g., 236 = *C A p ps-2 ps-3 i-3* and 501 = *c A P ps-2 ps-3 i-3 I-b* (Table 2).
- Varieties showing pink color at the apiculus may be assumed to have C^{Br}, while plants with C^{Br}/C^{Br} or C^{Br}/c and *A P* are thought to express no stigma color even if they have a gene for stigma color (Takahashi 1958; 1964, p. 217). T65 is known to have C^{Br}*a P* for apicuius color (Hsieh 1960; 1961, p. 85); varieties 535 and 552 with pink apiculus may also be assumed to have C^{Br}. This study suggests, however, that the suspending effect of C^{Br} on stigma coloration is limited to *Ps-3,* leaving *Ps-2* unaffected. For instance, the F_2 from 108/T65 segregating for *Ps-2* showed a 3:1 ratio (95:32) for stigma color. On the assumption that the genotype of T65 is C^{Br}*a P ps-2 Ps-3 i-3 I-a,* its crosses with groups B and C are expected to show a 15:1 F_2 ratio for stigma color. This agrees well with the data

(121:9, 2 homogeneous crosses pooled, c^2 = 0.1, P>0.5). Together with the complementary inhibitors, the suspending effect of C^{Br} on *Ps-3* brings about a complication in F_2 ratios. For instance, the F_2 of 219/T65 involving C^{Br}/C and *I-a, I-b* is expected to give a 21:235 ratio (Table 2, footnote *i*), which agrees with the data (12:108). Variety 022, which showed the 0:1 pattern in its F_2 with T65, would have *ps-2 ps-3 I-3.* Variety 535 may be considered to have C^{Br}*A P Ps-2 Ps-3 i-3,* because its F_2 with 647 showed a 15:1 ratio (82:3). Its cross with 545 is expected to give an F_2 ratio of 201:55 (Table 2, footnote *j*), which agrees with the data (139:46; c^2 = 1.3, P>0.25).

- Variety 563 shows purple-tawny color spread over the hulls at maturity, which is expressed by gene *Pr* (linkage group II, Yen and Hsieh 1968), and pink color in the stigma. The gene for stigma color of this variety could not be identified, although more than 20 crosses with different varieties were observed. It seems that *Pr/pr* has some effect on stigma color, but the variation in color tone is continuous from pink to colorless or to purple. Accordingly, the data for 563 crosses are not presented in this paper.
- The crosses of W106 with different cultivars all produced colored F_1 plants, and when the F_2 segregated for apiculus color, all colored-apiculus segregants had colored stigmas (1:0) in most crosses. When crossed with varieties having *I-3,* there were a few segregants with colorless stigmas (+ – type). W106 is then considered to have *C A P Ps-2 Ps-3 i-3.* In addition, it may have *Ps-1,* because its cross with varieties having *C A p* produced a few F_2 segregants of – + type. Accordingly, the occurrence of many + + and a few + – F_2 plants is assumed to represent a 63:1 or 61:3 ratio (Table 2). Several other wild strains (e.g., W120, W134, and W145) also showed the same segregation patterns, suggesting that their genotypes for stigma coloration genes are similar to that presumed for W106.

Crosses of Gaisen-mochi (532) with other varieties and linkages

Variety 532, having colorless apiculus and purple stigma (– + type), is thought to have *Ps-1* (called *Ps* by Takahashi [1958]), which causes stigma pigmentation without *P* as long as *C* and *A* are present. This variety, having *C A p Ps-1,* was crossed with several other varieties. The F_2 patterns observed were complex (Table 4) but could be analyzed by using the linkage of relevant genes with *Ph* for phenol reaction (532 had *Ph*).

P and *Ph* are linked with a mean recombination value of 27.2+5.6% (Table 5). Also, *Ps-2* and *Ph* are linked with a mean recombination value of 21.1 ± 4.0% (Table 6). Accordingly, *P* and *Ps-2* are linked (9.0 ± 5.4%).

The F_2 data obtained in 545/532 and 219/532 suggest that gene *Ps-1* carried by 532 is also linked with *Ph,* as most segregants of – + type showed positive phenol reaction (Table 4). In both F_2 populations, + + plants were fewer than + – plants, suggesting that an inhibitor for *Ps-1* (*I-Ps-1*) is present in 545 and 219. However, – + segregants are more numerous (19% in 545/532) than expected when *Ps-1* is completely suppressed by *I-Ps-1* (about 6%). To account for the observed patterns, an assumption is needed

Table 4. F_2 segregation patterns for apiculus (Ap) and stigma (St) color and phenol reaction (Ph) observed in crosses with Gaisen-mochi (532).

Cross	F_1[a]		Plants (no.) with given segregation pattern								Plants (no.)
	Ap	St	F_2 Ap:	+	+	+	+	–	–	–	–	
			St:	+	+	–	–	+	+	–	–	
			Ph:	+	–	+	–	+	–	+	–	
545/532	P	(R)		26	6	64	31	45	1	48	22	243
219/532	P	(R)		67	5	77	33	55	2	0	4	243
108/532	P	P		102		0		12		123		237
414/532	P	P		67		3		8		94		172
(pooled)				169		3		20		217		409
521/532	P	P		20	14	9	7	16	0	53	9	128
647/532	P	P		47	31	11	3	24	3	41	14	174

[a] P = purple to dark red, (R) = faint pink

Table 5. Linkage relations estimated between loci *P* and *Ph*.[a]

Cross (P_1/P_2)	Parental genotype	F_2 phenotype[b]					Plants (no.)	Recombination value (%)	Distortion considered for
		Ap:	+	+	–	–			
		Ph:	+	–	+	–			
001/703									
108/703	P_1: *cAP-Ph*	Obs.	149	25	266	126	566	22.5 ± 4.5	*C:c*
414/703									
(pooled)	P_2: *Cap-ph*	Exp.	150.5	23.6	274.0	117.9	c^2 = 0.9,	P > 0.75	
545/532	P_1: *CaP-ph*	Obs.	90	37	93	23	243	35.8 ± 9.9	
	P_2: *CAp-Ph*	Exp.	97.0	39.7	85.3	21.0	c^2 = 1.4,	P > 0.5	
219/532	P_1: *CAP-ph*	Obs.	144	38	59	2	243	24.5 ± 6.0	*Ph:ph*
	P_2: *CAp-Ph*	Exp.	136.4	36.5	66.5	3.6	c^2 = 2.0,	P > 0.5	
647/532	P_1: *CaP-ph*	Obs.	58	34	65	17	174	26.4 ± 15.7	
	P_2: *CAp-Ph*	Exp.	67.5	30.4	63.0	13.1	c^2 = 3.0,	P > 0.25	

[a] Mean = 27.2 ± 5.6%, weighted according to the number of plants observed. Standard deviation for mean = standing for the variation among estimates. Ap = apiculus color, Ph = phenol reactlon, "-" shows linkage. [b] Obs. = observed, Exp. = expected.

that *I-Ps-1* requires *P* for its function. The F_2 data for 219/532 are also explained by the same assumption favorably.

In 545/532, – – segregants would be mostly the *aa* plants: in 219/532, in which no *aa* plant occurs, – – segregants were few (Table 4). This suggests the linkages of *p* with *Ps-1* as expected from the linkage of *Ph* with *P* and *Ps-1*. Thus, the parental genotypes are assumed to be: 532= *C A p—Ph—Ps-1—ps-2 ps-3 i-3 i-Ps-1* ("—"shows linkage), 545 = *C a P—ph—ps-1—ps-2 ps-3 I-3 I-Ps-1*, and 219 = *C A P—ph—ps-1—ps-*

Table 6. Linkage relations estimated between loci *Ps-2* and *Ph*.[a]

Cross P_1/P_2	Parental genotype	F_2 phenotype					Plants (no.)	Recombination value (%)
		St:	+	+	–	–		
		Ph:	+	–	+	–		
219/160	P_1: *ps-2—ph Ps-3 I-3*	Obs.	279	31	36	43	389	21.0 ± 3.8[b]
219/435 (pooled)	P_2: *Ps-2—Ph Ps-3 i-3*	Exp.	285.9	40.2	29.1	33.8	c^2=6.4, P > 0.05	
647/108	P_1: *ps-2—ph Ps-3 i-3*	Obs.	113	20	15	25	173	20.7 ± 4.3
647/143 (pooled)	P_2: *Ps-2—Ph Ps-3 I-3*	Exp.	116.5	21.3	13.2	21.9	c^2=0.9, P > 0.75	
545/001	P_1: *ps-2—ph*	Obs.	100	14	9	22	145	18.0 ± 3.6
545/124 545/414 (pooled)	P_2:*Ps-2—Ph*	Exp.	96.9	11.9	11.9	24.4	c^2=1.4, P > 0.5	
219/108	P_1: *ps-2—ph*	Obs.	241	55	34	47	377	27.8 ± 2.8
219/124 219/414 (pooled)	P_2:*Ps-2—Ph*	Exp.	237.4	45.4	45.4	48.8	c^2= 5.0, P > 0.1	
647/435	P_1: *ps-2—ph Ps-3 i-3*	Obs.	38	8	4	9	59	18.0 ± 7.3
	P_2: *Ps-2—Ph Ps-3 I-3*	Exp.	40.6	7.3	3.6	7.5	c^2=0.58 P > 0.9	

[a]Mean = 21.1 ± 4.0%, weighted according to the number of plants observed. Standard deviation for mean = standing for the variation among estimates. St = stigma color, Ph = phenol reaction, "–"shows linkage. Obs. = observed, Exp. = expected. [b]Segregation distortion for *Ph:ph* was considered in computation (cf. Oka 1989c).

2 Ps-3 I-3 I-Ps-1. The inhibitors are assumed to be independent of coloration genes so as to better explain the data.

In 545/532, since both parents have *ps-3* (recessive), this gene and its inhibitor can be neglected in analyzing the segregation pattern. Letting the recombination value between *P* and *ps-1* (repulsion) be *p*, the frequencies of genotypes for four color classes are expected to be as in Table 7.

The maximum likelihood estimate of *p* is 36.1 ± 14.6%, and the expected values show no significant deviation from observed ones. Letting the recombination value between *Ps-1* and *Ph* (coupling) be *q*, the expected frequencies are as shown in Table 8, in which the genes controlling color phenotype are the same as in Table 7. In estimating *q*, the *P—ps-1* recombination (*p*) is assumed to be 0.30 to be close to the mean for different crosses (Table 9).

The *q* value for *Ps-1* and *Ph* recombination was 14.1 ± 5.7%. Similarly, the recombination value between *P* and *Ph* was 35.8 + 9.9% (Table 5). Thus, the observed segregation pattern could be explained by analyzing linkage relations.

In 219/532, the computation procedures were more intricate, since *I-3* for *Ps-3* was involved in addition to *I-Ps-1*, and distorted segregation for *Ph/ph* had to be considered. It must be determined whether *I-Ps-1* is independent of *I-3* or whether they are the same

Table 7. Frequencies of genotypes for 4 color classes in 545/532.

Phenotype (Ap)	(St)	Genes concerned	Frequency expected		Plants (no.) Observed	Expected
+	+	*A P-Ps-1 i-Ps-1*	$\frac{3}{4}\ \frac{1}{4}\ \frac{1}{4}$	$(2+p^2) = 0.0938 + 0.0496p^2$	32	24.3
+	–	*A P-ps-1*	$\frac{3}{4}\ \frac{1}{4}$	$(1-p^2)$		
		A P-Ps-1 I-Ps-1	$\frac{3}{4}\ \frac{3}{4}\ \frac{1}{4}$	$(2+p^2)$	95	112.4
				$= 0.4687 - 0.0496p^2$		
–	+	*A p-PS-1*	$\frac{3}{4}\ \frac{1}{4}$	$(1-p^2) = 0.1875 - 0.1875p^2$	46	39.6
–	–	*A p-ps-1* and *aa*	$\frac{3}{4}\ \frac{1}{4}$	$p^2 + \frac{1}{4} = 0.25 + 0.1875p^2$	70	66.7
Total				1.0 ($p = 0.361$)	243	$c^2 = 6.9$, $P > 0.05$

Table 8. Expected frequencies in 545/532.

Phenotype (Ap)	(St)	(Ph)	Expected frequency[a] ($p = 0.30$)	Plants (no.) Observed	Expected	Remarks
+	+	+	$0.0980\ (3-2q+q^2) \cdot 1/3$	26	21.7	Pooled in computing c^2 value
+	+	–	$0.0980\ (2q+q^2) \cdot 1/3$	6	2.1	
+	–	+	$(0.2939\ (3-2q+q^2) \cdot 1/3)$	64	71.9	
			$(0.1706\ (2q-q^2)$			
+	–	–	$(0.2939\ (2q-q^2) \cdot 1/3$	31	41.0	
			$(0.1706\ (1-2q+q^2)$			
–	+	+	$0.1706\,(3-2q+q^2) \cdot 1/3$	45	37.9	Pooled in computing c^2 value
–	+	–	$0.1706\ (2q-q^2) \cdot 1/3$	1	3.6	
–	–	+	$0.0169\ (2q-q^2) + 0.1875$	48	46.6	
–	–	–	$0.0169\ (1-2q+q^2) + 0.0625$	22	18.2	
Total			1.0 ($q = 0.141$)	243	$c^2 = 7.3$, $P > 0.1$ (df = 5)	

[a] 1/3 is a multiplier necessary for estimating the second recombination value by using the estimate of first recombination value. To forget this is a pitfall in computing 2 linkage relations consecutively.

gene (or closely linked). The expected numbers for eight phenotypic classes gave a good fit to the observed numbers when *I-Ps-1* and *I-3* were assumed to be independent, but deviated significantly from the observed numbers when they were assumed to be closely linked, as shown in Table 10.

Table 9. Recombination values obtained between *Ps-1* and 3 other loci.[a]

Cross	Recombination value (%)	Plants (no.)	Genes controlling F_2 phenotypes (showing dominant genes only)
	Ps-1—P (repulsion)		Apiculus color : stigma color
545/532	36.1 ± 14.6	243	*A P—Ps-1 I-Ps-1*
219/532	26.4 ± 6.2	243	*P—Ps-1 I-Ps-1 Ps-3 I-3*
647/532	31.3 ± 14.5	174	*A P—Ps-1 Ps-3*
521/532	31.4 ± 21.9	128	*C A P—Ps-1*
Mean	31.3 ± 3.9 (standard deviation for variation among estimates)		
	Ps-1—Ph (coupling)		Apiculus/stigma color : phenol reaction
219/532	14.9 ± 6.1	243	*P—Ph—Ps-1 I-Ps-1 Ps-3 I-3* (distortion for *Ph:ph* considered)
545/532	14.1 ± 5.7	243	*A P—Ph—Ps-1 I-Ps-1*
Mean	14.5 ± 4.2 (standard error from two standard deviations)		
	Ps-1—Ps-2 (repulsion)		Apiculus color : stigma color
108/532 414/532 (pooled)	23.9 ± 6.8	172	*C P–Ps-2–Ps-1* (distortion for *C:c* considered)
	P–Ps-2 (repulsion) 9.0 ± 5.4	172	*C P—Ps-2—Ps-1* (distortion for *C:c* considered)

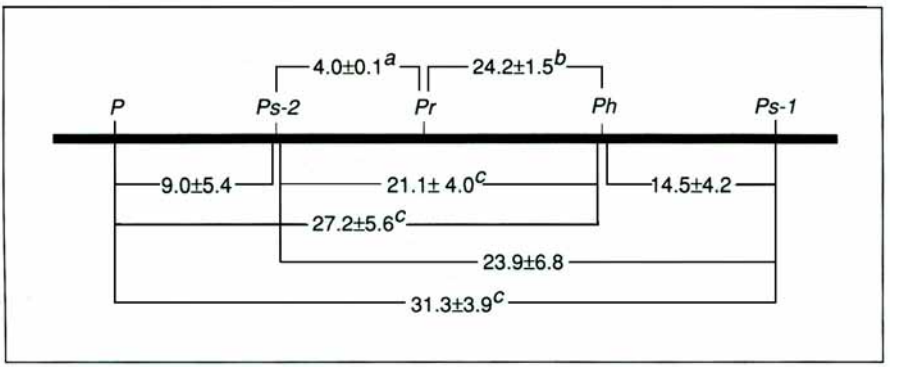
[a] "–" shows linkage.

Table 10. Phenotypic classes of 219/532.

Apiculus	+	+	+	+	−	−	−	−	Total (no.)
Stigma	+	+	−	−	+	+	−	−	
Phenol	+	−	+	−	+	−	+	−	
Observed no.	67	5	77	33	55	2	0 (pooled)	4	243
Expected no., when *I-Ps-1* and *I-3* were assumed:									
Independent	53.2	8.0	90.5	26.8	58.2	3.2	1.1	2.0	$c^2 = 8.3$, $P > 0.1$
Closely linked	35.0	7.0	104.9	27.1	56.5	4.3	1.4	1.8	$c^2 = 41.3$, $P < 0.01$

This comparison shows that the two inhibitors are independent. The *Ps-1–Ph* recombination value was estimated to be 14.9 ± 6.1%. The *P—Ps-1* and *P–Ph* values were also estimated in this cross as shown in Tables 5 and 9. The good fit of expected to observed numbers may serve as a verification of the assumption of relevant genes.

Similarly, the segregation patterns observed in 108/532, 414/532, 521/532, and 647/532 were analyzed. In these crosses, inhibitor *I-Ps-1* does not seem to be involved (it is recessive in both parents). The data from 108/532 and 414/532 were pooled, since they were homogeneous. In these two crosses, stigma color is controlled by *Ps-1* and *Ps-2,* which are linked, *Ps-3* being recessive in both parents. The *Ps-1–Ps-2* recombination value is estimated to be 23.9 ± 6.8%. The *P–Ps-3* recombination is estimated to be 9.0 ± 5.4%. All these genes belong to linkage group II; their sequence is mapped in Figure 1.



1. Mapping of 5 genes in linkage group II.[a]Yen and Hsieh (1968).[b] Takahashi (1964, p. 224).[c] Standard deviation standing for variation among several estimates derived from different crosses.

Discussion

This study has shown that there are three genes for stigma coloration: *Ps-1, Ps-2,* and *Ps-3.* This is in agreement with the description in Kinoshita (1984, p. 30), which is based on a survey of literature by T. Kinoshita. *Ps-1,* a special gene detected in Gaisen-mochi, confers stigma color even when *P* is recessive if *C* and *A* are dominant, as reported by Takahashi (1958). *Ps-2* and *Ps-3* require the dominant combination of *C, A,* and *P* for expressing stigma color. *Ps-2* seems to be relatively frequent in indica varieties (86%; Table 2, group A to 619), while *Ps-3* is common in japonicas (50%; group C to T65), although many colored varieties of indicas and japonicas (groups B and C) have both genes.

This study has also shown that *Ps-1* and *Ps-2* belong to linkage group II and are linked with *Ph* for phenol reaction. In Kinoshita (1984, p. 30) and Committee on Gene Symbolization, Nomenclature, and Linkage Groups (1987, p. 15), *Ps-2* and *Ps-3* are considered to belong to linkage group II, referring to Hsieh (1961) and Yen and Hsieh (1968). But Hsieh gives no such information on *Ps-3,* mentioning, on the contrary, that "presumably, Ps_2 [corresponding to *Ps-3*] is not linked with Ps_1, [corresponding to *Ps-2*] nor with the phenol reaction gene" (Hsieh 1961, p. 128); he reported the linkage of Ps_1 (= *Ps-2*) with *A* (corresponding to *P*) and *Ph.* In this study, also, no linkage was found between *Ps-3* and other genes, and the location of *Ps-3* remains unknown.

Ps-1 in Gaisen-mochi is thought to belong to linkage group V (Kinoshita 1984, p. 30), based mainly on the linkage of *Ps* (= *Ps-1*) with *I-Bf* (inhibitor for brown furrows on glume, Nagao and Takahashi 1963; Takahashi 1964, p. 225). But the recombination value of 42.1±3.3% is too high to demonstrate the linkage decisively. Referring to this linkage, Shastry et al (1975) reported that *Ps* (= *Ps-1*) is also linked with *sd* (*sd-1* in IR20, for semidwarfism), but *sd-1* is known to belong to linkage group III (Committee on Gene Symbolization, Nomenclature and Linkage Groups 1987, p. 17). The present study has clearly shown that *Ps-1* is linked with *Ph* and several other loci belonging to linkage group II.

In this study, inhibitors for stigma color were detected for *Ps-1* and *Ps-3,* but not for *Ps-2.* The inhibitors appeared to be independent of each other and of other coloration genes, although their loci remain unknown. The inhibitor for *Ps-3, I-Ps-3* (or *I-3*), seemed to have a different locus in a few varieties, giving a 1:15 ratio for stigma color in crosses with varieties having *I-3* at the ordinary locus. The dual locations of *I-3* may be regarded as corresponding to $1^{ps}2a$ and $1^{ps}2b$ assumed by Hsieh (1961, p. 129). Furthermore, Hsieh (1960; 1961, p. 87) has considered that there is a set of complementary inhibitors, one of which is carried by T65. In the present study, no critical evidence was obtained for this, but this assumption was adopted for T65 and a few other varieties to account for F_2 ratios that were otherwise not accountable. It seems certain that there are two inhibitors, *I-Ps-3* and *I-Ps-1,* but the assumption of inhibitors beyond these two is still provisional. In this kind of genic analysis, we must elucidate all observed segregation patterns by assuming a minimum number of genes. The web of inhibitors for *Ps-3* is left for more elaborate analysis in the future. It is also an unanswered question why the genes for stigma color are more complicated than those for apiculus color.

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Gene mapping of some morphological traits and chlorophyll deficiency in rice

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Six new loci responsible for morphological traits and chlorophyll deficiency were used for gene mapping. Reduced culm number (*rcn-2*) was located in linkage group II; sheathed panicle (*Shp-1*) in linkage group III; two types of long grain (*lk-i* and *Lk-f*) in linkage groups II and XI+XII, respectively; zebra necrosis (*zn*) in linkage group I; and zebra-6 (*z-6*) in linkage group IV. New linkage relationships were found between ebisu dwarf (*d-2*) and stripe (*st-5*), and between *d-2* and sheathed panicle-2 (*shp-2*) in linkage group II. Furthermore, a new depressed palea (*dp(t)*) locus was found to be linked with *I-Bf* in linkage group V. In addition, gene Ig^a, which is responsible for both short ligule and short auricle, was found to be allelic to *Ig* (liguleless), and the order of dominancy was $Ig^+ > Ig^a >$ Ig.

Refinement of linkage maps is important as a basis for rice genetics and breeding. Both genetic and cytogenetic approaches have been applied to mapping since the construction of 12 groups by Nagao and Takahashi (1963). We are mapping new mutant genes that have been analyzed mainly in our laboratory. We have detected 13 kinds of new genes through the genetic analysis of morphological and physiological traits and the inheritance mode; allelism and inter- or intra-loci interaction are described together with the character expressions of the genes.

Recently, restriction fragment length polymorphism linkage maps have been constructed by two research groups (Kishimoto et al 1989, McCouch et al 1988). An integration of conventional and molecular linkage maps is urgently needed.

Materials and methods

The strains used in this study are presented in Table 1 together with their sources. Character expressions are as follows:

- *Reduced culm number-1.* Number of panicles is extremely reduced, and plant height is about 50% that of A-5 Akamuro, the original variety. The character reverts to normal under high temperature.

- *Reduced culm number-2.* Panicle number is usually reduced to 50% of that of Shiokari, the original variety, and plant height is slightly reduced. The character shows no response to temperature. The gene responsible for this character is different from *rcn-1,* which was previously designated (Takamure and Kinoshita 1985) and is highly sensitive to low temperature.
- *Reduced culm number-3.* Panicle number is extremely low, and plant height is reduced to 50% of that of AC-15, the original strain. The character shows sensitivity to low temperature.
- *Reduced culm number-4.* Panicle number is highly reduced, and plant height is about 70% of that of AC-34, the original strain. The character is influenced by low temperature.
- *Sheathed panicle-1.* This is caused by non-elongated first and second internodes. The panicle is enclosed by the sheaths of the flag leaf and first leaf. The lengths of the internodes, except for the first and second, are normal. A panicle base is not recognizable. This character is dominant.
- *Sheathed panicle-2.* Although this character is almost the same as sheathed panicle-1, the panicle base is discernible. This is a recessive character.
- *"Fusayoshi" long grain.* This character is governed by an incompletely dominant gene. A mutant induced from the variety Shiokari possesses the same gene (Sato et al 1989).
- *"IRATI3" long grain.* The long grain of IRAT13 is caused by a single recessive gene (Takamure and Kinoshita 1986). The homozygote for *lk-i* shows a grain length increase of more than 20% over that of the homozygote.

Table 1. Strains used.

Strain	Phenotype	Gene	Source
		Morphological traits	
N-133	Reduced culm number	*rcn-1*	M_2 plant of γ-ray-treated A-5 Akamuro
N-174	Reduced culm number	*rcn-2*	M_2 plant of ethylmethylsulfonate-treated Shiokari
N-176	Reduced culm number	*rcn-3*	M_2 plant of γ-ray-treated AC-15 (doubled haploid of F_1 [H-59/H-120])
N-175	Reduced culm number	*rcn-4*	M_2 plant of γ-ray-treated AC-34 (doubled haploid of F_1 [A-5/H-69])
H-340	Sheathed panicle	*Shp-1*	Progeny from L-26/A-5 Akamuro
No. 201	Sheathed panicle	*shp-2*	M_2 plant of γ-ray-treated A-5
Fusayoshi	Long grain	*Lk-f*	Local variety in Aomori Prefecture
IRAT13	Long grain	*lk-i*	IRRI accession
MA-85152	Liguleless	lg^a	M_2 plant of EMS-treated Akihikari
H-151	Depressed palea	*dp(t)*	Mutant from A-14 Chabo/E-44 Pirurutong
		Chlorophyll deficiency	
H-61-s	Stripe	*st-5*	Spontaneous mutant of H-61
AC-100	Zebra	*z-6*	Mutant found in the doubled haploid of F_1 (H-50/N-71)
M-52	Zebra necrosis	*zn*	M_2 plant of γ-ray-treated AC-581 (doubled haploid of F_1 [A-5/H-69])

- *Liguleless-a.* The auricle and ligule of this mutant are very short, and the leaf collar becomes narrow.
- *Depressed palea.* H-151 possesses both malformed spikelets and normal spikelets on a single panicle. Malformed spikelets show complete sterility, while normal ones are fertile.
- *Stripe-5.* This mutant has very fine white stripes on the leaf blades, leaf sheaths, and glumes at all growth stages.
- *Zebra-6.* This mutant expresses irregular yellow bands on leaf blades and leaf sheaths at all stages.
- *Zebra necrosis.* This mutant manifests irregular zebra necrosis bands on leaf blades and leaf sheaths.

Recombination values were calculated by the maximum likelihood method (Allard 1956).

Results

The results obtained in this study are reported below.

Reduced culm number

Four reduced culm number mutants were found. Based on inheritance modes and allelism tests among the four, they are apparently governed by different recessive genes, designated *rcn-1, rcn-2, rcn-3,* and *rcn-4.* As shown in Table 2, their expressions were examined in a ricefield (low temperature) and a plastic house (high temperature). The panicle number (PN) in three mutants increased in the plastic house, but did not significantly differ from those of the original strains. The PN of N-174 with *rcn-2* was only 71% of the original strain. N-175 possessing *rcn-4* showed significantly longer

Table 2. Culm length and panicle number in 4 reduced culm number mutants in a ricefield and a plastic house.[a]

		Ricefield		Plastic house	
Strain	Gene	Culm length (cm)	Panicles (no.)	Culm length (cm)	Panicles (no.)
N-133	*rcn-1*	36.2** (48.0)	2.3** (27.4)	73.4 (92.7)	10.0 (104.2)
N-174	*rcn-2*	61.8 (85.7)	6.7** (43.0)	67.1 (100.5)	9.8** (71.0)
N-176	*rcn-3*	55.2** (55.7)	1.9** (16.1)	83.2 (94.2)	4.0 (90.9)
N-175[b]	*rcn-4*	66.0** (69.0)	1.7** (6.7)	86.6** (110.7)	7.0 (86.4)

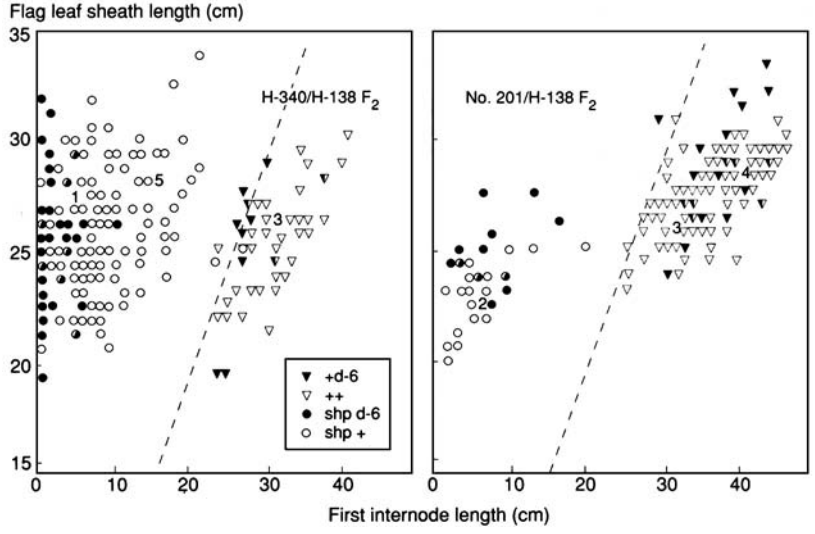
[a]** = significantly different from the original strain at the 1% level. Numbers in parentheses are culm length or panicle number of mutants expressed as percentages of those characters in the original strains. [b]Plant height in N-175 is substituted for culm length.

culm than the original strain; no significant difference was observed between the other mutants and their original strains under high temperature. High sensitivity to low temperature by *rcn-1* had previously been reported (Takamure and Kinoshita 1985). In this experiment, *rcn-2* showed almost no sensitivity to low temperature, but both *rcn-3* and *rcn-4* were highly sensitive.

Takamure and Kinoshita (1985) determined the location of *rcn-1* in linkage group I. Gene *rcn-2* was newly found to be linked with *Ph* and *Pr* in linkage group II, while the locations of *rcn-3* and *rcn-4* remain unknown.

Sheathed panicle

To examine the expression of sheathed panicle in H-340 and No. 201, the relationship between the uppermost internode length (In 1) and flag leaf sheath length (Ls 1) in the F_2s of crosses No. 201/H-138 and H-340/H-138 are presented in Figure 1. Sheathed panicle is dominant over normal panicle in the F_2 of H-340/H-138, while a single recessive gene is responsible for F_2 segregation of sheathed panicle in No. 201/H-138. For an allelism test between two sheathed panicles, H-340 was crossed with No. 201. The two types of sheathed panicle were not discerned because of a similar visible character in the F_2 population. However, the F_2 population was grouped into sheathed panicle type (278 plants) and normal type (58 plants). This segregation ratio was in good agreement with the 13:3 estimate based on the independent relationship of dominant and recessive genes. It was assumed that the sheathed panicle genes in H-340 and No. 201 differed from other sheathed panicle genes reported hitherto by Sethi et



1. Relation between uppermost internode length and flag leaf sheath length In F_2 of crosses between H-340 (*Shp-1*) and H-138 (+), and between No. 201 (*Shp-2*) and H-138. Numbers indicate mean of H-340 (1), No. 201 (2), H-138 (3), F_1 of No. 201/H-138 (4), and F_1 of H-340/H-138 (5).

Table 3. Linkage between sheathed panicle (*shp-2*) and ebisu dwarf (*d-2*) belonging to linkage group II.

Marker	Linkage phase	Normal		Sheathed		Total	Recombination value (%)	Goodness of fit	
		Dominant	Recessive	Dominant	Recessive			c^2	P
d-2		255	122	120	9	506		33.7[a]	<0.001
	Repulsion	261.4	118.1	118.1	8.4		25.7 ± 4.1	0.4	0.50-0.75
lg	Repulsion	289	88	93	36	506	>50.0	1.2[a]	0.25-0.50

[a]Chi-square value for 9:3:3:1.

al (1937), Jones (1952), and Heu and Shrestha (1983, 1986), based on F_2 segregations. Therefore, the new gene involved in H-340 was designated *Shp-1,* and that in No. 201 was designated *shp-2.*

Shp-1 was found to be closely linked with *A* and *Rd* in linkage group III. On the other hand, a new linkage relationship between *shp-2* and *d-2* was estimated in linkage group II, though no linkage relationship between *shp-2* and either *lg, P,* or $P1^i$ was observed in linkage group II (Table 3).

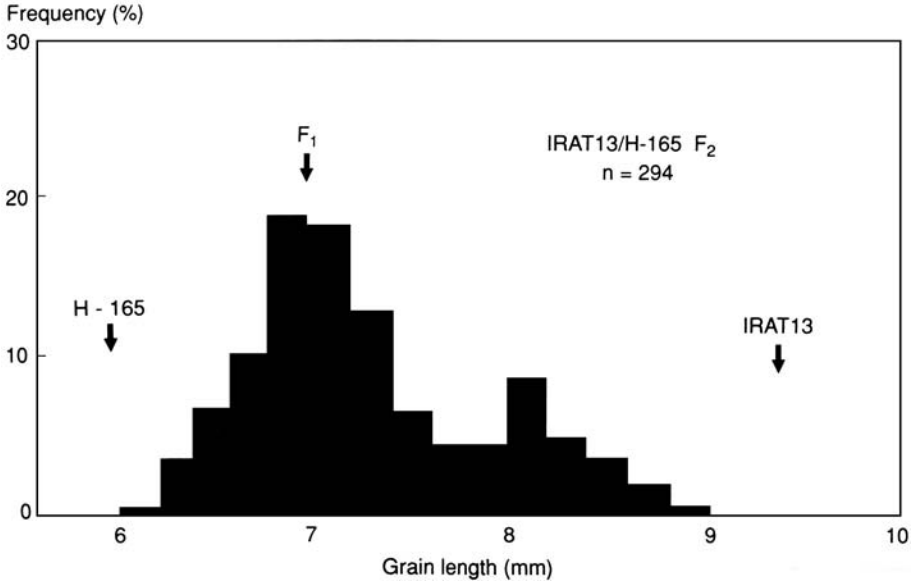
Long grain

Takeda and Saito (1980) reported that long grain in Fusayoshi is controlled by the major gene *Lk-f,* which is linked with the awning gene. *Lk-f is* also linked with *Mi* (Takamure and Kinoshita 1983), *bc-1, lhs-1,* and *dl* belonging to linkage group XI+XII.

From the F_2 of IRAT13/H-165, it was deduced that long grain in IRAT13 is governed by a major gene. The grain size of the F_1 plants was intermediate; in the F_2 population, normal grain type was dominant over long grain type (Fig. 2). F_2 segregation for long grain fitted the single recessive gene model (Table 4). However, considerable variation existed in F_2 grain size, and minor genes might also be responsible for grain size. In an allelism test for long grain between IRAT13 and Fusayoshi, F_1 plants showed grain size similar to that of Fusayoshi, and continuous variation of grain size beyond the parents was observed. From this, it was deduced that the long-grain gene in IRAT13 is different from that in Fusayoshi. The long-grain gene in IRAT13 was designated *lk-i;* it was found to be linked with *Ph* and *Pr* belonging to linkage group II.

Liguleless

When MA-85152 with short ligule and short auricle was crossed with normal strains, the F_2 segregation ratio of normal vs short ligule fit a monogenic segregation, indicating that short ligule is recessive (Table 5). On the other hand, F_1 plants of crosses between MA-85152 and liguleless strains expressed short ligule and short auricle, as shown in Table 5. In the F_2 and BC_1 populations, no normal-ligule plants were observed, and the populations segregated into short ligule and liguleless at the ratios of 3:1 and 1:1, respectively (Table 5). These results suggest that short ligule is dominant



2. Frequency distribution of grain length in F_2 of cross between IRAT13 (long grain) and H-165 (normal).

Table 4. F_2 segregation for grain type in IRAT13/H-165.

Grain type			Goodness of fit		
Normal	Long	Total	Ratio	c^2	P
219	75	294	3:1	0.0	0.75–0.90

Table 5. F_2 and BC_1 segregations for short ligule in crosses between MA-85152 and normal strains and between MA-85152 and liguleless strains.

Cross	Phenotype of F_1	Ligule				Goodness of fit (3:1)	
		Normal	Short ligule	Liguleless	Total	c^2	P
MA-85152/normal strain	Normal	1232	456		1688[a]	3.7	0.05–0.10
MA-85152/liguleless strain	Short		981	328	1309[b]	0.0	0.90–0.95
MA-85152/liguleless strain//liguleless strain			278	261	539[c]	0.5[d]	0.25–0.50

[a]Pooled data of 7 F_2 populations. [b]Pooled data of 5 F_2 populations. [c]Pooled data of 3 F_2 populations. [d] Chi-square value for 1:1.

Table 6. Linkage between short ligule and *Pl* (purple leaf) in F_2 of H-126/MA-85152.

	Normal		Short		Total	Recombination value (%)	Goodness of fit (9:3:3:1)	
	Pl	+	*Pl*	+			c^2	P
Observed	76	17	15	14	122		10.3	0.01–0.025
Calculated	75.4	16.1	16.1	14.4		31.4±5.3	0.1	0.50–0.75

Table 7. Linkage between depressed palea and dark furrows (*I-Bf*) found in F_2 of Shiokari/H-151.

Marker	Linkage phase	Normal palea		Depressed palea		Total	Recombination value (%)	Goodness of fit	
		Normal	Furrowed	Normal	Furrowed			c^2	P
I-Bf		202	12	16	58	288		158.2[a]	<0.001
	Coupling	202.1	13.9	13.9	58.1		10.2±1.9	0.6	0.25-0.50

[a]Chi-square value for 9:3:3:1.

over liguleless and is allelic to gene *lg.* Furthermore, the recombination value of 31.4% between short ligule and purple leaf (*Pl*) in linkage group II was calculated in the F_2 of H-126/MA-85152 (Table 6). This result also supports the hypothesis that short ligule is one of the alleles at the *lg* locus, since the recombination value is close to that between *Pl* and *lg* (Kinoshita and Takamure 1986). Consequently, the gene responsible for short ligule and short auricle was designated lg^a, with dominance $lg > lg^a > lg$.

The presence of the auricleless gene that causes the same character expression as lg^a was reported by Librojo and Khush (1986), who demonstrated that the mutant with *aul* lacked auricles, and that *aul* was nonallelic to *lg.* However, it was assumed that *aul* belonged to linkage group II. Since *aul* and lg^a manifested a similar character, further allelism tests are needed to confirm the possibility of structural change between both materials.

Depressed palea

F_1 plants of H-151/Shiokari showed normal palea, and the F_2 population segregated into normal and depressed palea at a ratio of 3:1. Thus, depressed palea is controlled by a single recessive gene, and the gene was found to be linked with *I-Bf* with recombination value 10.4% in linkage group V, as shown in Table 7. Iwata and Omura (1971) reported that depressed palea-2 (*dp-2*) is located in linkage group VII. Although depressed palea in H-151 is distinguishable from depressed palea-2 by sterile spikelets with depressed palea, the gene was tentatively designated *dp(t).*

Chlorophyll deficiency

Chlorophyll deficiencies have been used as useful markers because of expression at the young seedling stage. A single gene, *st-5,* is responsible for stripe in H-61-s and is

Table 8. Linkage between stripe (*st-5*) and 4 markers belonging to linkage group II.

Marker	Linkage phase	Normal		Stripe		Total	Recombination value (%)	Goodness of fit	
		Dominant	Recessive	Dominant	Recessive			χ^2	P
d-2		747	150	134	155	1186[a]		156.5[c]	<0.001
	Coupling	747.5	141.9	141.9	154.6		27.8±1.6	0.9	0.75–0.90
lg	Repulsion	150	60	52	17	279	47.1±4.6	1.4[c]	0.50–0.75
P	Coupling	347	114	86	40	587[b]	45.4±2.9	6.6[c]	0.05–0.10
Pl	Coupling	36	215	9	48	308	>50.0	10.6[d]	0.01–0.05

[a]Pooled data of 4 F_2 populations. [b]Pooled data of 2 F_2 populations. [c]Chi-square value tor 9:3:3:1. [d]Chi-square value for 9:39:3:13.

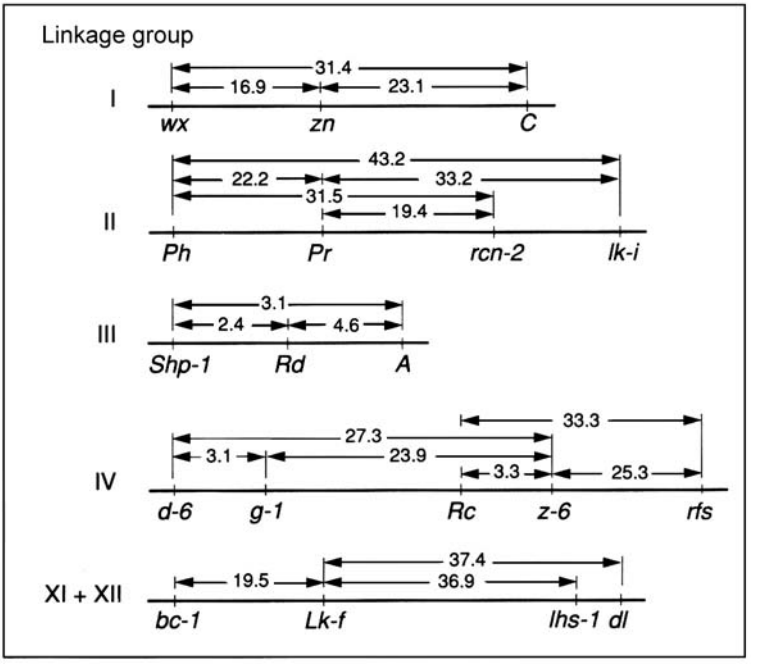
linked with *d-2* with a recombination value of 27.8% in linkage group II (Table 8). Zebra-6 (*z-6*), induced during anther culture, was found to be linked with *d-6, g-1, Rc,* and *rfs* in linkage group IV. Furthermore, zebra necrosis (*zn*) was detected to be linked with *wx* and *C* in linkage group I.

Linkage relationships

Linkage relationships found between the abovementioned mutant genes and marker genes are shown in Figure 3. Gene *zn* is located in the order *wx - zn - C* in linkage group I. The order of *rcn-2* and *lk-i* is estimated to be *Ph - Pr - rcn-2 - lk-i* in linkage group II. However, the recombination value between *rcn-2* and *lk-i* was not obtained. *Shp-1* is determined in the array *Shp-1 - Rd - A,* based on the recombination values of 2.4% between *Shp-1* and Rd and 3.1% between *Shp-1* and *A,* although the recombination value between *Rd* and *A* was slightly larger than the 0.3% found by Nagao and Takahashi (1963). Gene *z-6* is arranged in the sequence *d-6 - g-1 - Rc - z-6 - rfs. Lk-f* is mapped in the series *bc-1 - Lk-f - lhs-1 - dl* in linkage group XI+XII, based on the recombination values of 36.9% between *Lk-f* and *lhs-1* and 37.4% between *Lk-f* and *dl,* although the exact array of *lhs-1* and *dl* remains unknown because of the very close linkage between the two genes (Khush and Singh 1986).

Discussion

In this study, six new mutant genes were mapped in linkage groups I, II, III, IV, and XI+XII, as described in Figure 3. Additionally, *shp-2* and *st-5* were found to be linked with *d-2* in linkage group II, although only the linkage relationship between *d-3* and *d-2* is known. The linkage between *dp(t)* and *I-Bf* was estimated in linkage group V, which is poorly mapped. In 1987, it was reported that *lam(t)* for low amylose content endosperm is closely linked with *I-Bf* (Kikuchi and Kinoshita 1987). Thus, these genes are expected to be applicable as useful new marker genes to complete linkage groups II and V.



3. Linkage relationships between new genes and linkage markers.

On the other hand, with the recent development of the analytic methods of molecular genetics, linkage maps of restriction fragment length polymorphism in rice were constructed by McCouch et al (1988) and Kishimoto et al (1989). If combined linkage maps of molecular markers and conventional genetic markers are established (Muehlbaner et al 1988), the combined maps will be quite useful in breeding programs, and the applications of the combined linkage maps should permit studies of gene expression and regulation to develop rapidly.

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Linkage relationships between genetic male sterile and marker genes in rice

H.S. Suh, J.D. Kim, M.H. Heu, and G.S. Chung

Identification of more sources of genetic male sterility would be helpful for hybrid rice breeding. We studied the linkage relationships between the genetic male sterile (GMS) genes of the spontaneous male sterile rices Milyang 67ms (japonica) and Milyang 77ms (Tongil type), and marker genes. The male sterile rices were crossed with linkage testers having marker genes. From the F_2s, the GMS gene of Milyang 67ms, *ms-m67(t),* appeared to be linked with marker genes *lax,* eg, *d-10,* and *A,* all belonging to linkage group III, with recombination values of 0, 13.7, 23.6, and 34.0%, respectively. The locus of *ms-m67(t)* was arranged in the sequence *eg - ms-m67(t) - d-10 - A* and was linked completely with *lax.* The GMS gene of Milyang 77ms, *ms-m77(t),* was linked with the marker gene *mp-1* with a recombination value of 14.9%.

Since genetic male sterile (GMS) rices sensitive to photoperiod were reported by Chinese scientists (Li et al 1988, Lu and Wang 1988), increasing interest has been focused on GMS rice. GMS rices have been developed mostly by artificial treatment (Fujimaki and Hiraiwa 1986; Fujimaki et al 1977; Ko and Yamagata 1980, 1987; Singh and Ikehashi 1981), and a few by spontaneous mutation (Lu and Wang 1988, Suh et al 1989).

Close linkage between GMS genes and marker genes in rice could be used for identifying homozygous male sterile individuals from heterozygous progenies. Information on linkage between GMS genes and marker genes has been reported by only a few researchers (Ko and Yamagata 1985).

Suh et al (1989) reported four GMS genes: *ms-ir36(t)* from IR36ms, *ms-m67(t)* from Milyang 67ms, *ms-m77(t)* from Milyang 77ms, and *ms-m55(t)* from Milyang 55ms. The present study investigated linkage relationships between the GMS genes of Milyang 67ms and Milyang 77ms, and marker genes.

Materials and methods

Two spontaneous male sterile rices, Milyang 67ms and Milyang 77ms, were tested. The japonica Milyang 67ms was crossed with 20 japonica marker lines obtained from N.

Iwata, Kyushu University: F1 60, F1 85, F1 204, F1 214, F1 215, F1 217, F1 222, F1 232, F1 233, F1 240, F1 244, F1 260, F1 283, F1 288, F1 293, F1 297, F1 304, F1 310, F1 312, and F5 814. Tongil-type Milyang 77ms was crossed with 10 Tongil-type marker lines (Suh and Heu 1978): 1001, 1003, 1005, 1006, 1007, 1009, 1010, 2021, 1023, and 1036, and with IR42667-36-mpl having the *mp-1* gene. Linkage relationships were investigated from the F_2s. Recombination values were calculated by the minimum discrepancy method (Murty 1954).

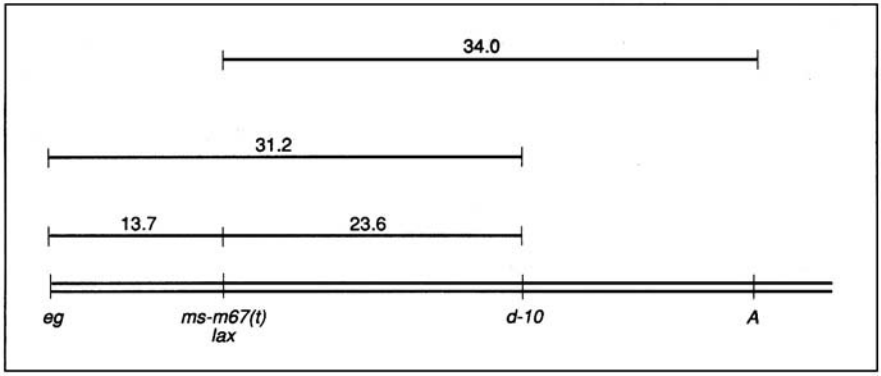
Male sterility of Milyang 67ms

The linkage relationships between the GMS gene of Milyang 67ms and the marker genes belonging to linkage group III are shown in Table 1. Of 22 marker genes (linkage group given in parentheses)— *C* (I), *1g* (II), *nal-1* (II), *eg* (III), *lax* (III), *d-10* (III), *A* (III), *d-18* (III), *fs-2* (III), *spl-5* (IV), *rfs* (IV), *spl-7* (VI+IX), *nl-1* (VI+IX), *la* (VIII), *d-k-4* (X), *ch-1* (XI), *ch-2* (XI), *ch-3* (XI), *fc-1* (XI), *dl* (XI), *fl* (fgl), and *rk-2* (fgl)— *eg,* lax, *d-10,* and *A* belonging to linkage group III, from marker lines F1 204 and F1 297, appeared to be linked with *ms-m67(t).* The other markers tested were independent of *ms-m67(t).* Complete linkage was found between *ms-m67(t)* and the *lax* (lax panicle) gene in the repulsion phase. Relatively close linkages were found between *ms-m67(t)* and *eg* (extra glume), *d-10* (dwarf-10), and *A* (anthocyanin activator), with recombination values of 13.7, 23.6, and 34.0%, respectively. Marker genes *d-18* (dwarf-18) and *fs-2* (fine stripe-2) of linkage group III were independent of *ms-m67(t).* Recombination values between *eg* and *d-10, d-10* and *A,* and *eg* and *A* were calculated as 31.2, 47.2, and 50.5%, respectively. From these results, we can assume that *ms-m67(t)* is located on linkage group III in the sequence *eg-ms-m67(t) - d-10 - A,* and is located distantly from *d-18* and *fs-2* (Fig. 1).

Table 1. Linkage relationships between the male sterility gene *ms-m67(t)* of japonica male sterile rice Milyang 67ms and marker genes *eg* (extra glume), *lax* (lax panicle), *d-10* (dwarf-10), A (anthocyanin activator), *d-78* (dwarf-18), and *fs-2* (fine stripe-2) belonging to linkage group III.

Genes combined		Linkage phase	F_2 phenotype					c^{2a}	Recombination value (%)
A	B		AB	Ab	aB	ab	Total		
ms-m67(t)	*eg*	Repulsion	449	211	216	4	880	80.15**	13.7
ms-m67(t)	*lax*	Repulsion	389	204	208	0	801	100.00**	0.0
ms-m67(t)	*d-10*	Repulsion	455	205	208	12	880	57.75**	23.6
ms-m67(t)	*A*	Coupling	402	258	93	127	880	23.29**	34.0
ms-m67(t)	*d-18*	–	214	66	68	19	367	1.07	Independent
ms-m67(t)	*fs-2*	–	214	66	71	16	367	2.54	Independent
d-10	*eg*	Coupling	568	115	117	100	880	76.70**	31.2
d-10	*A*	Coupling	347	316	148	69	880	16.62**	47.2
eg	*A*	Coupling	352	313	143	72	880	12.14**	50.5

[a] ** = significant at the 1% level.



1. Linkage map of 5 genes including male sterility gene *ms-m67(t)* of male sterile rice Milyang 67ms.

Table 2. Linkage relationships between the male sterility gene *ms-m77(t)* of Tongil-type rice Milyang 77ms and marker genes *C* (chromogen), *Pr* (purple hull), *A* (anthocyanin activator), *Rc* (brown pericarp), *gh-1* (gold hull-1), *nl-1* (neck leaf-1), gl-1 (glabrous-1), *Hg* (hairy glume), and *mp-1* (multiple pistil-1).

Genes combined		F_2 phenotype					c^{2a}	Recombination value (%)
A	B	AB	Ab	aB	ab	Total		
ms-m77(t)	*C*	148	134	54	38	374	1.892	Independent
ms-m77(t)	*Pr*	148	130	60	49	387	3.142	Independent
ms-m77(t)	*A*	430	142	148	54	774	0.839	Independent
ms-m77(t)	*Rc*	211	82	76	28	387	2.179	Independent
ms-m77(t)	*gh-1*	217	66	85	19	387	3.838	Independent
ms-m77(t)	*nl-1*	230	59	68	30	387	4.924	Independent
ms-m77(t)	*gl-1*	228	63	63	34	388	6.969	Independent
ms-m77(t)	*Hg*	197	81	81	28	387	4.509	Independent
ms-m77(t)	*mp-1*	224	94	101	2	421	32.224**	14.9

[a] ** = significant at the 1% level.

Iwata et al (1984) reported that the *spl-6* (spotted leaf-6) and *ch-5* (chlorina-5) genes are closely linked with *lax*. Close linkage is expected between *ms-m67(t)* and *spl-6* or *ch-5*, which should be tested. If we could identify seedling marker genes located close to the GMS gene, they would be helpful in identifying male sterile individuals from heterozygous progenies.

Male sterility of Milyang 77ms

The linkage relationships between the GMS gene *ms-m77(t)* of Milyang 77ms and nine marker genes—*C* (I), *Pr* (II), *A* (III), *Rc* (IV), *gh-1* (VI+IX), *nl-1* (VI+IX), *gl-1* (XI+XII), *Hg* (XII), and *mp-1* (III or VIII)—are shown in Table 2. The *mp-1* (multiple pistil-1) gene was linked with *ms-m77(t)* with a recombination value of 14.9%; the 8 other marker genes were independent of *ms-m77(t)*.

Librojo and Khush (1986) reported that *mp-1* is located in chromosome 1; however, Heu et al (1987) reported that the multiple pistil originating from Double Rice is identical to those of Nepali varieties Laila Majanu and Sautenia Dhan, and is linked with the *la* (laziness) gene of linkage group VIII with a recombination value of 29%. Further studies are necessary to identify the linkage group of *mp-1* as well as that of *ms-m77(t).*

Almost no seedling marker genes have been reported to be linked with *ms-m77(t)* and *mp-1*. Further studies to find seedling marker genes linked closely with *ms-m77(t)* would be useful for hybrid rice breeding.

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Notes

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Linkage analysis and application of isozyme genes in rice

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Linkage analysis of seven rice isozyme genes is presented. *Pgd-2* showed linkage to *Est-2* and *Pgi-2,* which belong to linkage group I. *Pgd-1* and *Adh-1* were found to be linked to *la* (lazy habit) and *v-4* (virescent leaf) in linkage group VIII. *Pgi-1* showed linkage to *chl-1* (chlorina) and a gene for glutamate dehydrogenase (*Gdh-1*). *Acp-1* and *Pox-2* showed linkage to *d-33* (bonsaito dwarf). In total, 23 genes were allocated to 9 rice chromosomes, and 12 of them were mapped on conventional linkage maps. The expression and stability of 15 isozyme genes in calli were investigated; at least 8 proved useful as gene markers at the cellular level. Isozymes could serve as useful gene markers in genetic studies at the plant and cellular levels, as well as for uniting conventional linkage and restriction fragment length polymorphism linkage maps.

Isozyme work in rice, initiated in the mid-1960s, has been used in studies of evolution, gene regulation, and developmental genetics, reviewed by Endo and Morishima (1983). Inheritance of isozymes has also been investigated in 14 enzyme species, and 39 genes have been recognized (Table 1) (Ishikawa et al 1987,1988; Morishima and Glaszmann 1986; Ranjhan et al 1988; Wu et al 1988). Among these isozyme genes, locations on linkage maps have been determined only for *Amp-3, Pgi-2,* and *Est-2,* which belong to linkage group I (Nakagahra and Hayashi 1976, Sano and Barbier 1985). Mapping of these isozyme genes on conventional and restriction fragment length polymorphism (RFLP) linkage maps is needed to use them as markers for genetic study and plant breeding.

Isozyme genes could be useful gene markers not only at the plant level but also at the cellular level. For instance, they could be used to select somaclonal hybrids produced by cell fusion or to detect somaclonal variations. Somaclonal variations that are detected by morphological changes in regenerated plants may be more efficiently studied at the callus level by using isozyme markers. However, isozyme genes are well known for having stage- or organ-specific expression. Therefore, the stability of gene expression at the cellular level should be examined at the respective loci during subculture.

Table 1. Rice isozyme genes and corresponding chromosomes.

Enzyme species	Gene type	Trisomic	Linkage group
Acid phosphatase (E.C.3.1.3.2)	*Acp-1*	A	d-33
	ACP-2	A	d-33
	ACP-3		
	ACP-4		
Alcohol dehydrogenase (E.C.1.1.1.1)	*Adh-1*	G	VIII
	Adh-2		
Aminopeptidase (E.C.3.4.11.-)	*Amp-1*	N	X
	Amp-2	D	*sug*
	Amp-3	B	I
	Amp-4	D	*sug*
Catalase (E.C.1.11.1.6)	*Cat-1*	B	I
Endopeptidase (E.C.3.4.22.-)	*Enp-1*	B	I
Esterase (E.C.3.1.1.-)	*Est-1*		
	Est-2	B	I
	Est-3	H	VII
	Est-4		
	Est-5	O	III
	Est-6		
	Est-7		
	Est-8		
	Est-9	F	IV
Glutamate dehydrogenase (E.C.1.4.1.2)	*Gdh-1*	M	XI+XII
Aspartate amino transferase (E.C.2.6.1.1)	*Got-1*	O	III
	Got-2	B	I
	Got-3		
Isocitrate dehydrogenase (E.C.1.1.1.37)	*Icd-1*	O	III
Malate dehydrogenase (E.C.1.1.1.37)	*Mdh-1* (NAD)		
(E.C.1.1.1.40)	*Me-1* (NADP)		
Phosphogluconate dehydrogenase (E.C.1.1.1.43)	*Pgd-1*	G	VIII
	Pgd-2	B	I
Phosphoglucose isomerase (E.C.5.3.1.9)	*Pgi-1*	M	XI+XII
	Pgi-2	B	I
	Pgi-3		
Peroxidase (E.C.1.11.1.7)	*Pox-1*		
	Pox-2	A	*d-33*
	Pox-3		
	Pox-4		
	Pox-5	B	I
Shikimate dehydrogenase (E.C.1.1.1.25)	*Sdh-1*	A	*d-33*

In this paper, we first present linkage data for seven isozyme genes located in three linkage groups. The expression of 15 isozyme genes is then examined at the cellular level, and examples of somaclonal variations occurring during subculture are described.

Materials and methods

Ten cultivars including three linkage testers (*Oryza sativa*), and an F_4 plant derived from the cross *O. sativa/O. rufipogon* were used for linkage analysis (Table 2). F_2 populations derived from eight crosses listed in Table 3 were examined. Samples were prepared using the upper parts of plumules and mature leaves. Isozyme assay was by the method of Ishikawa et al (1989). Recombination values were calculated using the formula given by Allard (1956).

For the study of cellular expression of isozyme genes, calli were induced from seeds of four strains—IR36 (indica), FL 47, KL 609, and Acc 224 (japonica)—and F_1 hybrids of three crosses (Acc 224/IR36, FL 47/IR36, and IR36/KL 609). After 2 mo, calli were cut off from respective seeds and transferred to fresh Murashige and Skoog's medium. They were maintained in the light at 25°C by transferring to fresh medium every month. The first 3 mo from callus induction was called the initial stage, and the stage after that the late stage.

The calli were examined every month for the 15 isozymes listed in Table 4. At 12 mo after callus induction, cell lines from 2 F_1 hybrids (FL 47/IR36 and IR36/KL 609) were each divided into about 100 subcell lines for examination of the expression of 5 isozyme loci at which parents of the critical crosses carry different alleles (*Amp-2, Cat-1, Pgi-1, Pgi-2,* and *Sdh-1*).

Table 2. Materials used in linkage analysis.

Cultivar[a]	Remarks[b]
IR36	Indica
FL 27	Japonica, linkage tester (*d-33, d1*)
FL 280	Japonica, linkage tester *(la, v-4*)
FL 330	Japonica, linkage tester (*chl-1, v-8*)
Acc 001	Indica
Acc 435	Indica
Acc 437	Indica
Acc 224	Japonica
Acc 259	Japonica
Acc 647	Japonica
F_4 plants	F_4 progeny derived from Acc 504/W120 (*Oryza rufipogon*)

[a] Acc = accession number at the National Institute of Genetics, Japan. [b] Linkage testers from Kyushu University.

Table 3. F_2 segregations and recombination values for 9 isozyme genes.

Cross	Aa:Bb	Recombination value (%)	F_2 segregation									c^2 (fitness due to recombination value)[a]
			AABB	AABb	AAbb	AaBB	AaBb	Aabb	aaBB	aaBb	aabb	
F_4/IR36	*Pgd-2:Pgi-2*	29.6±4.59	6	9	3	6	22	6	1	10	14	6.13 (0.5<p<0.7)
	Pgd-2:Est-2	24.2±4.10	7	9	2	6	23	5	2	5	18	9.38 (0.3<p<0.5)
	Est-2:Pgi-2	9.6±2.59	11	4	0	2	32	3	0	5	20	4.75 (0.7<p<0.8)
Acc 001/Acc 259	*Gdh-1:Pgi-1*	8.4±1.58	33	7	0	5	76	5	1	8	34	3.53 (0.8<p<0.9)
Acc 224/Acc 647	*Adh-1:Pgd-1*	19.4±3.32	21	9	1	9	25	5	1	6	15	5.75 (0.5<p<0.7)
			A-BB	A-Bb	A-bb	aaBB	aaBb	aabb				
FL 27/Acc 435	*Pox-2:Acp-1*	23.9±3.59	46	78	19	3	12	21				2.78 (0.7<p<0.8)
	d-33:Acp-1	29.2±3.93	47	74	24	2	16	16				4.19 (0.5<p<0.7)
FL 280/IR36	*la:Pgd-1*	14.2±4.89	13	27	3	2	1	12				12.51 (0.02<p<0.05)
	v-4:Pgd-1	5.4±3.03	15	27	2	0	1	13				0.40 (0.9<p)
FL 280/Acc 437	*v-4:Adh-1*	26.8±5.27	27	33	6	3	11	12				5.47 (0.3<p<0.5)
FL 330/IR36	*chl-1:Pgi-1*	27.2±3.24	64	87	28	8	21	39				6.52 (0.2<p<0.3)
			A-B-	A-bb	aaB-	aabb						
FL 27/Acc 435	d-33:Pox-2	7.6±2.07	138	7	5	29						3.43 (0.3<p<0.5)

[a]Significant at the 5% level.

Table 4. Expression of 15 isozyme genes in plant tissues and in stages of callus, and allelic expression in calli derived from F_1 hybrids.[a]

Locus	Whole plant		Callus stage			Allelic expression in calli derived from F_1 hybrids
	Plumule	Mature leaf	Initial	Late	Expression type[b]	
Acp-1	+	+	+	–	3	–
Adh-1	+	+	+	+	1	+
Adh-2	–	–	+	+	1	NT
Amp-1	+	+	+	+	1	NT
Amp-2	+	–	+[m]	+[m]	2	+
Amp-3	+	+	+[m]	+[m]	2	+
Cat-1	+	–	+	+	1	+
Est-2	+	+	+	+	1	+
Est-5	+	+*	+*	+*	3	NT
Est-9	+	–	+	–	3	–
Pgd-1	+	–	–	–	3	–
Pgd-2	+	+	+	+	1	NT
Pgi-1	+	+	+	+	1	+
Pgi-2	+	+	+	+	1	+
Sdh-1	+	+	+	+	1	+

[a] + = expression present, +[m] = expression present but modified, +* = one of the pair bands absent, – = expression absent, NT = not tested. [b] Expression type 1 = stable expression, 2 = modified expression, 3 = nonexpression or unstable expression during subculture.

Results

First, the linkage relationships of isozyme genes on the conventional linkage map were examined to establish their genetic basis. Relationships were found among nine isozyme genes and four morphological markers on the respective chromosomes.

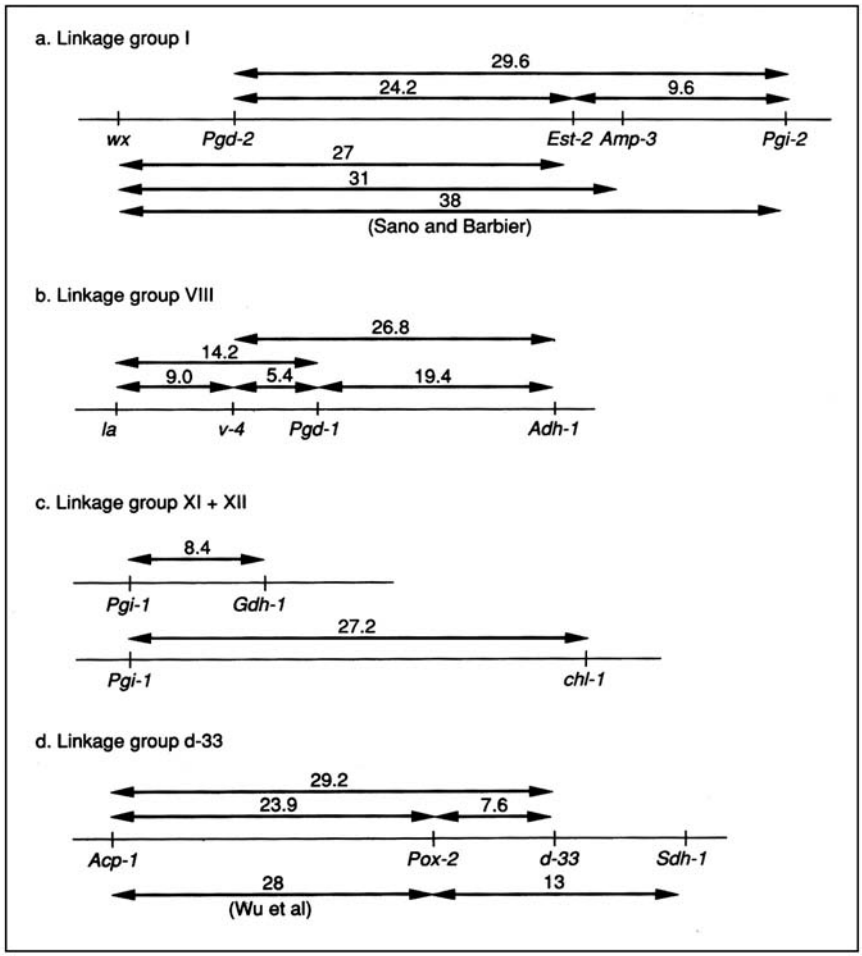
Linkage analysis

Analyses of four linkage groups are presented here.

Linkage group I. Phosphogluconate dehydrogenase has two bands controlled by *Pgd-1* and *Pgd-2.* A variant allele at *Pgd-2* was found in wild rice (*O. rufipogon* strain W120). An F_4 plant derived from Acc 504/W120 was crossed with IR36. In the F_2 population, 15 isozyme genes were segregated simultaneously. Locus *Pgd-2* was found to be linked to *Amp-3, Pgi-2,* and *Est-2.* It showed recombination values of 29.6±4.59% for *Pgi-2* and 24.2±4.1% for *Est-2* (Table 3). *Pgi-2* was linked to *Est-2* with a recombination value of 9.6±2.59%. The order of these three isozyme genes was confirmed as *Pgi-2 - Est-2 - Pgd-2* (Fig. 1a). *Pgd-2* may be located near the *wx* locus.

Linkage group VIII. Adh-1 and *Pgd-1* were assigned to linkage group VIII through trisomic analysis (Ishikawa et al 1986, Ranjhan et al 1988) and were linked with a recombination value of 19.4±3.32% (Ishikawa et al 1989).

In the cross FL 280/IR36, *Pgd-1* showed linkages with two marker genes, *la* (lazy growth habit) and *v-4* (virescent leaf). The recombination values were 14.2±4.89% for *la* and 5.4±3.03% for *v-4* (Table 3). In the cross FL 280/Acc 437, *Adh-1* was also found



1. Linkage maps of 11 isozyme genes.

to be linked to *v-4* with a recombination value of 26.8±5.27%, but not to *la.* It is known that la is linked to *v-4* with a recombination value of 11%. Based on these results, the order of these genes is assumed to be *la - v-4 - Pgd-1 - Adh-1* (Fig. 1).

Linkage group XI+XII. Acc 259 was found to have a rare allele for glutamate dehydrogenase, which is encoded by *Gdh-1.* In an F_2 population of Acc 001/Acc 259, eight isozyme genes segregated simultaneously. *Pgi-1* showed a linkage relation with *Gdh-1,* with a recombination value of 8.4±1.58 (Table 3).

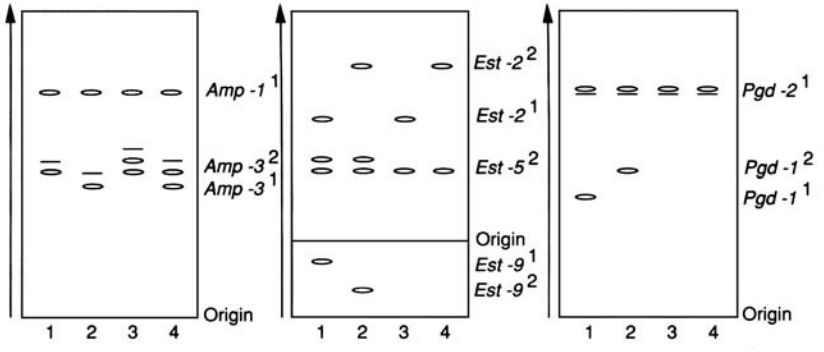
In the cross FL 330/IR36, *Pgi-1* also showed linkage with *chl-1* (chlorina), which is located on linkage group XI+XII. The recombination value was 27.2±3.24% (Table 3). The linkages between *Pgi-1* and the other loci near *chl-1* are under study. Therefore, *Pgi-1* and *Gdh-1* are considered to belong to linkage group XI+XII (Fig. 1c).

Linkage group d-33. In the cross FL 27/Acc 435, both *Acp-1* and *Pox-2* showed linkages with *d-33* (bonsaito dwarf), with recombination values of 29.2±3.93% and 7.6±2.07%, respectively (Table 3). The recombination value between *Acp-1* and *Pox-2* was estimated as 23.9±3.59%, which was lower than the value obtained by Wu et al (1988), possibly because of the distorted segregations at these isozyme loci that are frequently observed in indicaljaponica crosses. The order of these three genes is estimated as *Acp-1 - Pox-2 - d-33* (Fig. 1d).

Cellular expression

At first, the cellular expression of 15 isozyme genes was surveyed. Zymograms obtained from plants and calli derived from japonica and indica strains are shown in Figure 2. Based on the stability of the bands during the callus stage, the isozyme loci examined were classified into three types: a) stable expression type, b) modified expression type, and c) nonexpression or unstable expression type (Table 4). Nine genes were classified as type 1 and two—for aminopeptidase—as type 2. Four genes—*Acp-1, Est-5, Est-9,* and *Pgd-1*—were type 3. In *Acp-1,* three major and minor bands were found at the initial stage, though these bands became obscure at the late stage (about 3 mo after callus induction). *Est-5* lost one of the pair bands during subculture. In *Est-9,* expression was found at the initial stage, but not at the late stage. The band of *Pgd-1* was not observed throughout all callus stages, while that of *Pgd-2* was. Thus, the patterns of expression in calli differed according to their respective genes.

Gene expression in cell lines of F_1 hybrids was surveyed to determine whether both alleles derived from the parents were equally expressed during subculture. Expression of both alleles was consistently found at 8 of 11 loci, which were classified as types 1 and 2 (Table 4). Their stable expression continued for 1 yr after callus induction. These genes may be available as genetic markers at the cellular level. About 100 subcell lines were also surveyed for isozyme expression of 5 genes—*Amp-2, Cat-1, Pgi-1, Pgi-2,* and *Sdh-1*—which were heterozygous in the initial hybrids. Two subcell lines of FL 47/IR36 and five of IR36/KL 609 showed loss of one of the parental bands, as shown

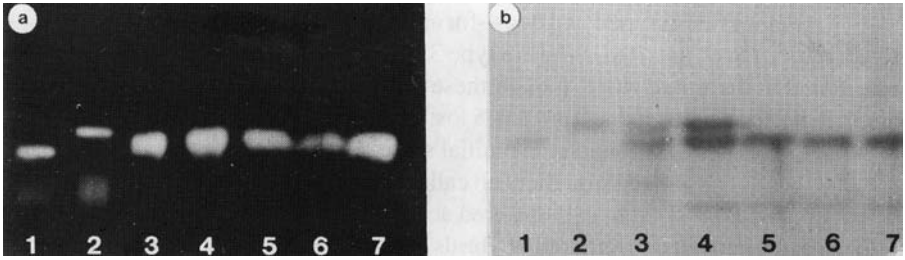


2. Expression of 7 genes in plumules of Acc 224 and IR36 (lanes 1 and 2) and in calli of these strains (lanes 3 and 4).

Table 5. Number of variant subcell lines in which the expression of 1 parental allele was lost.

Cross[a]	Subcell lines examined (no.)	Variant subcell lines (no.)	Variant phenotype
IR36/KL 609	108	5	Amp-2^2 (V1) Cat-1^2 (V2) Cat-1^2 (V3) Cat-1^2 (V4) Sdh-1^2 (V5)
FL 47/1R36	98	2	Amp-2^1 (V6) Sdh-1^1 (V7)

[a] IR36 has *Amp-2*^{2}, *Cat-1*^{1}, *Pgi-1*^{1}, *Pgi-2*^{2}, and *Sdh-1*^{1} alleles. KL 609 and FL 47 have *Amp-2*^{1}, *Cat-1*^{2}, *Pgi-1*^{2}, *Pgi-2*^{1}, and *Sdh-1*^{2} alleles.



3. Expression of both *Cat-1* (a) and *Sdh-1* (b) in plumules of parental strains (IR36 and FL 47) and in subcell lines of their F_1 hybrids (FL 47/IR36). Lanes 1 and 2: expression of both *Cat-1* and *Sdh-1* in plumules of IR36 and FL 47, respectively. Lanes 3 and 4: normal expression of both genes in subcell lines of F_1 hybrids. Lanes 5–7: variant expression of *Sdh-1* found in subcell lines while showing normal phenotype for *Cat-1*.

in Table 5 and Figure 3. Four such variant subcell lines (V1, V2, V5, and V7) were divided into many pieces of calli, and each was re-examined for phenotype. All calli of V2 and V5 showed the same single band found in the original variant lines, while V1 and V7 showed segregations for phenotype (Table 6). Twelve of 14 calli examined in V1 variant lines showed normal expression of the heterozygote, and 2 showed only a single band derived from the indica strain (IR36). Among 62 calli of V7, 58 showed a single band from the indica strain, 3 showed that from the japonica strain, and 1 showed normal expression. Therefore, V5 and V7 might be chimeric calli containing both cells showing normal and cells showing variant phenotypes. The mechanism of these variations might be mutation, methylation, or mitotic recombination.

Discussion

Isozyme genes have several unique characteristics.

- They are direct products of genes, so that they are expressed not only at the plant level but also at the cellular level.

Table 6. Segregation of phenotypes among calli derived from variant subcell lines.

Variant examined	Original phenotype[a]	Segregation of phenotypes		
		Japonica type	Heterozygous type	Indica type
V1	*Amp-2*^{2} (I)	0	12	2
V2	*Cat-1*^{2} (J)	14	0	0
V5	*Sdh-1*^{2} (J)	14	0	0
V7	*Sdh-1*^{2} (I)	3	1	58

[a] I = allele derived from indica parent, J = from japonica parent.

- As their alleles show codominance. the heterozygote can be detected directly.
- If appropriate parents are crossed, segregation at a number of loci can be observed in a single cross.

Therefore, isozyme genes are thought to be useful genetic markers as well as RFLP markers at both the plant and cellular levels (Rick and Yoder 1988, Tanksley and Rick 1980).

Mapping of new genes will be accelerated using linkage relations with isozyme genes. In the present study, the chromosomal locations of *Gdh-1* and *Pgd-2* could be detected in only one F_2 (Table 3). In particular, the availability of isozyme markers will facilitate identification and linkage analysis of genes such as quantitative trait loci, which are difficult to detect by other markers because of their complicated inheritance.

The construction of RFLP maps in rice and their integration into conventional linkage maps is an urgent need of geneticists and breeders. Isozymes can serve as useful molecular markers to bridge two linkage maps of different levels.

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Notes

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Genetic mapping in rice using isozyme and RFLP markers

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Four isozyme loci were assigned to linkage groups through primary trisomic analysis: *Got-3* on chromosome 2, *Enp-1* on chromosome 6, *Est-1* on chromosome 7, and *Fdp-1* on chromosome 11. *Amp1* and *Got-3,* located on chromosome 2, were found to be independent of each other. *Enp-1* was independent of *Est-2* and *Pgi-2,* and *Est-1* of *Est-9.* Linkage relationships between isozyme and RFLP markers were determined from IR36/Ma Hae F_2 segregation. IR36 and Ma Hae show polymorphism for *Adh-1, Amp-1, Amp2, Amp-3, Amp4, Est-1, Est-2, Est-9, Fdp-1, Mal-1, Pgd-1, Pgi-1, Pgi-2,* and *Sdh-1.* The single-copy DNA probes of the rice genomic library (RG probes) were labeled with nonradioactive digoxigenin-deoxyuridine triphosphate. The DNA was digested with seven restriction enzymes (*Eco*RI, *Eco*RV, *Bam*H1, *Pst*1, *Hind*lll, *Xba*1, *Dra*1) and hybridized with six polymorphic RG probes belonging to chromosomes 6, 7, and 11. RG213 was shown to be linked with *Est-2, Amp3,* and *Pgi-2,* with distances of 1.7, 1.7, and 7.9 map units, respectively. RG64 was linked with these 3 markers with distances of 22.5, 22.5, and 11.5 map units, respectively. RG118 was linked with *Adh-1* with a distance of 7.3 map units. *Mal-1* was located on chromosome 7 through linkage with RG173.

Knowledge of the genetic architecture of the rice plant is essential for applying the new tools of biotechnology to rice improvement. Three kinds of markers—morphological (plant traits), isozyme (biochemical), and restriction fragment length polymorphism (RFLP) (DNA)—are being used for genetic mapping in rice. Genetic mapping provides useful information about the relative distances and positions of various genes on the chromosome map. This knowledge forms the basis for tagging genes governing agronomic traits with biochemical and molecular markers; it is useful for practicing marker-based selection for traits that are difficult to select through conventional procedures. To date, about 150 morphological markers have been assigned to 12 linkage groups through conventional genetic segregation analysis (Khush and Kinoshita 1992). The genetic map of rice is poorly marked with morphological markers. Moreover, most of the morphological markers are deleterious, are influenced by the

environment, have pleiotropic effects, and are thus not very useful in rice breeding. Isozymes have several advantages over morphological markers. They show codominance (all the genotypes can be classified); a large number of samples can be analyzed; they require limited plant tissue for analysis; analytical procedures are nondestructive; and they can be scored in the laboratory, thus eliminating the need for growing large populations in the field. However, the number of isozyme loci is not sufficient to saturate the genetic map. Recently, very useful molecular markers—RFLPs—have become available; they have all the advantages of isozyme markers, but are so numerous that a saturated genetic map can be prepared.

All 12 linkage groups have been assigned to respective chromosomes through primary trisomic analysis (Iwata et al 1984, Khush et al 1984). Twenty-two isozyme loci have been assigned to respective linkage groups through trisomic analysis (Pham et al 1990, Ranjhan et al 1988, Wu et al 1988). An RFLP map of rice consisting of 230 DNA markers has been prepared (McCouch et al 1988, McCouch and Tanksley 1992). We have continued our efforts to saturate the genetic map with additional isozyme loci and to determine linkage relations between isozyme and RFLP markers. Isozyme polymorphism was identified for eight new loci (de los Reyes et al 1989, IRRI 1990). The chromosomal locations of isozyme loci were determined through trisomic analysis. Linkage relations between isozyme and RFLP markers were investigated using nonradioactive digoxigenin DNA-labeled probes (Ishii et al 1990).

Materials and methods

The materials comprised the IR36 primary trisomic series: three isozyme marker stocks; and their F_1, F_2, and backcross populations. These populations were used to determine the chromosomal location of isozyme loci. The F_2 population of IR36/Ma Hae was used to study linkage relationships between isozyme and RFLP markers.

Chromosomal location of isozyme loci

Four isozyme loci (*Got-3*, *Enp-1*, *Est-1*, and *Fdp-1*) were investigated. Primary trisomics of IR36 and the isozyme tester stocks were examined for isozyme polymorphism. Crosses were made using trisomics as female parents and the tester stocks as male parents. The tester stocks and their allelic constitution for a specific isozyme locus are shown in Table 1. The trisomic F_1s were either backcrossed or selfed to obtain BC_1 and F_2 populations, respectively. Gene dosage effects in F_1 trisomics and segregation in the BC_1 or F_2 populations were studied. Segregation ratios were calculated to determine the critical trisomics carrying the isozyme loci. The *Mal-1* locus was located through linkage analysis with RFLP markers.

Linkage relationship between isozyme and RFLP markers

Rice cultivars IR36 (Acc 30416) and Ma Hae (Acc 23754) have contrasting alleles at 14 isozyme loci. A cross was made between IR36 and Ma Hae and the F_1 and F_2 populations were examined for their isozyme patterns. The F_2 plants were also

Table 1. Allelic composition of IR36, Ma Hae, Ptb 30, and *O. nivara* at 5 isozyme loci.

Material	Acc. no.	Allelic constitution at locus under investigation				
		Got-3	*Enp-1*	*Est-1*	*Fdp-1*	*Mal-1*
IR36	30416	1	1	1	1	2
Ma Hae	23754	1	1	0	2	1
Ptb 30	6304	2	1	1	1	
O. nivara	104443	1	2			

Table 2. Segregation for *Got-3* in the F_2/BC_1 progenies from the crosses of 8 trisomics with Ptb 30 (Acc. 6304).

Trisomic	Generatior	Plants (no.) with designated isozyme genotype					c^{2a}
		11	112	122	12	22	
Triplo-2	BC_1	0		40		12	15.076** (0.002)[b]
Triplo-5	F_2	8		17		8	0.030
Triplo-6	BC_1	0		53		51	0.038
Triplo7	F_2	11		26		9	0.955
Triplo-8	F_2	10		12		6	1.715
Triplo-9	F_2	27		54		28	0.010
Triplo-11	F_2	10		17		9	0.167
Triplo-12	F_2	7		18		5	1.466

[a]Mendelian segregations are 0:1:1 for BC_1 and 1:2:1 for F_2. ** = significant at the 1% level. [b]Trisomic segregations are 0:3.28:1 for BC_1 and 4.86:7:1 for F_2 assuming 30% female transmission of the extra chromosome.

examined for RFLP pattern. Cosegregation for the isozyme and RFLP markers was analyzed in the F_2 population to determine linkage relationships. Linkage values were calculated using the LINKAGE 1 program (Suiter et al 1983).

The DNA isolated from the leaves of F_2 plants was digested with seven restriction enzymes (*Eco*RI, *Eco*RV, *Pst*1, *Bam* H1, *Hin*dIII, *Xba*1, *Dra*1). The Southern blots were hybridized using nonradioactive digoxigenin-deoxyuridine triphosphate-labeled DNA probes (Boehringer-Mannheim, Genius kit) following the procedure of Ishii et al (1990). Of the 11 rice genomic library (RG) probes, belonging to 3 chromosomes (6, 7, and 11), 6 showing polymorphism (RG64, RG172, RG213, RG173, RG2, and RGl18) were used in hybridization with the DNA of the F_2 population.

Results and discussion

The chromosomal location of five isozyme loci (*Got-3, Enp-1, Est-1, Fdp-1,* and *Mal-1*) and the linkage relationships of *Est-2, Amp-3, Pgi-2, Adh-1,* and *Mal-1* with RFLP markers are discussed in the following sections.

Table 3. Segregation for *Enp-1* in BC_1 progenies of 7 trisomics with *O. nivara* (Acc. 104443).

Trisomic	Plants (no.) with designated isozyme genotype					c^2[a]
	11	112	122	12	22	
Triplo-2	0		18		13	0.83
Triplo-4	0		32		50	3.96
Triplo-5	0		19		15	0.50
Triplo-6	0		38		12	13.54** (0.01)[b]
Triplo-1 0	0		26		22	0.35
Triplo-11	0		42		51	0.88
Triplo-12	0		22		17	0.67

[a]Mendelian segregations are 0:1:1 for BC_1. ** =significant at the 1% level. [b] Trisomic segregations are 0:3.28:1 for BC_1 assuming 30% female transmission of the extra chromosome.

Table 4. Segregation for *Fdp-1* in F_2 progenies from the crosses of 7 trisomics with Ma Hae (Acc. 23754).

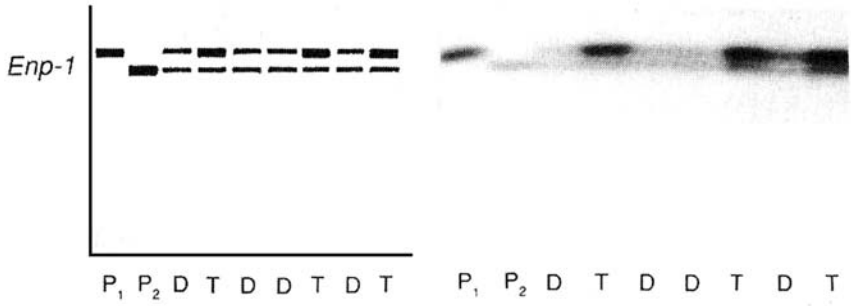
Trisomic	Plants (no.) with designated isozyme genotype					c^2[a]
	11	112	122	12	22	
Triplo-1	50		98		32	5.18
Triplo-2	26		59		24	0.82
Triplo-4	24		46		19	0.66
Triplo-6	23		46		23	0.00
Triplo-7	24		41		20	0.47
Triplo-10	15		37		15	0.72
Triplo-11	89		111		13	54.61** (1.87)[b]

[a]Mendelian segregations are 1:2:1 for F_2. ** =significant at the 1% level. [b]Trisomic segregations are 4.86:7:1 for F_2 assuming 30% female transmission of the extra chromosome.

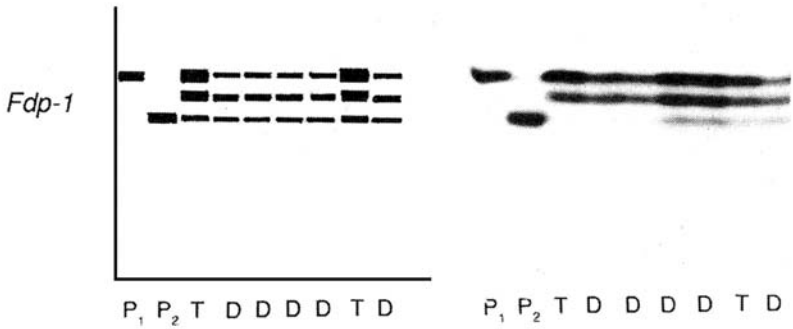
Table 5. Segregation for *Est-1* in F_2 progenies from the crosses of 7 trisomics with Ma Hae (Acc. 23754).

Trisomic	Plants (no.) with designated isozyme genotype					c^2[a]
	11	10	100	110	22	
Triplo-1		53			19	0.05
Triplo-4		73			26	0.06
Triplo-6		49			18	0.09
Triplo-7		73			10	7.79** (1.65)[b]
Triplo-9		86			28	0.01
Triplo-10		44			17	0.21
Triplo-11		58			14	1.35

[a]Mendelian segregations are 3:1 for F_2. ** = significant at the 1% level.[b]Trisomic segregations are 11.86:1 for F_2 assuming 30% female transmission of the extra chromosome.



1. Gene dosage for *Enp-1* isozyme (monomeric) in F_1 of trisomic 6 (T).



2. Gene dosage for *Fdp-1* isozyme (dimeric) in F_1 of trisomic 11 (T).

Chromosomal location of isozyme loci

The segregation data from crosses of primary trisomics with isozyme marker stocks having contrasting alleles are given in Tables 2, 3, 4, and 5. Segregation for *Got-3* (glutamate oxaloacetate transaminase) was studied in the F_2 or BC_1 progenies from crosses of Ptb 30 with eight trisomics. The segregation data (Table 2) show that *Got-3* is located on chromosome 2. Linkage analysis revealed that *Got-3* is independent of *Amp-1*.

Segregation for *Enp-1* (endopeptidase) was studied in BC_1 populations derived from crosses of *O. nivara* with seven trisomics. Gene dosage in the F_1 of trisomic 6 (Fig. 1) showed that *Enp-1* is located on chromosome 6. Segregation in the BC_1 confirmed triplo-6 as the critical trisomic having the Enp-1 locus (Table 3). In an independent study, Pham et al (1990) also located *Enp-1* on chromosome 6.

Gene dosage for *Fdp-1* (fructose-1,6-diphosphatase) was studied in seven F_1 trisomics. Gene dosage for this dimeric enzyme was observed in trisomic 11 (Fig. 2). Segregation analysis in the F_2 (Table 4) confirmed these results. So far only two alleles of *Est-1* (esterase) are known (null and allele 1); hence only two classes could be observed in the F_2 progenies of seven trisomics. The segregation data (Table 5) show that *Est-1* is located on chromosome 7.

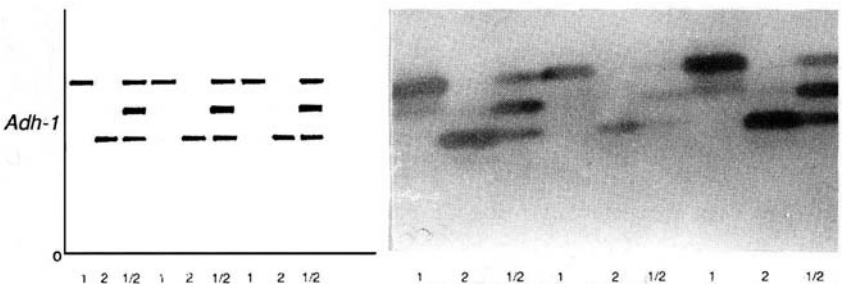
Linkage relationship between isozyme and RFLP markers

The allelic constitution of IR36 and Ma Hae for five isozyme loci and six RFLP markers is shown in Table 6. Linkage relations were studied from the F_2 segregation data of the cross IR36/Ma Hae (Fig. 3). RFLP segregation was analyzed using non-radioactive digoxigenin-dUTP-labeled RG probes (Fig. 4). The results of co-segregation for

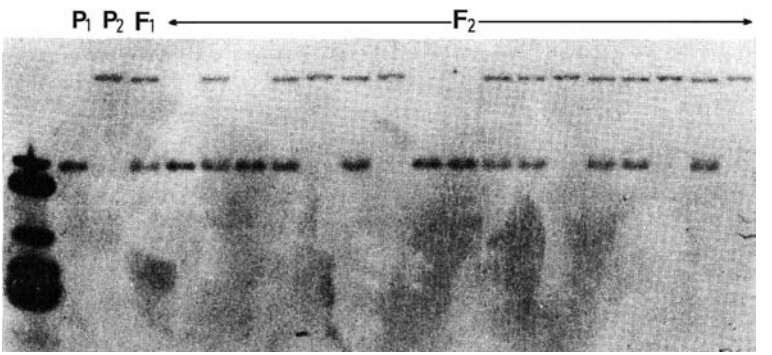
Table 6. Allelic constitution of IR36 (Acc. 30416) Ma Hae (Acc. 23754) at 5 isozyme[a] and 6 RFLP[b] loci.

Cultivar	Isozyme					RFLP markers with restriction enzymes					
	Est-2	Amp-3	Pgi-2	Mal-1	Adh-1	RG64 (*Hin*d III)	RG172 (*Hin*d III)	RG213 (*Xba*1)	RG173 (*Hin*d III)	RG2 (*Hin*d (III)	RG118 (*Pst*1)
IR36	2	1	2	2	1	1	1	1	1	1	1
Ma Hae	1	2	1	1	2	2	2	2	2	2	2

[a]Isozyme allele designation based on Glaszmann et al (1988). [b]RFLP allele designation: 1 (IR36), 2 (Ma Hae).



3. Segregation for *Adh-1* in F_2 of IR36/Ma Hae; IR36 (1), Ma Hae (2), and heterozygote (1/2).



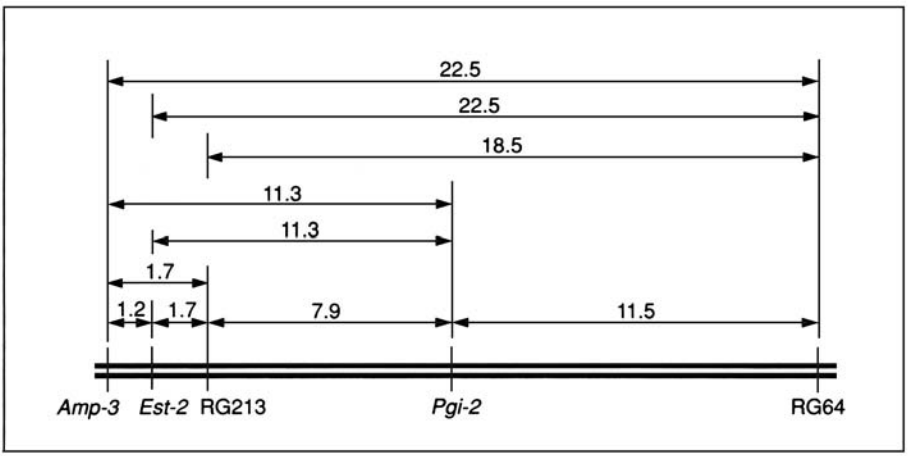
4. RFLP pattern of *Pst-1*-digested DNA from IR36 (P_1), Ma Hae (P_2), IR 36/ Ma Hae (F_1), and the F_2 population hybridized with RG118 (2.0 kbp) previously mapped on chromosome 11.

Table 7. Cosegregation for isozyme and RFLP markers in F_2 of IR36/Ma Hae.

Marker	Allelic constitution	Plants (no.) with marker genotype			Recombination (%) ±SE	P
		11	12	22		
			Pgi-2			
Amp-3	11	0	3	14		
	12	10	38	4	11.29 ± 2.59	0.000
	22	16	1	0		
Est-2	11	16	2	0		
	12	10	37	4	11.29 ± 2.59	0.000
	22	0	2	14		
RG 64	11	0	1	2		
	12	1	22	2	11.49 ± 3.92	0.000
	22	7	2	1		
RG 213	11	0	1	8		
	12	3	32	4	7.89 ± 2.57	0.000
	22	11	1	0		
			Amp-3			
Est-2	11	1	0	17		
	12	0	51	0	1.19 ± 0.84	0.000
	22	16	0	0		
RG 213	11	9	0	0		
	12	12	38	0	1.70 ± 1.19	0.000
	22	0	1	11		
RG 64	11	1	2	0		
	12	4	20	1	22.53 ± 5.60	0.056
	22	1	5	4		
			Est-2			
RG 213	11	0	0	9		
	12	0	38	1	1.69 ± 1.19	0.000
	22	11	1	0		
RG 64	11	0	2	1		
	12	1	20	4	22.53 ± 5.60	0.056
	22	4	5	1		
			RG 213			
RG 64	11	1	2	0		
	12	3	21	1	18.51 ± 5.03	0.011
	22	1	4	5		
			Mal-1			
RG 1734	11	7	2	0		
	12	4	16	5	22.74 ± 5.29	0.001
	22	2	2	5		
			Adh-1			
RG 118	11	9	1	1		
	12	2	23	1	7.29 ± 2.71	0.000
	22	0	1	12		

Table 8. Summary of linkage relationships among isozyme and RFLP markers.

Marker combination		Chromosome	P	Recombination (%) ± SE
Amp-3	*Est-2*	6	0.000	1.19 ± 0.84
Amp-3	RG 213	6	0.000	1.70 ± 1.19
Est-2	RG 213	6	0.000	1.69 ± 1.19
Pgi-2	RG 213	6	0.000	7.89 ± 2.57
Pgi-2	RG 64	6	0.000	11.49 ± 3.92
Pgi-2	*Amp-3*	6	0.000	11.28 ± 2.59
Pgi-2	*Est-2*	6	0.000	11.29 ± 2.60
RG 213	RG 64	6	0.011	18.51 ± 5.03
Amp-3	RG 64	6	0.056	22.53 ± 5.70
Est-2	RG 64	6	0.056	22.53 ± 5.60
Mal-1	RG 173	7	0.001	22.74 ± 5.29
Adh-1	RG 118	11	0.000	7.29 ± 2.71



5. Chromosome 6 showing linkage relationships among isozyme and RFLP markers.

isozyme and RFLP markers are given in Table 7. The RFLP marker RG213 was found to be linked with *Est-2, Amp-3,* and *Pgi-2* with distances of 1.7, 1.7, and 7.9 map units, respectively (Table 8). The three isozyme loci *Est-2, Amp-3,* and *Pgi-2* are linked with RG64 with a distance of 22.5, 22.5, and 11.5 map units, respectively. Similarly, RG118 is linked with *Adh-1* with a distance of 7.3 map units. The linkage relationships among isozyme and RFLP markers belonging to chromosome 6 are shown in Figure 5. In addition to the four isozyme loci reported above, *Mal-1* was located on chromosome 7 through linkage analysis with RFLP markers. It is situated at a distance of 22.7 crossover units from RG173. The results show that RFLP and isozyme analysis is useful in mapping the rice genome.

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Notes

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Production of monosomic alien addition lines of *Oryza sativa* having a single *O. punctata* chromosome

H. Yasui and N. Iwata

To study the possibility of gene transfer from wild rice to cultivated rice, monosomic alien addition lines (MAALs) of *Oryza sativa* having a single chromosome of *O. punctata* were produced. Autotetraploid plants of *O. sativa* L. japonica cultivar Nipponbare (genome AAAA), of which the primary trisomic series had been established, were crossed with *O. punctata* Kotschy (BB). Three hybrid plants (AAB) were developed after embryo culture. These hybrids were completely self-sterile and were backcrossed with Nipponbare. In the BC_1F_1, BC_1F_3, BC_2F_1, and BC_2F_2 progenies, 46 monosomic addition plants (2n=25) were detected, 37 of which could be grouped into 1 of 11 types based on their morphological resemblance to one of the primary trisomic lines for the 11 chromosomes of the Nipponbare haploid genome. The nine other MAALs could not be grouped because their morphological features had no resemblance to any of the primary trisomics. Of the 11 grouped types, 9 were fertile, and the next generation could be obtained; but the 2 others were completely sterile. This paper discusses the morphological and cytological characteristics of the *O. sativa* MAALs having a single chromosome of *O. punctata*.

The genus *Oryza* has about 20 species, 2 of which are cultivated. Asian cultivated rice *Oryza sativa,* African cultivated rice *O. glaberrima,* and their closely related wild species such as *O. nivara, O. rufipogon,* and *O. longistaminata* share a common genome, designated AA. All species with the AA (2n=24) genome have good crossability and show regular pairing and recombination. Thus gene transfer between these species can be achieved without much difficulty.

The more distantly related species have nonhomologous genomes, designated BB (*O. punctata*), CC (*O. officinalis*), or EE (*O. australiensis*). These species are difficult to cross with species possessing the AA genome, and the F_1 hybrids are completely male sterile.

Gene transfer from the distantly related wild species to cultivated species has been achieved through the establishment of monosomic alien addition lines (MAALs). MAALs have been produced in wheat (Alston 1970, Riley and Chapman 1958, Sears 1956), oats (Thomas 1968), tobacco (Gerstel 1945), and beet (Savitsky 1978, Speck-

mann and Debock 1982). Recently, MAALs were also produced in rice (Jena and Khush 1989, Shin and Katayama 1979). These MAALs have the complete chromosome complement of *O. sativa* and single chromosomes of *O. officinalis.* The 12 morphological types corresponding to rice haploid chromosome number were classified by Jena and Khush (1989).

In Tateoka's (1965) classification of genus *Oryza, O. punctata* includes diploid and tetraploid strains. The genome constitution of the latter was designated BBCC by Hu and Chang (1965), and that of the former was designated BB by Katayama (1967). In this study, we investigated the possibility of establishing MAALs having the complete chromosome complement of *O. sativa* and the single chromosomes of the *O. punctata* diploid strain.

Materials and methods

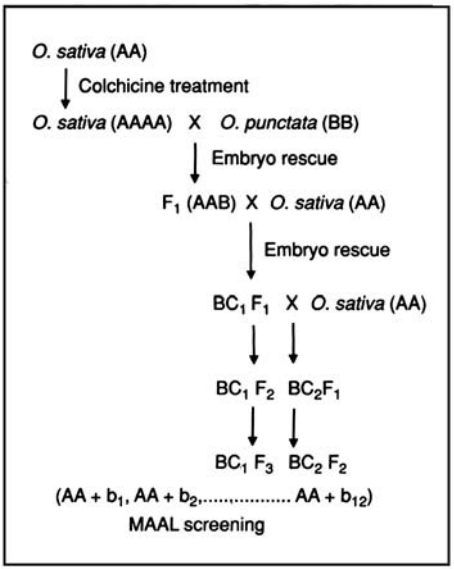
O. punctata strain W1514, a diploid having the BB genome, was obtained from the National Institute of Genetics in Japan. It was crossed with autotetraploids induced by colchicine from *O. sativa* japonica cultivar Nipponbare.

We attempted a number of crosses between the tetraploids of *O. sativa* as the female and the strain of *O. punctata* as the male, but the seeds were always imperfectly developed. To overcome this problem, immature embryos were excised under the stereomicroscope under aseptic conditions and cultured on half-strength Murashige and Skoog's (MS) medium devoid of auxin. The embryos were incubated in the dark until germination and were subsequently transferred to a light incubation chamber. Seedlings at the five- or six-leaf stage were transferred to soil under controlled humidity.

The F_1 plants were pollinated with pollen from diploids of Nipponbare to obtain BC_1F_1 plants following the same embryo rescue technique as above. The seeds were germinated on half-strength MS medium. The BC_1F_1 plants were again crossed with the same male parents to obtain BC_2F_1 progenies. The BC_1F_1 and BC_2F_1 plants were self-pollinated to obtain BC_1F_2 and BC_2F_2 progenies, respectively. The BC_1F_2 plants were also self-pollinated to obtain BC_1F_3 progenies. The procedure used for the development of MAALs is shown in Figure 1.

The F_1, BC_1F_1, BC_1F_2, BC_1F_3, BC_2F_1, and BC_2F_2 plants were cytologically examined to determine their chromosome number. Mitotic chromosome analysis was conducted on the progenies of the cross between autotetraploids of Nipponbare and *O. punctata* by the enzyme maceration technique (Kurata and Omura 1978, Nishibayashi and Kaeriyama 1986).

Plants having 25 chromosomes were selected and grouped on the basis of their morphological features. The morphologies of MAALs were compared with those of the primary trisomics of *O. sativa.* The MAAL resembling trisomic A was designated MAAL A; that resembling trisomic B, MAAL B; and so on. Seed fertility and the transmission rates of the alien chromosomes were also studied by examining the progeny of each MAAL.



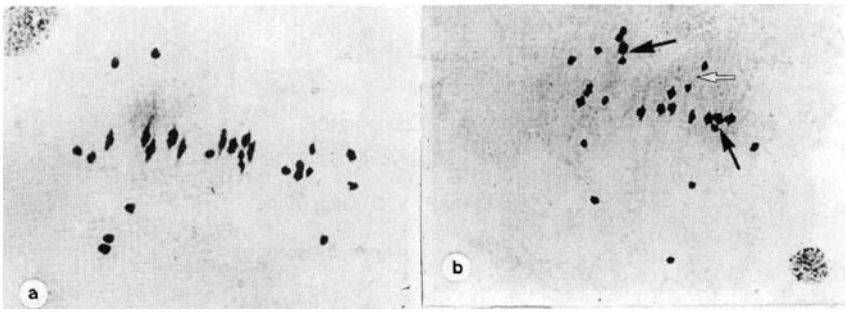
1. Development of *O. sativa* MAALs having single *O. punctata* chromosome.

Results

From 75 imperfectly developed seeds we obtained 3 F_1 hybrids between autotetraploid Nipponbare and W1514. The crossability of autotetraploids of *O. sativa* with *O. punctata* was under 1%.

The hybrids expressed the characteristics of both parents to a certain degree. The purple pigmentation of the basal leaf sheath, flexible midrib, presence of awn, and purple stigma were the same as in *O. punctata;* and the shape and color of the spikelets were the same as in the autotetraploids of *O. sativa.* The three F_1 plants were robust and extremely vigorous, but completely sterile. All of their somatic cells had 36 chromosomes. Most of their pollen mother cells (PMCs) had 12 bivalents and 12 univalents (Fig. 2a). The mean frequency of trivalents per PMC was 10.5% (Table 1, Fig. 2b).

The number of chromosomes in the 103 BC_1F_1 plants obtained varied from 24 to 31 (Table 2). Two plants among the BC_1F_1 had a chromosome fragment. Plants with 26, 27, 28, and 30 chromosomes, which were highly sterile when crossed with Nipponbare, produced plants with 25 chromosomes. Thus, plants with 25 chromosomes (i.e., having the complete chromosome complement of *O. sativa* and a single chromosome of *O. punctata*) represented the MAALs, of which we obtained 46. Eleven morphological types were identified among the 2n+l plants. These MAALs had a striking resemblance to the *O. sativa* primary trisomics. Seven plants each resembled trisomics A, F, and G; 4, trisomic N; 3, trisomic E; 2 each, trisomics B, C, and M; and 1 each, trisomics D, L, and O (Table 3). A plant resembling trisomic A was designated MAAL A; a plant resembling trisomic B, MAAL B; and so on, for 11 different MAALs, except for



2. Chromosome association at metaphase I in F_1 plants. a) Pollen mother cell (PMC) showing 12 II + 12 I. b) PMC showing 2 III (black arrows) + 10 II + 8 I + 1 secondary association (open arrow).

Table 1. Chromosome association at metaphase I in the allotriploids (AAB).

F_1 plant	Pollen mother cells observed (no.)	Cells (no.)		
		12 II + 12 I	1 III + 11 II + 11 I	Secondary association
1	98	84	14	17
2	42	41	1	4
3	3	3	0	0
Total	143	128	15	21

Table 2. Plants derived from crosses between allotriploids (AAB) and Nipponbare (AA).

Year	Plants (no.) with given no. of chromosomes								
	24	25	26	27	28	29	30	31	Not confirmed
1985	0	1	1	1	1	0	0	1	18
1986	3	6	4	0	1	1	0	0	47[a]
1987	2	14	3	4	2[a]	0	1	0	38
Total	5	21	8	5	4	1	1	1	103

[a]Including a plant with a chromosome fragment.

Table 3. Classification and number of MAALs of *O. sativa* having a single chromosome of *O. punctata.*

Type	MAALs (no.)	Type	MAALs (no.)
A	7	G-I	1
B	2	G-II	6
C	2	L	1
D	1	M	2
E	3	N	3
F-I	1	N-telo.	1
F-II	3	0	1
F-III	3	Others	9

MAAL H, which corresponded to the haploid chromosome complement of *O. punctata.* Slightly different types were obtained from three types of MAALs: MAALs F-II and F-III from the progenies of MAAL F-I; and MAALs G-II and N-telo. from MAALs G-I and N, respectively. Among seven plants of MAAL F, one was grouped with MAAL F-I, and 3 each with MAALs F-II and F-III. Among seven plants of MAAL G, one was grouped with MAAL G-I and 6 with MAAL G-II. Among four plants of MAAL N, three were grouped with MAAL N and one with MAAL N-telo. Another three plants somewhat resembled trisomics C, F, and N, respectively, although each of them showed different features from their respective MAALs. The remaining six plants could not be assigned to any specific group.

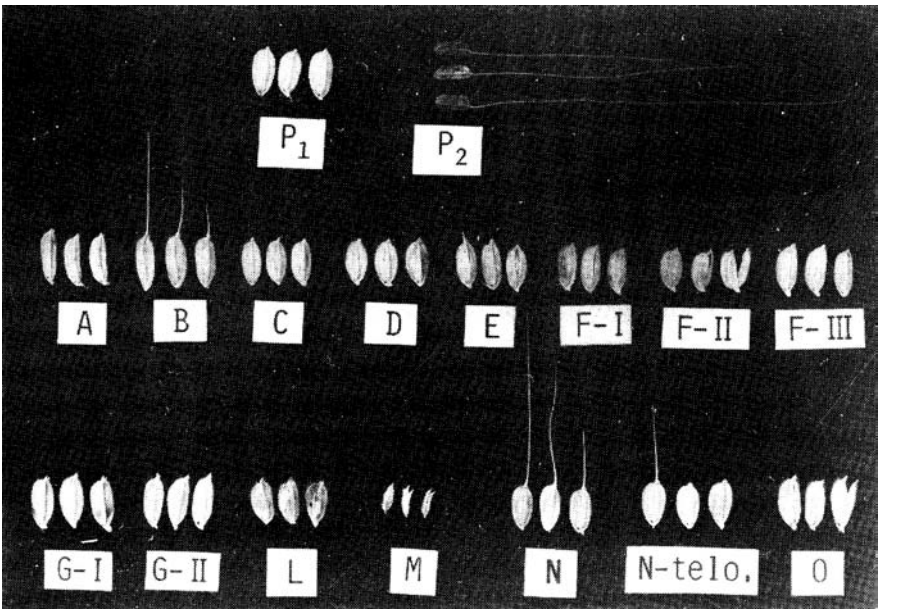
Morphological and reproductive features of MAALs

MAAL M was easily identified at the seedling stage by its dark green leaves and dwarf plants. MAALs A, B, D, F, L, N, and O were easily distinguishable at maximum tillering. MAALs C, E, and G could be identified only at flowering. MAALs L, M, N, and O were dwarfs, while MAALs D and E were tall. MAALs B and N had spikelets with long awns. MAAL F-I had a red pericarp, a trait inherited from *O. punctata.* MAAL F-II had a brown pericarp, a modified trait inherited from *O. punctata.* A few plants derived from MAAL F-I showed the same morphological features as MAAL F-I, although they had normal color pericarp, so we tentatively named them MAAL F-III. MAAL G-II was obtained from the progenies of MAAL G-I, but differed morphologically. Therefore, the additional chromosomes of MAALs F-II, F-III, and G-II seemed to be modified. MAAL M had a purple stigma, a trait inherited from *O. punctata.*

All of the MAALs except MAALs A and M were fertile and produced the same type of MAAL in the self-pollinated progenies. The pollen and spikelet fertilities of the MAALs were lower than in the primary trisomics. MAALs A and M were completely sterile. Otherwise trisomic A showed normal fertility, and trisomic M showed complete male sterility. Each MAAL had a characteristic grain size and shape that resembled that of the respective primary trisomic (Fig. 3).

Cytology of MAALs

Chromosome pairing in the MAALs was examined at pachytene, diakinesis, and metaphase I. The modal chromosome association in all MAALs was 12 II + 1I (Fig. 4a). Only the karyotypes of MAALs E and N-telo. were detected in the somatic cells. MAAL E had three long, subtelocentric chromosomes in its somatic cell, one of which seemed to originate from *O. punctata* and two from *O. sativa* (Fig. 4b). Thus, the extra chromosome of MAAL E is probably the same type as that of trisomic E. In contrast, MAAL N-telo. had a telocentric chromosome in its somatic cell, so it had better recovery of seed fertility than did MAAL N. This telocentric chromosome was formed by the breakage of an additional chromosome during meiosis in MAAL N (Fig. 4c) and was transmitted in this form to its progeny (Fig. 4d).



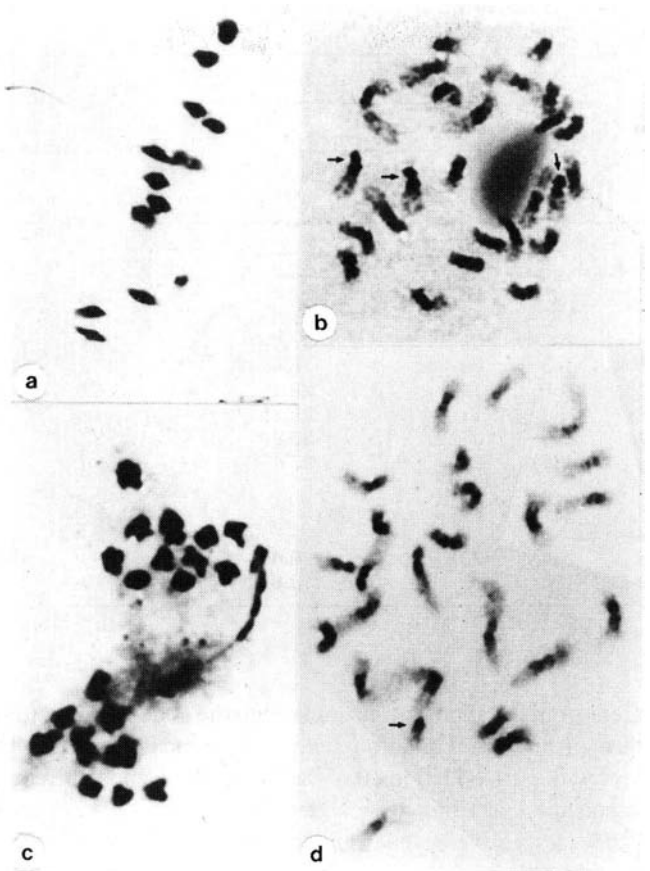
3. Grains of *O. sativa* (P_1), *O. punctata* (P_2), and 15 MAALs.

Transmission rates of alien chromosomes

Transmission rates of alien addition chromosomes through the female were fairly high except in MAALs A and M (Table 4). MAAL A was sterile with regard to both female and male gametes, and MAAL M was extremely weak and completely male sterile. Thus, we could not obtain any progeny of these two MAALs. Female transmission of alien chromosomes could be studied in nine MAALs: B, C, E, F-I, F-II, G-I, N, and N-telo. had transmission rates above 20%; MAAL O gave the lowest transmission rate (7.6%). Disomic alien addition lines were obtained in the self-pollinated progenies of MAALs B, C, D, E, F-I, F-II, G-I, and G-II, so alien addition chromosomes were also transmitted through the male gamete in these MAALs.

Discussion

MAALs are a useful tool to examine the effects of additional chromosomes. Levy et al (1987) proposed an experimental model using addition lines to evaluate the effects of alien chromosomes on quantitative traits. Their model was based on the comparison between disomic addition lines and tetrasomic lines. both of which are in the background of the common wheat cultivar Chinese Spring. In rice, Jena and Khush (1989) established MAALs of *O. sativa* having single chromosomes of *O. officinalis.* Khush et al (1984) compared the MAALs with the primary trisomics in the same background of indica rice cultivar IR36. They evaluated each chromosome of the C genome by comparison with the primary trisomics.



4. a) PMC of MAAL N showing 12 II + 1 I. b) Somatic chromosomes of MAAL E. Arrows show 3 subtelocentric chromosomes. c) Chromosome breakage at anaphase I in MAAL N. d) Somatic chromosomes of MAAL N-telo. Arrow shows a telocentric chromosome.

In the present study, we developed MAALs of *O. sativa* having single chromosomes of *O. punctata* in the background of japonica rice cultivar Nipponbare, for which Iwata and Omura (1984) had developed the trisomic series. In the comparison of the trisomics and MAALs, trisomic A was fertile, but MAAL A was completely sterile. We suppose that some sterility factor is located on the alien chromosome of MAAL A in the cross *O. sativa/O.punctata.* Similarly, there were pericarp pigmentation genes located on the alien chromosome of MAAL F-I, and stigma pigmentation genes located on that of MAAL M. The MAALs that could not be assigned to any specific group probably had an interchanged chromosome. These chromosomes seemed to originate from the secondary association between nonhomologous chromosomes of the B genome at meiosis in the allotriploids.

Table 4. Transmission rates of the extra chromosome in MAALs of *O. sativa* having a single chromosome of *O. punctata*.[a]

Type of MAAL	Transmission rate								
	(2n+1) self-pollinated					(2n+1)/(2n)			
	Total (no.)	2n (no.)	2n+1 (no.)	2n+2 (no.)	% AD	Total (no.)	2n (no.)	2n+1 (no.)	% AD
A	–	–	–	–	–	–	–	–	–
B	101	68	32	1	32.7	93	68	23	24.7
C	102	69	28	5	32.4	200	134	63	31.5
D	277	172	95	9	37.5	–	–	–	–
E	415	298	109	3	27.0	64	51	13	20.3
F-I	435	343	79	1	18.4	200	157	42	21.0
F-II	409	285	112	7	29.1	64	47	16	25.0
F-III	202	150	51	0	25.2	–	–	–	–
G-I	457	299	137	18	33.9	46	32	13	28.3
G-II	576	435	128	7	23.4	–	–	–	–
L	–	–	–	–	–	–	–	–	–
M	–	–	–	–	–	–	–	–	–
N	8	5	3	0		348	254	80	23.0
N-telo.	276	184	73	0	26.4	636	493	140	22.0
O	149	120	28	0	18.8	119	110	9	7.6

[a]AD = alien addition lines.

Gene transfers from the alien chromosomes into the genomes of cultivated species are achieved through rare recombinational events. Shin and Katayama (1979) observed microsporocytes with 1 III + 11 II in 10 of the 12 MAALs, and the frequency of cells with such an association was quite low (under 15%), with the exception of 2 MAALs of 66.7 and 47.5%, respectively. Jena and Khush (1989) observed only 3 of 95 cells with trivalents of MAAL 3.

In the present study, the mean frequency of trivalents per PMC was 10.5% in the F_1s. The trivalent formation suggests that rare recombinational events possibly occurred between the *O. sativa* and the *O. punctata* chromosomes. Moreover, we found disomics with modified morphology in the progenies of some MAALs. Detailed analysis of the progenies is now being done.

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Notes

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Discussion

Session 2: Genetic markers, linkage groups, and aneuploids

Q–*Glaszmann:* To what extent will segregation distortions affect estimates of recombination ratios?

A–*Oka:* Empirically, the largest difference between estimates considering and not considering F_2 distortion was 5% in both the positive and the negative directions. Usually it was less than 5%. Certation (for pollen only) does not affect estimates of recombination values.

Q–*Chaudhary:* Is the cleistogamous character earlier reported in rice the same as the sheathed panicle you reported? The *cl* gene symbol already exists for such a character.

A–*Maekawa:* The genes for sheathed panicle are different from *cls,* which was assigned to cleistogamous panicle connected with the dwarf gene *d-7.*

Q–*Multani:* What was the objective of conducting isozyme studies on callus cultivars?

A–*Ishikawa:* When we investigate somaclonal variation, we should look for variation not only at the plant level by using regenerated plants, but also at the cellular level, because the plant is not able to regenerate some variations from callus. Besides, cells having some variation may be distinguished during cell culture because of competition with normal cells. Therefore, we must look for somaclonal variation at the cellular level. On the other hand, a survey for cellular expression of the isozyme may be necessary to use them for early selection in cell fusion experiments. In that case it would be better to choose isozyme genes that show stable expression during cell culture.

Q–*Hsin-Kan Wu:* Where does the additional chromosome segment come from in your N-telo. type alien addition line?

A–*Yasui:* This telocentric chromosome in monosomic alien addition line (MAAL) N-telo. may have resulted from spontaneous breakage of an alien chromosome during meiosis in MAAL N, followed by transmittal to MAAL N-telo.

Q–*Zhang Qifa:* Monosomic by definition refers to a chromosome complement of 2n-1. Would it be better to call the addition line something else instead of "monosomic addition"?

A–*Yasui:* The term "monosomic addition line" is popular in polyploid species such as wheat, tobacco, and cotton. I followed Jena and Khush (1989) with regard to the terminology of monosomic addition lines in rice.

Q–*Chaudhuri:* It seems that there are additions of *O. punctata* chromosomes in the *O. sativa* genome. Can you clearly identify your *punctata* chromosomes from *sativa* chromosomes?

A–*Yasui:* Yes, I observed meiosis in these MAALs. The modal chromosome association was 12II + 1I in 11 morphological types of MAALs. So I decided these univalent chromosomes were derived from *O. punctata.*

SESSION 3

Genetics of Stress Tolerance

Genetics of leaf rolling under drought stress

B.N. Singh and D.J. Mackill

A genetic study of five drought-resistant and two susceptible lines was carried out during the 1989 and 1990 dry seasons at IRRI under dry seeded upland conditions. Parents, F_2, and F_3 lines from 10 resistant/susceptible crosses were grown in a randomized complete block design. The crop was raised for 40 d with irrigation and then subjected to a 30-d dry period followed by rewatering. Plants with rolled leaves were classified as susceptible and those with unrolled leaves (with dark green foliage and no leaf drying) as resistant. F_3 lines segregated into a ratio of 1 rolled: 2 segregating (3:1): 1 unrolled, suggesting the effect of a major dominant gene on leaf rolling under drought stress. Plants with rolled and unrolled leaves were clearly differentiated between 1600 and 1750 h at –0.1 MPa soil water potential. Plants that showed early leaf rolling during stress had poorer drought resistance as indicated by level of leaf drying. Lines with good drought resistance and delayed leaf rolling recovered faster after the stress was removed.

Drought is a major constraint to rice production on 40 million ha of rainfed upland, lowland, and deepwater rice environments. It also affects the productivity of irrigated areas, because the groundwater table drops and less water from reservoirs is available for irrigation and hydroelectric power. Resistant varieties can withstand drought for longer periods, and, when sufficient rain comes, may show little decline in yield when properly managed.

Developing improved drought-resistant lines has been a major breeding objective in rainfed rice improvement programs. Field screening techniques for evaluating rice germplasm and breeding lines were developed at IRRI for mass screening (De Datta et al 1988). Over 6,000 accessions are screened every year during the dry season (DS) under dryland conditions, and resistant lines are selected for further use (IRRI 1988). Resistance donors are usually traditional varieties from drought-prone environments; their resistance needs to be incorporated into the modern high-yielding genetic background.

Knowledge of the genetics of drought resistance or its component traits is important for a successful breeding program. At IRRI, genetic research on traits associated with drought resistance has been conducted by Armenta-Soto et al (1983) and Chang et al (1986), who studied different traits related to root systems in aeroponics. Ekanayake et al (1985a, b) studied inheritance of drought resistance in lowland rice by the root pulling force method, as well as root traits in hydroponic culture solution in the phytotron. Inheritance was polygenic for different root traits, with both additive and dominance gene effects.

Leaf rolling during drought stress may prevent transpiration loss but leads to closure of stomata, so that gaseous exchange and CO_2 entry into cells are reduced and photosynthesis is decreased. Rolling also reduces the photosynthetic surface and light absorption area and thus leads to reduced assimilate levels. Leaf rolling as a drought symptom occurs because of the inability of leaves to sustain the evapotranspiration demand of the plant (Blum 1988). Selection for delayed leaf rolling may result in a range of drought resistance mechanisms (Hsiao et al 1984). In the present study, the genetics of leaf rolling under drought stress was studied in F_2 and F_3 lines under dryland field conditions in 1989 and 1990 DS.

Materials and methods

Five resistant lines—IR33353-64-1-2-1 (IR33353), IR26702-155-2-3 (IR26702), Salumpikit, MGL 2, and ITA 186—representing improved and traditional types, and two susceptible lines—TR29692-65-2-3 (IR29692) and IR29725-22-3-3-3 (IR29725)—were used (Table 1). Upland drought score over 3 yr was the main criterion for selection. Ten crosses involving the five resistant lines crossed with each of the two susceptible lines were made in the 1987 wet season (WS). The F_2 populations were grown in 1988 WS under irrigated conditions, and over 100 random plants were

Table 1. Lines used in the study.

Designation	Parentage	Origin	Plant type[a]	Cultural type[b]	Drought score[c] (1 to 9) at –0.2 MPa	–0.5 MPa	–1.0 MPa
Salumpikit	Land race	Philippines	T	TL	1	1	1
IR33353-64-1-2-1	IR52/IR36//IR52	IRRI	I	IL	1	1	2
MGL 2	Land race selection	India	T	TL	1	1	3
ITA186	Moroberekan/Juma 1, TOX7-3-2-3-2, SE 3636	IITA Nigeria	T	IU	1	1	3
IR26702-155-2-3	FR13A/IR48//IR42	IRRI	SD	IL	1	3	5
IR29692-65-2-3	IR17494-32-3-1-1-3/ IR9129-209-2-2-2-1	IRRI	I	IL	3	5	6
IR20 (check)	IR262-24-3/TKM6	IRRI	SD	IL	5	7	9

[a] T = tall, I = intermediate, SD = semidwarf. [b] TL = traditional lowland, IL = improved lowland, IU = improved upland.
[c] Average of 1989 and 1990 dry season field tests at different soil water potentials.

harvested to generate F_3 lines. The field trials for genetic studies were conducted at IRRI during 1989 and 1990 DS.

During 1989 DS each cross was grown in a randomized complete block design with four replications in a drought screening nursery at IRRI (De Datta et al 1988). One row of each parent, and eight rows of F_2 (grown together) were included in each block. Additionally, 20 F_3 lines were randomly selected in each of 4 replications, making a total of 80 F_3 lines per cross. The 33 lines in each replication were grown in 3 tiers. Salumpikit and IR20 were the resistant and susceptible checks planted together after every 11 rows in each replication. During 1990 DS, 75 F_3 lines and 18 F_2 rows were randomized in 3 replications to minimize the effect of differences in soil moisture.

Five grams of seed of each line was seeded in 2.5-m-long rows spaced 25 cm apart. Seeding was done on 2 and 3 Feb 1989 in granular Maahas clay soil, an Andaqueptic Haplaquoll with 45% clay, 40% silt, and 15% sand in the top 20 cm layer, and a pH of 5.8 (De Datta et al 1988). During 1990, seeding was done on 6 and 7 February in an upland field, where the soil is 33% clay, 47% silt, and 20% sand in the top 15 cm and the pH is 6.0. Sprinkler irrigation was used every 4 d until 40 d after seeding. Nitrogen fertilizer was applied twice, 30 kg/ha each time, at 10 and 20 d after emergence. P at 13 kg/ha and K at 25 kg/ha were applied basally. Herbicide was sprayed initially to control weeds. Plants were thinned twice to achieve a population of 20–30 plants/row. F_3 lines having fewer than 15 plants/row were ignored in genetic analysis.

During 1989 DS, after irrigation, soil moisture status at 10-, 15, 20-, and 30-cm depth was monitored by 3 tensiometers in the 500-m^2 experimental area. Rainless periods created drought stress of –0.l, –0.2, –0.5, and –l.0 MPa on 8 ,18, 21, and 24 April, respectively. Soil samples were taken every other day for moisture determination at 15-, 20-, and 30-cm depth by the gravimetric method. During 1990 DS, 10 tensiometers were placed at 15- and 30-cm depths. The free water table was below 1 m at the beginning of drought. Soil samples were also taken at 15- and 30-cm depth to monitor soil moisture. Soil water potentials of –0.1, –0.2, –0.5, and –1.0 MPa were observed on 15, 21, and 26 April, and 2 May, respectively.

Leaf rolling was the first symptom of drought stress. The susceptible check IR20 and the line IR29725 rolled first, at around –0.1 to –0.2 MPa soil water potential, approximately 2 wk after drought stress. The other susceptible parent and the five resistant parents showed leaf rolling symptoms at different soil water potentials. MGL 2 and IR26702 rolled in the third week, and ITA186 and IR33353 in the fourth week.

In the first week after stress, rolling occurred between 1300 and 1600 h; in the second week it occurred from 0900 to 1600 h. In the third and fourth weeks, the susceptible and highly susceptible plants, lines, and check IR20 remained rolled even in the late afternoon or morning hours. Rolling in resistant plants started at around 0900 h as the evapotranspiration demand increased, and unrolling started at around 1600 h. During 1989 DS, rolling and unrolling were recorded from 0900 to 1750 h. In 1990 DS, however, the differences were more visible from 1600 to 1750 h, when evapotranspiration and temperature decreased. The susceptible plants remained rolled, and the

resistant plants unrolled. The resistant plants remained unrolled even during the day and had dark green leaves, normal tillering, and no leaf tip drying.

Scores for drought resistance ranged from 1 (resistant) to 9 (highly susceptible) and were taken from 0600 to 0900 h based on leaf tip drying, leaf and plant color, and desiccation. In susceptible and highly susceptible plants (scores 7 and 9), at least 75% of the leaf blades of younger leaves were dried, tightly rolled, and yellowish. Many of them died after the third week of stress. Plants of the intermediate category (score 5) were rolled in the evening and had partial unrolling in the morning. All their lower leaves and half of the younger leaf blades were dried. The scores were taken 30–36 d after stress, when moisture status was –1.0 MPa or higher. Unrolled plants were tagged for observation. As IR20 and IR29725 were the first to roll, they showed higher desiccation and leaf drying as drought stress increased.

Results and discussion

Of the two susceptible checks, IR29725 showed leaf rolling symptoms between –0.1 and –0.2 MPa, and IR29692 showed symptoms between –0.2 and –0.5 MPa moisture stress. The resistant parents showed leaf rolling symptoms at later periods of stress. IR26702 was first to roll at –0.5 MPa, followed by MGL2 and ITA 186. IR33353 started showing leaf rolling at around –1.0 MPa, while Salumpikit did not show any symptoms even after 5 d at –1.0 MPa. Rolling or unrolling behavior was not correlated with plant type (Table 1).

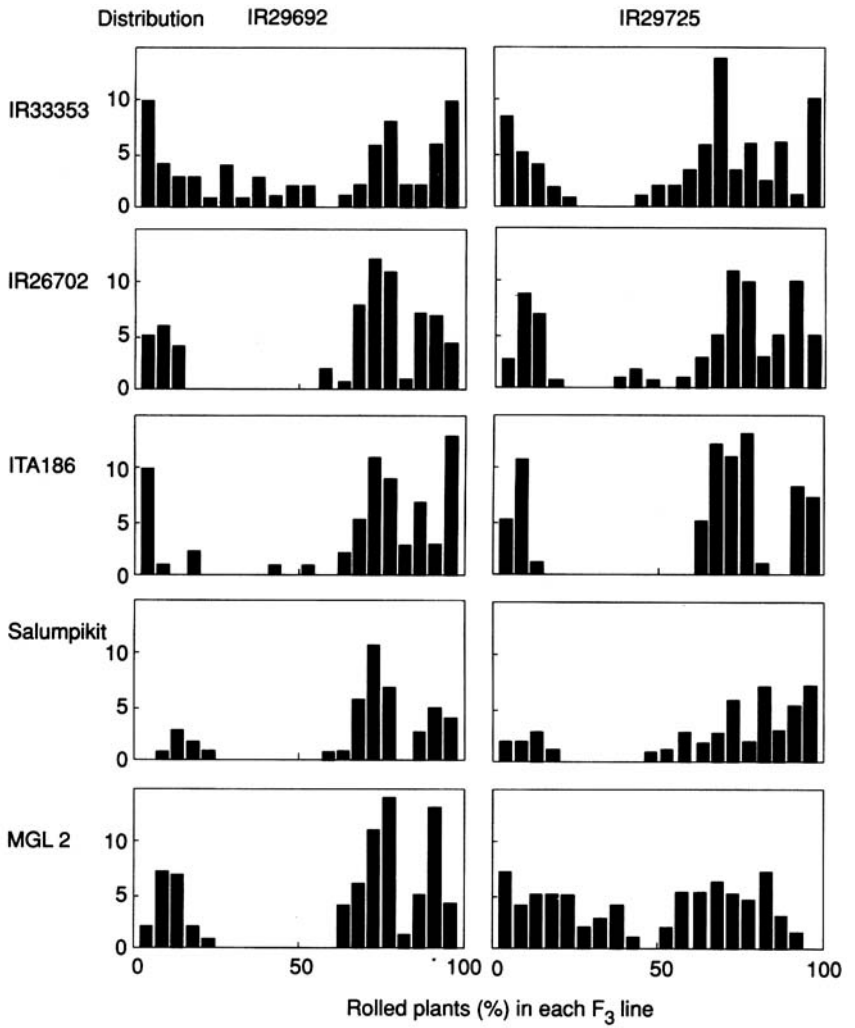
In April, rolling started after 0900 h on sunny days when evapotranspiration demand increased. After 1600 or 1700 h the rolled leaves unrolled. Many of the tolerant lines or plants in segregating generations did not roll, and their leaves remained unrolled during the day. Lines that rolled earlier had early symptoms of drying of lower leaves, followed by drying of upper leaves. They also had poor drought resistance at higher moisture stress.

Table 2. Leaf rolling and unrolling of F_2 populations and F_3 families, 1989 dry Season.

Cross	F_2 plants			F_3 families			
	Rolled (no.)	Unrolled (no.)	P (3:1)	Rolled (no.)	Segregating (no.)	Unrolled (no.)	P (1:2:1)
IR33353/IR29692	415	199	<0.01	20	32	23	0.40
IR26702/IR29692	605	251	<0.01	17	35	18	0.99
ITA186/IR29692	621	212	0.79	18	39	16	0.80
IR29692/Salumpikit	246	147	<0.01	16	31	17	0.95
IR29692/MGL 2	688	282	<0.01	20	39	19	0.99
IR33353/IR29725	771	280	0.23	19	37	19	0.99
IR26702/IR29725	899	294	0.80	20	35	24	0.49
ITA186/IR29725	777	215	0.02	18	42	16	0.72
IR29725/Salumpikit	319	161	<0.01	18	35	16	0.94
IR29725/MGL 2	393	282	<0.01	15	37	24	0.34

All the F_3 populations of 70–80 lines in each cross showed a ratio of 1 homozygous unrolled: 2 segregating: 1 homozygous rolled, with a probability level of 0.34–0.99 (Table 2). When the percentage of rolled plants per family was considered, a discrete distribution was apparent in almost all crosses (Fig. 1).

During 1990 DS, to lessen the effect of soil moisture variability, the F_2 rows were randomized and the rolling scores were taken at 1600–1750 h, when the difference in rolled and unrolled leaves was clearly visible. By this time the leaves of some of the



1. Distribution of plants of F_3 lines with rolled leaves under drought stress for crosses between 5 resistant (IR33353, IR26702, ITA186, Salumpikit, MGL2) and 2 susceptible (IR29692, IR29725) rices. IRRI, 1989 dry season.

Table 3. Leaf rolling and unrolling of F_2 populations and F_3 families, 1990 dry season.

Cross	F_2 plants			F_3 families			
	Rolled (no.)	Unrolled (no.)	P (3:1)	Rolled (no.)	Segregating (no.)	Unrolled (no.)	P (1:2:1)
IR33353/IR29692	252	104	0.08	24	30	20	0.22
IR26702/IR29692	249	102	0.09	16	42	17	0.59
ITA186/IR29692	175	74	0.09	14	31	21	0.43
IR29692/Salumpikit	181	81	0.04	12	42	18	0.22
IR29692/MGL 2	253	96	0.30	12	37	21	0.28
IR33353/IR29725	284	107	0.31	17	35	21	0.76
IR26702/IR29725	295	114	0.20	13	45	15	0.17
ITA186/IR29725	242	100	0.08	21	30	17	0.49
IR29725/Salumpikit	116	62	<0.01	19	30	21	0.47
IR29725/MGL 2	257	99	0.17	19	27	22	0.21

moderately resistant plants had unrolled, while those of the intermediate and susceptible types were still rolled. At 0600–0900 h, intermediate type plants showed partial rolling. The tolerant types were counted between 0900 and 1600 h. During 1990 DS, 8 of 10 crosses had amonogenic segregation pattern in the F_2 (Table 3). The F_3 showed a segregation ratio of 1 rolled: 2 segregating: 1 unrolled in all crosses.

Lines like IR29725 and IR20 (check) that started rolling earlier at –0.1 MPa had a score of 9 for drought resistance at –1.0 MPa drought stress. IR29692 started rolling later and had a score of 7. IR26702, the resistant parent, also rolled as stress continued, and had a desiccation score of 5 at –1.0 MPa stress. MGL 2 and ITA186 rolled after – 0.5 MPa of stress, and they had scores of 3 at – 1.0 MPa stress. Salumpikit and IR33353 still had scores of 1 and did not roll at – 1.0 MPa stress even at midday; but as the drought continued after this, IR33353 started rolling within 1 wk, but Salumpikit still did not roll. This suggests a close relationship between leaf rolling and drought resistance score. Plants showing leaf rolling at early stages of stress appear to have poor drought resistance.

During 1989 DS, marked differences were observed in the parents of crosses ITA186/IR29692, ITA186/IR29725, IR26702/IR29692, and IR26702/IR29725 in drought score at –1.0 MPa stress or higher. Plants whose leaves rolled at the initiation of drought had a susceptible score (5, 7, or 9), and the unrolled plants had a tolerant score (1 or 3). Similar observations were made during 1990 DS, when the stress was more severe and more uniform than in 1989, since there was no rain during the stress period.

Transgressive segregants (TS) for drought resistance were observed after –1.0 MPa soil moisture stress in all the crosses except those with Salumpikit. These plants or lines had better leaf rolling characteristics and drought resistance than the resistant parents. In the Salumpikit crosses, no TS were observed, because this parent had a high score for resistance. Susceptible and highly susceptible TS were also observed compared

with the parents. TS in the present study might be due to accumulation of more favorable genes for traits related to drought resistance. The two susceptible parents used in the study were not highly susceptible as observed in TS, and they might have some minor genes for resistance.

Loresto et al (1976) and Murty and Ramakrishnayya (1982) reported marked differences in drought recovery 12–20 h after rain or irrigation. During observation of individual plants, tagged plants with unrolled leaves recovered more rapidly after relief of stress than did plants with rolled leaves. Crosses with Salumpikit had more lines with high recovery ability. A positive relationship between resistance and recovery ability was also observed by Malabuyoc et al (1985). The drought-susceptible line IR29692 showed better recovery ability than IR20 or IR29725. Other resistant parents had quick recovery.

An earlier study at IRRI showed that a drought-tolerant variety (IR52) had lower canopy temperature (lower by 2°C) at the vegetative stage than IR36; it also had 24% higher root length density in the upper 30cm of soil (IRRI 1983). This indicates that leaf rolling is a symptom that plant leaves are unable to meet the evapotranspiration demand of the plant and to maintain low canopy temperature and high plant water potential under stress. This may also be attributed to the shallow rooting behavior of the plant, which prevents it from extracting moisture from the soil when stress increases. These plants show the early symptoms of tissue death and have higher desiccation, leading to poor drought resistance. In unrolled leaves, on the other hand, photosynthesis continues, and plant metabolism remains normal. Thus, leaf rolling is an early and clearly visible indicator of drought susceptibility.

This study suggests that the effects of major genes on leaf rolling can be detected in segregating populations. More studies are needed to determine if the genes in different parents are allelic. As delayed leaf rolling is related to drought resistance, these genes need to be incorporated into higher yielding varieties.

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Notes

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Genetic analysis for salt tolerance in rice

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Two pot culture diallel experiments, using six rice varieties with different levels of salt tolerance, were carried out, one in normal and the other in salinized soil. A comparison of the estimates of genetic parameters under the two situations provided information on the influence of salinity stress on gene action for various traits. Additive and dominance gene effects were significant for days to flowering, plant height, tiller number, panicle length, number of spikelets per panicle, 1000-grain weight, and dry matter accumulation under both normal and salinized conditions. However, the additive effect was significant for grain yield only in the salinized situation. Salinization suppressed to a greater extent the dominance effect, contributing to the expression of grain yield, suggesting that varieties with predominantly more additive genes for grain yield would perform better in saline soils. Selection on the basis of traits like number of spikelets per panicle, 1000-grain weight, and dry matter accumulation, which showed high heritability values, could lead to the development of salt-tolerant cultivars.

The presence of excess salts in the soil is one of the most serious problems limiting agricultural production. The threat of soil salinity looms large in the 230 million ha of the world's irrigated area (McWilliam 1986) that produces almost half of the world's food (Christiansen 1982). With the increasing need to use brackish water for irrigation, production of the rice crop, the staple food of the majority of the world's population, is considerably affected by salinity. About 27 million ha of potential riceland in the humid tropics of Asia lies uncultivated because of salinity (Akbar and Ponnamperuma 1980). In India, nearly 4 million ha of riceland is affected by salinity (Paul and Ghosh 1986).

A sure and cost-effective means of tackling the salinity problem is the use of salt-tolerant varieties, but only recently have rice breeders evidenced interest in their development. Studies on the genetics of salt tolerance have been limited, inhibiting the realization of breeders' goals. The present study was therefore planned to discover the nature of gene action governing salt tolerance in rice by examining various traits under normal and saline environments through diallel analysis of parents with different levels of tolerance for salt stress.

Materials and methods

Genetic analysis for salt tolerance was done using six rice varieties of differing salt tolerance levels: IR20 and IR50 (susceptible), CO 43 and Manoharsali (moderately tolerant), and Dasal and Pokkali (highly tolerant). After confirming their tolerance levels using the salinized soil method and the hydroponic method, they were crossed in all possible combinations to get a complete 6 × 6 diallel set comprising 30 F_1s. The F_1s and parents were screened for salt tolerance using the salinized soil method and were scored for salt tolerance using a 1–9 scale. The experiment was laid out in a complete randomized block design with six replications in two sets, one in normal soil (control) and the other in salinized (stressed) soil, in plastic tubs (45 × 30 × 45 cm). In the set evaluated under salt stress, 10 kg of soil was placed in each plastic tub and was salinized with 6 liters of 0.3% NaCl solution, so that the electrical conductivity was raised to 4.9 mmho/cm uniformly in all the tubs. The tubs of the control and the stressed diallel sets were kept in the nethouse and subjected to similar light and other environmental conditions. Labeled 20-d-old seedlings of the parents and the different crosses were planted singly and randomly in the tubs at a spacing of 15 × 10 cm so that each tub carried 6 seedlings, Normal cultural practices were followed, and the tubs were irrigated daily to maintain a water level of 1 cm above the soil level. Once a week, the soil between the plants was carefully raked to facilitate mixing and aeration. The plants were grown to maturity, and data were recorded for 10 characters. The salinized and stressed diallels were analyzed separately according to Hayman's approach (Hayman 1954a,b; 1957; 1958). The same data were also subjected to analysis as a factorial experiment to estimate the extent of genotype × salinity interaction (Gomez and Gomez 1984).

Results and discussion

There was marked suppression of growth in the salinized plants as compared with those growing in normal soil. A number of characters were studied in the normal and salinized diallel sets, and the results obtained are detailed below.

Screening of F_1s

The average susceptibility scores of the F_1s and parents comprising the 6 × 6 diallel set are given in Table 1. In general, the hybrids scored lower than at least one of their parents, indicating a tendency for salt tolerance expression to have a dominant bias in the F_1. All the crosses involvhg highly tolerant Pokkali and Dasal as parents scored between 1.2 and 3.1 except Manoharsali/Dasal (5.7). Among all the parents and hybrids, IR20 was the most susceptible genotype, with a score of 8.2, while the hybrids Dasal/IR50, DasalKO 43, Pokkali/Manoharsali and Pokkali/Dasal were the most tolerant with the score of 1.0. All crosses between moderately tolerant CO 43 and Manoharsali and susceptible IR20 and IR50 had scores ranging from 2.2 (IR20/CO 43) to 5.7 (Manoharsali/IR50); the hybrids were more tolerant when the susceptible

Table 1. Mean salt susceptibility scores[a] of parents and F_1 hybrids from the 6 × 6 diallel set at the seedling stage.

Parent	Susceptibility score					
	IR20	IR50	C043	Manoharsali	Dasal	Pokkali
IR20	8.2	6.9	2.2	2.3	2.1	1.7
IR50	7.2	8.0	2.4	2.7	1.8	3.1
CO 43	3.0	3.7	5.2	3.1	2.3	1.3
Manoharsali	4.3	5.7	2.3	6.3	5.7	3.1
Dasal	1.7	1.0	1.0	1.2	1.7	2.3
Pokkali	3.0	1.7	2.3	1.0	1.0	1.2

[a]1–9 rating scale: 1 = tolerant, 9 = susceptible.

Table 2. Mean sums of squares for characters under control and stressed conditions.

Character	Mean sum of squares[a]		
	Control	Stressed	Genotype × salinity interaction[b]
Days to flowering	106.49**	81.00**	28.09**
Plant height	214.14**	210.79**	557.64**
Tiller number	17.49**	23.32**	52.45**
Percentage productive tillers	5.39**	1.44ns	98.94**
Panicle length	91.32**	26.57**	11.75**
Number of spikelets per panicle	14.37**	35.27**	1153.67**
Percentage seed set	14.43**	87.72**	262.24**
Grain yield per plant	49.97**	49.73**	661.24**
1000-grain weight	166.70**	173.87**	4.48**
Dry matter accumulation per plant	78.53**	126.48**	2429.90**

[a]ns = nonsignificant, ** = significant at the 1% level. [b]Obtained from factorial analysis.

varieties were used as female parents. These results are consistent with those obtained by Akbar and Yabuno (1977) and Moeljopawiro and Ikehashi (1981). Paramasivam (1979) also reported that most hybrids between tolerant and susceptible rice varieties were tolerant; it was thus possible to incorporate salt tolerance through hybridization between tolerant and susceptible parents.

Gene action for different traits

The mean sums of squares for the various characters under control and stressed conditions from the analysis of variance, and those for genotype × salinity interaction from the factorial analysis are given in Table 2. All characters except percentage productive tillers under stressed conditions showed significant variation between

Table 3. Estimates of genetic parameters for characters in the control diallel set.[a]

Character	D	F	H_1	H_2	h^2	$(H_1/D)^{1/2}$	$H_2/4H_1$	K_D/K_R[b]	h^2/H_2	Narrow sense heritability (%)
Days to flowering	422.47**	137.36**	178.14**	138.81**	8.82	0.65	0.20	1.67	0.06	81.7
Plant height	1247.23**	339.18	1078.85**	720.10**	41.21	0.93	0.17	1.34	0.06	77.5
Tiller number	27.34**	20.10	45.01**	37.62**	35.35**	1.28	0.21	1.80	0.94	40.5
Percentage productive tillers	1.57	0.94	48.58**	40.08**	19.61	5.57	0.21	1.11	0.49	22.3
Panicle length	18.27**	16.80**	18.47**	11.46**	0.11	1.01	0.16	2.68	0.01	59.1
Number of spikelets per panicle	216.48**	67.36	872.23**	662.70**	778.53**	2.01	0.19	1.17	1.18	47.2
Percentage seed set	12.78**	8.62	44.65**	35.74**	51.09**	1.87	0.20	1.44	1.43	39.2
Grain yield per plant	62.27	102.09	714.71	580.26**	143.45	3.39	0.20	1.64	0.25	23.9
1000-grain weight	24.94**	4.65	9.13**	7.37	0.00	0.61	0.20	1.36	0.00	85.1
Dry matter accumulation per plant	1584.37*	742.66	5373.72**	3944.50**	1705.85	1.84	0.18	1.29	0.43	52.8

[a] Significant at the 5% (*) and 1% (**) levels. D = component of variation due to additive effect. F = mean value of Fr over arrays where Fr = the covariance of additive and nonadditive effects in the rth array. H_1 = component of variation due to dominance effect. $H_2 = H_1 (1- (u-v)^2)$, where u = proportion of positive genes in parents, v = proportion of negative genes in parents and (u-v) = 1. h^2 = dominance effect (as the algebraic sum over all loci in heterozygous phase in all crosses). $(H_1/D)^{1/2}$ = mean degree of dominance in F_1. $H_2/4H_1$ = proportion of genes with positive and negative effects in the parents. h^2/H_2 = number of groups of factors controlling the character and exhibiting dominance. [b] $K_D/K_R = (4DH_1)^{1/2} + F/(4DH_1)^{1/2} - F$.

genotypes. Estimates of the genetic parameters for the characters in the diallel set in normal soil and under stressed conditions are in Tables 3 and 4, respectively.

In this study, assumptions about the diallel set were fully satisfied by days to flowering, plant height, panicle length, number of spikelets per panicle, and 1000-grain weight in both control and stressed situations. Grain yield per plant satisfied the assumptions in the stressed situation alone. It therefore appears that the simple additive-dominance model is adequate to account for the expression of these traits. Expression of the remaining traits, namely tiller number, percentage seed set, and dry matter accumulation per plant under both situations, and grain yield in the nonstressed situation, could have involved some form of nonallelic gene interaction. Akbar et a1 (1985) made similar observations.

Of the 10 characters studied, all except percentage of productive tillers and grain yield per plant showed significant additive effects in the control diallel (Table 3). In the stressed diallel, all eight yield-related characters showed highly significant additive

Table 4. Estimates of genetic parameters for characters in the stressed diallel set.[a]

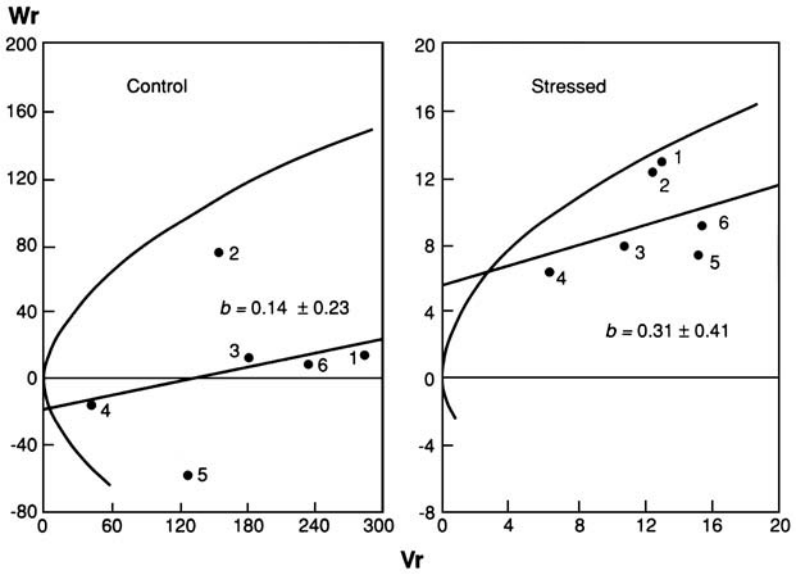
Character	D	F	H_1	H_2	h^2	$(H_1/D)^{1/2}$	$H_2/4H_1$	K_D/K_R[b]	h^2/H_2	Narrow sense heritability (%)
Days to flowering	360.52**	147.51*	227.06**	180.14**	–0.26	0.79	0.20	1.70	–0.00	73.4
Plant height	1180.52**	592.31*	1048.24**	746.35*	66.60	0.94	0.18	1.73	0.09	70.1
Tiller number	23.74**	21.87*	43.87	33.24**	15.78**	1.36	0.19	2.02	0.48	40.7
Panicle length	15.37**	22.37**	22.98**	12.65**	3.12	1.22	0.14	3.94	0.25	33.0
Number of spikelets per panicle	1794.99**	596.53**	1182.67**	1050.52**	2158.66**	0.81	0.22	1.52	2.06	69.7
Grain yield per plant	14.27**	–9.17	23.55**	19.84**	39.17**	1.28	0.21	0.60	1.97	71.7
1000-grain weight	18.56**	–0.47	4.61**	3.87**	4.71**	0.50	0.21	0.95	1.22	90.5
Dry matter accumulation per plant	1281.29**	–83.68	2025.67**	1448.00**	1242.19**	1.26	0.18	0.95	0.86	72.3

[a]Diallel anaiysls for percentage productive tillers and percentage seed set were not done in the stressed diallel. Significant at the 5% (*) and 1% (**) levels. D = component of variation due to additive effect. F = mean value of Fr over arrays where Fr = the covariance of additive and nonadditive effects in the rth array. H_1 = component of variation due to dominance effect. $H_2 = H_1(1–(u–v)^2)$, where u = proportion of positive genes in parents, v = proportion of negative genes in parents and (u–v) = 1. h^2 = dominance effect (as the algebraic sum over all loci in heterozygous phase in all crosses). $(H_1/D)^{1/2}$ = mean degree of dominance in F_1. $H_2/4H_1$ =proportion of genes with positive and negative effects in the parents. h^2/H_2 = number of groups of factors controlling the character and exhibiting dominance. [b]$K_D/K_R = (4DH_1)^{1/2} + F/(4DH_1)^{1/2} – F$.

effects (Table 4). When additive effects were nonsignificant for grain yield in the normal situation, they became highly significant in the salinized situation. At the same time, the magnitude of expression was greatly reduced because of salinity.

The Wr, Vr graphs for grain yield per plant (Fig. 1), although suggesting nonallelic interaction, showed the presence of overdominance in the control diallel, which was reduced to partial dominance in the stressed diallel, as revealed by the intercepts of the regression line with the Y-axis in the two situations. It can therefore be inferred that the part of grain yield accounted for by additive gene action was less affected by salinity than was the remaining part governed by nonadditive gene action. This inference is also supported by the fact that the narrow sense heritability for grain yield per plant increased from 23.9 to 71.7% on salinization. Therefore, it may be expected that in a variety with more additive genes for grain yield, yield suppression will be relatively less on salinization. Jones (1985) concluded that genetic variation for salt tolerance in rice is principally due to additive gene effects.

In the F_2, seeds from the same set of F_1s as the 6 × 6 diallel set were evaluated along with their parents in a pot culture diallel experiment with one set in normal soil and another set in salinized soil. The methodology was the same as that in the F_1. Genetic



1. Grain yield per plant. 1 = IR20, 2 = IR50, 3 = CO 43, 4 = Manoharsali, 5 = Dasal, 6 = Pokkali.

analysis of the nature of gene action in the F_2 revealed the predominant and significant nature of additive gene action for days to 50% flowering, percentage seed set, 1000-grain weight, and grain yield in salinized and normal soil, while days to flowering and 1000-grain weight showed significance for additive effects only in normal soil. Dominant gene action was significant for panicle weight in both situations, and for number of tillers per plant under saline conditions. In the F_2, additive gene action was consistently significant for grain yield under salinized conditions as in the F_1. In this aspect, the nature of gene action in the F_1 and the F_2 was similar for yield in saline soil (Krishnaraj and Sree Rangasamy 1989).

Furthermore, significant additive gene action was consistently significant for grain yield per plant in the F_2 under both normal and saline conditions as well as under field conditions (Krishnaraj and Sree Rangasamy 1989).

Estimates of narrow sense heritability were high for days to flowering, plant height, and 1000-grain weight under both normal and stressed environments. In addition, number of spikelets per panicle, grain yield per plant, and dry matter accumulation per plant had high heritability values in the stressed diallel. In saline environments, therefore, number of spikelets per panicle, 1000-grain weight, and dry matter accumulation, which are important components of yield, can be used as selection criteria to improve yield potential.

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Inheritance of panicle exsertion in *Oryza sativa* under low temperature

D.M. Mahishi, M. Mahadevappa, and P. Gopala Reddy

Hybridization of cold-tolerant cultivars Halubblu and Mangala with cold-sensitive varieties Jaya, IR8, and Prakash revealed the pattern of inheritance of panicle exsertion under low temperature. The F_1 plants of Jaya/Halubblu, Prakash/Halubblu, and IR8/Mangala showed well-exserted panicles as a dominant trait. In the F_2, the segregation for well-exserted vs partially exserted panicles fitted a 3:1 ratio, indicating monogenic inheritance of this trait. The gene symbols *Pe* (dominant) for well-exserted panicle and *pe* (recessive) for partially exserted panicle are proposed.

Nearly 1.8 million ha of India's rice crop is grown at high altitudes with average temperature below 18°C (Hamdani 1979). The rice plant in this climate is subjected to cold injury, leading to low germination, stunted growth combined with delayed heading, incomplete panicle exsertion, and sterility (Nanda and Seshu 1979). Most of the modern high-yielding varieties (HYVs) are not adapted to cold conditions, although one or two varieties like Mangala and Kalinga are grown at high altitudes during the winter season.

A hybridization program to transfer specific traits contributing to cold tolerance from local cultivars to HYVs was initiated. In the segregating generations derived from crosses between cold-tolerant and cold-susceptible genotypes, the pattern of inheritance for traits related to cold tolerance was studied. This paper reports on the inheritance of panicle exsertion as influenced by low temperature.

Materials and methods

Two locally grown varieties of rice were used: Halubblu (S-317), which is tall and photoperiod-insensitive and has a medium maturity period; and Managala, which is semidwarf, photoperiod-sensitive, and early maturing. HYVs Jaya, Prakash, and IR8, which are susceptible to cold injury, were crossed with Halubblu and Mangala in the following combinations: Jaya/Halubblu, Prakash/Halubblu, and IR8Mangala.

Table 1. Segregation pattern for panicle exsertion in rice under low temperature.

Cross	Expression in F_1	Expression (no.) in F_2		c^2 value[a] (3:1)
		Well-exserted	Partially exserted	
Jaya/Halubblu	Well-exserted	246	94	1.27ns
Prakash/Halubblu	Well-exserted	427	152	0.59ns
IR8/Mangala	Well-exserted	320	121	1.47ns

[a]Table value of c^2 at the 5% level is 3.84. ns = nonsignificant.

The F_1 and F_2 plants obtained from the crosses were grown during the 1986–87 winter season at the Main Research Station of the University of Agricultural Sciences, Bangalore, India, at an altitude of 899 m above mean sea level, 13°N and 77º31'E. The average temperature during the winter months (September to December) ranges from 15 to 19 °C (Ravikumar 1989).

Individual plants of the F_1 and F_2 were classified as well exserted or partially exserted. The expected and observed ratios were subjected to the chi-square test.

Results and discussion

In all three crosses involving cold-tolerant and cold-susceptible genotypes, the F_1 expressed well-exserted panicles (Table 1), indicating that the well-exserted panicle trait is dominant. In the F_2, wherein 246–427 plants were screened for each cross, panicle exsertion under low temperature showed clear segregation into a 3:1 ratio of well-exserted vs partially exserted. The chi-square test also confirmed the fitness of the ratio to 3:1, thus indicating monogenic inheritance of this trait. The following gene symbols are proposed for the trait of panicle emergence in rice under low temperature.

Well-exserted panicle = *Pe* (dominant)
Partially exserted panicle = *pe* (recessive)

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Discussion

Session 3: Genetics of stress tolerance

Q—*Narayanan:* Do you have any information on the salt tolerance of the drought-tolerant varieties you have included in your studies? One of the effects of salt stress being dehydration, one can expect a strong correlation between the two traits.

A—*Singh:* I have not studied the salt tolerance of these lines. But Dr. M. Akbar has observed that drought tolerance and salt tolerance are not related.

C—*Akbar:* In preliminary studies I could not find any correlation between drought tolerance and salt tolerance during the seedling stage.

Q—*Cruz:* You measured drought tolerance by using scores from 1 to 9, but it seems that there was continuous variation in the phenotypes of the materials, from completely tolerant to susceptible. How suitable is it, then, to classify the drought tolerance trait as a monogenic character?

A—*Singh:* Leaf rolling is the first symptom of drought tolerance at 0.05–0.1 MPa soil moisture tension. Plants with rolled leaves at this level show a susceptible reaction to drought. Unrolled leaves at this level are either resistant or moderately resistant. Continuous variation in the score may be due to other genes. I do not want to conclude that it is a monogenic trait, but rather that one or two recessive genes with major effect are involved.

Q—*Oka:* Have you tested wild rice strains for drought tolerance by the aeroponic or any other method?

A—*Singh:* We have tested *O. glaberrima* for drought tolerance in the field. One line, Alkana, is highly resistant. We have not tested drought tolerance by aeroponics.

Q—*Oka:* Are the genes you have detected for leaf rolling and recovery independent?

A—*Singh:* They seem to be interdependent. The unrolled types have better recovery ability than the rolled types.

C—*Ikehashi:* I am doubtful about the applicability of the diallel method for analysis of stress tolerance. Stressed plants show a small range of variation and contribute little to the whole variance. Less stressed plants occupy a large part of the whole variance. So variance analysis would reflect mostly the behavior of less stressed plants.

C—*Sree Rangaswamy:* I agree. There is a suppression of variability for some characters but not for all. Before doing the diallel analysis, the variance for all characters was estimated to ensure that the variance was significant. For percentage of productive tillers, variance was not significant under the stressed condition, and therefore analysis was not carried out for this trait.

Q—*Mishra:* Our experience with salinity tolerance has very clearly demonstrated the maintenance of a defined level of salinity stress as an essential component for such studies. Salinity stress as shown during your presentation is less—nearly

parallel to normal soil. Do you think that such genetic information derived from such low salinity will be helpful in our future breeding program for salt-affected soils? Second, what type of breeding strategy is recommended based on your genetic studies? Third, what is the agreement between vegetative phase score and reproductive phase tolerance? Our experience with a large spectrum of populations has shown no association of vegetative phase score with reproductive phase tolerance.

A–*Sree Rangaswamy:* I do not entirely agree with your observation. The normal electrical conductivity in Coimbatore is around 2.5 mmho/cm, while in our experiment the initial level was 4.9 mmho/cm, and by the end of the experiment it rose to 8.5 mmho/cm. Susceptible IR20 was greatly suppressed and stressed. We have not estimated how much the scores in the vegetative phase correlate with the scores in the reproductive phase. Our observation is that the plants become more tolerant as they reach maturity. For breeding we suggest the pedigree method.

Q–*Akbar:* Did you notice reciprocal differences for salt tolerance in your experiments?

A–*Sree Rangaswamy:* We noticed reciprocal differences for salt tolerance with respect to some traits.

Q–*Brar:* Can you draw any conclusion about the allelic relationship of genes governing salt tolerance in tolerant genotypes Pokkali and Dasal?

A–*Sree Rangaswamy:* Our observations suggest that the genes governing tolerance in Pokkali and Dasal are nonallelic.

C–*Singh:* In analysis of 0–9 scores, the value should be transformed to a suitable scale for variance-covariance analysis. Otherwise variance becomes zero in highly tolerant (0) types. The scale can be modified to a 1–9 scale for variance-covariance analysis.

C–*Sree Rangaswamy:* We have not done variance-covariance analysis for the 0–9 scores based on grades of salinity.

SESSION 4

Genetics of Morphological and Physiological Traits

Inheritance of grain size and its implications for rice breeding

K. Takeda

Because the rice kernel develops inside the hull, the size and shape of the kernel are determined by the hull. Grain size may be indicated by weight, volume, or length, but grain length is the most adequate character for analyzing the inheritance of grain size because of the high heritability of the trait. In normal cultivars, grain size is usually controlled by polygenes. In varieties with extraordinary large or small grains, and in mutants for grain size, grain size is controlled by a major gene or genes. The dominant small-grain gene *Mi* decreases kernel weight to 2/3 of normal. The incompletely dominant large-grain gene *Lk-f* develops kernels with 1.4 times normal weight. But because the large-grain type develops fewer spikelets per plant, it does not necessarily outyield the normal counterpart. Varieties with large grains frequently develop grains with white belly or white core. Uniformity of grain size is poor in large-grain isogenic lines. Cold tolerance during the reproductive phase is high in large-grain isogenic lines because they produce more pollen per anther.

Because the rice kernel develops inside the hull, the size and shape of the kernel are determined by the hull. Takeda and Takahashi (1970b) reported that the correlation coefficient between hulled and unhulled grain lengths was more than 0.99. But Morinaga et al (1939) showed that the size and shape of the kernel were different from those of unhulled grain when the tip of the hull was removed immediately after anthesis. Takeda and Takahashi (1970a) clarified that "free" kernels that developed without restriction by the hull were longer than those that developed encased in the hull, suggesting an imbalance in growth between hull and kernel. They found that varieties with a large imbalance between hull length and kernel length frequently develop notched grains or hull-cracked grains.

The proportion of hull weight to whole-grain weight ranged from 18.6 to 25.4%. Assuming a common hull thickness, hull percentage may increase with decrease in grain size, or as the grain shape varies from round to slender; but it was around 21% in most varieties, irrespective of grain size and shape (IRRI 1977). Hull percentage may be affected greatly by degree of grain filling.

Glutinous endosperm and kernel development

Because 90% of the rice kernel consists of endosperm, the nature of the endosperm may affect kernel development. Morinaga and Imai (1943), Suzuta (1954), and Takeda et al (1978) found that in the F_2 seed developed on F_1 plants of glutinous and nonglutinous hybrids, glutinous kernels were significantly lighter than nonglutinous ones, even though their floral glume sizes were the same. Takeda (1980) examined the grain filling process of isogenic lines and showed that glutinous kernels were equal to nonglutinous ones in fresh weight; however, glutinous kernels had more water content and, therefore, lower dry weight. He pointed out that the sink size for dry matter deposit is 7% less in glutinous kernels than in nonglutinous kernels, even if hull size is the same.

Components of grain size

Grain size may be indicated by weight, volume, or length, but weight and length are the commonly used indicators. Grain weight may correlate with the components of grain volume, i.e., length, breadth, and thickness. As Takeda (1986) showed, grain length is the strongest determinant of grain size (Table 1). These statistics are deduced from a sample of 88 rice varieties with a very wide range of variation in grain size and shape (Fig. 1). In many cases, correlations of length with breadth and thickness are not so close.

Because environmental variation in grain length is generally smaller than that in grain weight, the heritability value of grain length is usually higher. Variations in kernel breadth and thickness are much smaller than that in kernel length. Therefore, grain length may be the best character for analyzing the inheritance of grain size.

Quantitative inheritance of grain size

As grain size is one of the most important agronomic traits in rice, many studies have been done on its inheritance. Generally, grain size in commercial varieties with normal grain size is controlled by polygenes. In hybrids between varieties with similar grain size, transgressive segregation due to accumulation of plus or minus factors for grain

Table 1. Variation and correlation of kernel size characters in 88 varieties with a wide range of variation (Takeda 1986).

Kernel character	Correlation coefficients[a] with			Mean	Range
	Length	Breadth	Thickness		
Weight	0.916**	0.370**	0.596**	20.15 mg	5.6–43.7 mg
Length mm		0.044	0.295**	4.91 mm	2.3– 9.7
Breadth mm			0.651**	2.91 mm	2.5– 3.3
Thickness mm				2.04 mm	1.8– 2.4

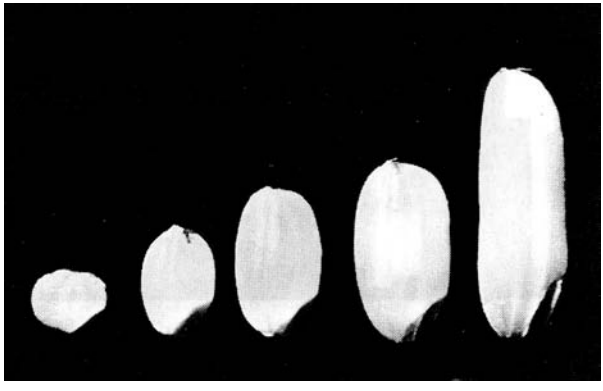
[a] **= significant at the 1% level.

size may occur. Takeda (1983) found significant transgressive segregation for kernel length: the variation in kernel length was 4.3–5.3 mm in an F_2 population from the cross between the traditional Japanese varieties Kamenoo-1 (4.9 mm) and Kairyo-aikoku (4.8 mm).

In crosses between varieties with very large or small grains, grain size is controlled by major gene(s) in some cases, but many cases involve multifactorial segregations. Morinaga et al (1943) analyzed the inheritance of the long-kernel variety Ko-to (9.3 mm) using several crosses with varieties having normal kernel length (around 5 mm). Observing the segregating populations up to the F_4, they concluded that the long kernel of Ko-to was controlled by five cumulative genes. Takita (1985) examined several F_2 populations of crosses between varieties with large and normal kernel size, and found that two to five cumulative genes were responsible for inheritance of kernel length. The author analyzed the inheritance of kernel length using an F_2 population and backcross F_1 plants from a cross between Oochikara (7.0 mm) and Taichung 65 (5.0 mm). In the backcross F_1 as well as in the F_2, kernel length varied continuously, suggesting it was controlled by polygenes. Diallel analyses revealed that grain size is controlled by quantitative genes that adapt to the additive-dominance model (Kato 1989a, Murai and Kinoshita 1986, Tseng 1977).

Chandraratna and Sakai (1960) found matroclinous inheritance for grain weight in indica varieties. The difference between reciprocal crosses was 4.5 mg in the F_1; it was 0.9 mg in the F_2. They analyzed the components of variance to estimate the heritability values, which were 0.77 (broad sense) and 0.40 (narrow sense).

Takeda and Saito (1983) conducted selection experiments to estimate the heritability value for kernel weight, examining hybrid populations from the cross between the large-kernel strain H-347 (27 mg) and the Japanese cultivar Yuukara (22 mg), and the reciprocal crosses up to the F_8. Heritability values estimated from the selection response were around 0.7, irrespective of the generation where selection for kernel weight was conducted. This suggests that kernel weight in this cross was controlled mostly by additive genes.

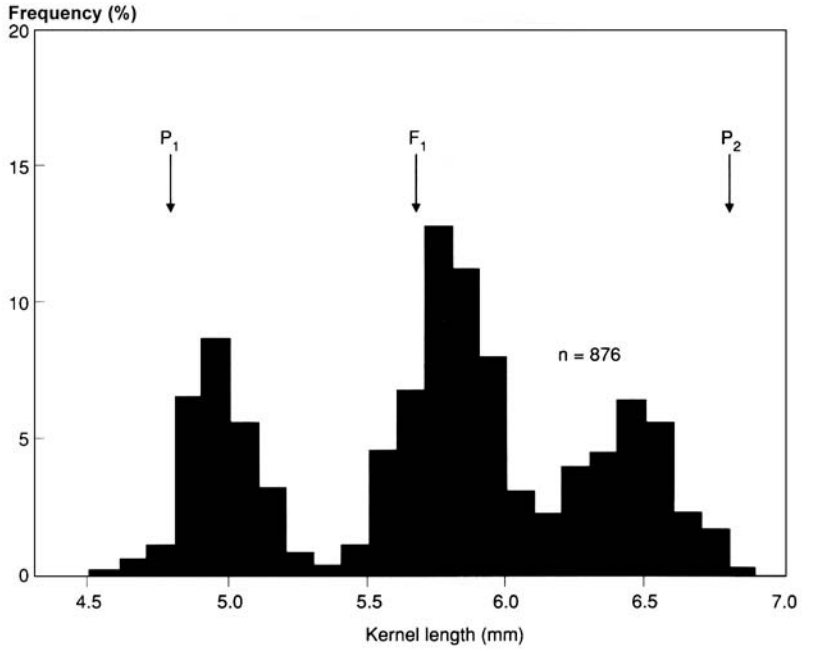


1. Variation in size and shape of rice kernels.

Major genes controlling grain size

In varieties with extraordinarily large or small grains, or in mutant lines for grain size, grain size variation is often controled by major genes. Chao (1928) examined the inheritance of spikelet length in the F_2 population from a cross between lines with short (4.1 mm) and long (8.8 mm) spikelets. The F_2 plants segregated into a 3:1 ratio. Takeda and Saito (1977) examined the inheritance of kernel length using Minute (3.2 mm) and detected a dominant major gene *Mi* controlling short kernel. Many dwarf genes pleiotropically develop short grains; however, *Mi* was independent of the gene controlling plant height, i.e., the average culm lengths of F_2 segregants were 95.3cm and 95.2cm for short and normal grain types, respectively. *Mi* decreases kernel weight to 2/3 of normal.

Shakudo (1951) detected in So-tairyu an incomplete dominant major gene pleiotropically controlling kernel length and plant height. Ikeda (1952) found in Tairyu-to an incomplete dominant major gene pleiotropically controlling kernel length and plant height. Takeda and Saito (1980) also detected in the local variety Fusayoshi an incompletely dominant major gene, *Lk-f,* controlling long kernel (Fig. 2). *Lk-f* also pleiotropically increases culm and panicle length. Judging from their gene action, these three genes could be the same. Takamure and Kinoshita (1985) found that a mutant gene controlling long grain induced in Shiokari was allelic to *Lk-f,* and Kato (1989b) demonstrated that seven long-grain mutants derived from Gin-bozu were controlled by



2. Segregation for kernel length in F_2 population from Shin-2/Fusayoshi.

genes allelic to *Lk-f*. These results indicate that *Lk-f* is a mutable allele. *Lk-f* was linked with *Mi* by a 24% linkage intensity (Takamure and Kinoshita 1983), and *Lk-f* was linked with the awn gene by a 7.6% recombination value (Takeda and Saito 1980). Takamure and Kinoshita (1986) found a recessive gene named *lk-i* in the large-grain variety IRAT13.

Grain size in relation to adaptation

Generally grain size correlates negatively with number of grains per plant, and seed weight correlates closely with seedling vigor (Takeda 1972). Therefore, grain size may affect the fitness of the genotype via seedling vigor and number of seeds. Data were limited, but the author has examined the fitness of the *Mi* gene. Hybrid populations from a cross between H-347 (*mimi*) and H-346 (*MiMi*) were grown up to the F_6 by the single seed descent method. The proportion of small-grain plants decreased with each generation. The observed proportion of plants with small grains was significantly lower than the theoretical value, assuming that the fitness of the small-grain type (*MiMi* and *Mimi*) was equal to that of the large-grain one (*mimi*), but it was very close to the calculated value when the selection coefficient was estimated at 0.17 for the small-grain type. Thus only 83% of the small-grain type may be able to survive in a community mixed with large-grain type. Generally, small-grain plants bear more seeds than large-grain plants, and the small-grain genotype and the large-grain genotype may equilibrate in the natural population. Detailed studies with an ecological perspective are needed in this area.

Grain size in relation to quality

The larger the grain is, the higher the frequency of grains with white belly or white core. Takeda and Saito (1983) analyzed the relationship between kernel weight and frequency of kernels with white belly using four F_2 populations with a large variation in kernel weight. The phenotypic correlation coefficients between the characters were 0.24–0.81. In an F_2 population from Shin-2/Fusayoshi, the long-kernel gene *Lk-f* pleiotropically increased the frequency of white belly.

In Japan, the Sakamai group of varieties is bred especially for brewing sake. Sakamai varieties are characterized by large, round grains (26–28 mg) with high frequency of white core. However, the grain weight of all Japanese cooking rice varieties is 22–24 mg, because the appearance of medium- to small-size kernels is better than that of large ones. Extraordinarily large kernels are easily broken during milling.

Since the small-grain gene *Mi* restricts hull length more severely than kernel length, the genetic imbalance between kernel length and hull length is more serious in small-grain plants than in normal ones (Takeda 1982). Notched grains frequently develop in small-grain plants because the imbalance between the lengths of kernel and hull is the primary factor determining the occurrence of notched grains. Breeders should take note of the genetic imbalance between hull size and kernel size when they breed for grain size.

Uniformity of grain size within a genotype is commercially important. The author examined the effect of *Lk-f* gene on the uniformity of grain weight using 12 isogenic pairs except for *Lk–f*. All fertile grains on the primary and secondary rachis branches were weighed, one by one, and the mean and the coefficient of variation (CV) were calculated. Irrespective of the isogenic line, the mean weight of grains on primary rachis branches was 10% higher than that on secondary rachis branches (Table 2). On the other hand, the CV of the weight of grains developing on secondary rachis branches was 30% larger than on primary rachis branches. This suggests that grain filling on secondary rachis branches is inferior to that on primary ones.

Irrespective of rachis branches, grains were 30% heavier in large-grain lines, and the CV was 24% larger. The frequency distribution of spikelet weight indicated that, in normal-grain lines, most kernels grew to the limit of grain size, but in large-grain lines, many kernels did not reach the potential upper limit. Thus, increasing the upper limit of grain size without improving the source of photosynthate may spoil the uniformity of kernel weight.

Grain size in relation to cold tolerance

Sterility due to cool weather during the reproductive phase may occur at high latitude or elevation. Because spikelet sterility due to cold injury is caused by pollen sterility, varieties with more pollen per anther and/or with long stigma to receive more pollen per ovary may be tolerant of cold injury. Examining a wide range of varieties, Suzuki (1981) reported that the correlation coefficient between anther length and number of pollen grains per anther was as high as 0.94. Suzuki (1982) found in an F_2 population that the sterility index under low temperature conditions correlated with anther length ($r = -0.36$) and stigma length ($r = -0.55$).

Koike et al (1990) examined the cold tolerance of 12 isogenic pairs except for *Lk-f* to check the effect of grain size. As shown in Table 3, the anther volume of the large-grain lines was 40% greater than their normal counterparts, while pollen size was the same in isogenic pairs. Thus the number of pollen grains per anther of the large-grain lines was also 60% greater than that of their normal counterparts. The cold tolerance of large-grain lines was remarkably higher than that of normal counterparts.

Table 2. Mean weight and CV of grain weight for primary or secondary rachis branch of 12 isogenic line pairs except for grain size.[a]

	Mean weight (mg)	CV of weight (%)
Normal		
Primary	26.74	8.58
Secondary	24.05	11.25
Whole	25.46	11.21
Large		
Primary	35.28	10.68
Secondary	31.23	13.97
Whole	33.50	13.52

[a] Normal vs large, and primary vs secondary groups are significantly different at the 1% level.

Grain size in relation to yield

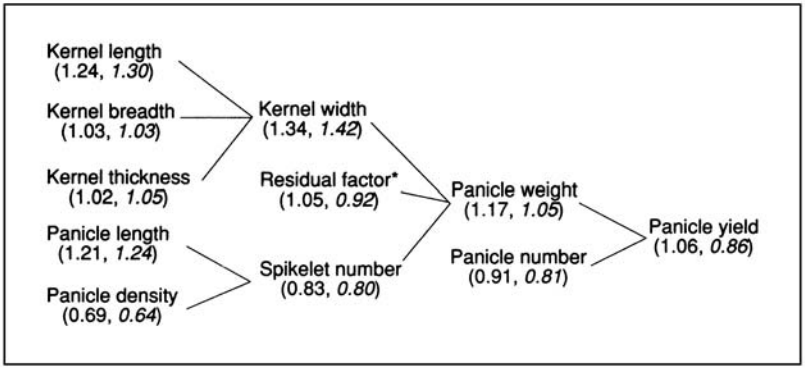
To investigate the effect of grain size on various agronomic traits including yield potential, Takeda et al (1987) examined 38 isogenic pairs except for *Lk-f* in southern and northern Japan. Plants with *Lk-f* developed 1.4 times heavier kernels than did their isogenic counterparts, but plants with large grains bore fewer spikelets per panicle and fewer panicles per plant (Fig. 3).

Genotype (*Lk-f* vs *lk-f*) × environment (location of cultivation) interaction was significant for grain yield. In northern Japan, most large-grain lines showed performance inferior to that of their normal-grain counterparts, while many large-grain lines performed well in southern Japan. Even though yield is a product of the number and weight of kernels, large kernels do not necessarily cause high yield because of the general negative association between kernel number and weight. For increasing yield potential, the source of photosynthate as well as the sink capacity (size × number of grains) must be improved. As grain size per se does not determine yield potential, breeders should select a kernel size that takes into consideration commercial value and local cultivation practices.

Table 3. Mean size of anther, number of pollen grains, and cold tolerance in 12 isogenic line pairs except for grain size.[a]

Grain size	Anther length (mm)	Anther breadth (mm)	Anther volume (mm^3)	Pollen diameter (μm)	Pollen per anther (no.)	Cold tolerance[b] at 3d[c]	Cold tolerance[b] at 5d[d]
Normal	2.0	0.45	0.41	49	978	67	33
Large	2.7	0.46	0.57	49	1573	92	63

[a] Normal- vs large-grain groups are significantly different at the 1% level, except in pollen diameter. [b]Spikelet fertility of the treated plants relative to the control. [c]3-d chilling at 12°C. [d] 5-d chilling at 12ºC.



3. Relative performance of large-grain lines for yield components in 38 isogenic pairs except for grain size, examined in the southern (roman) and northem (italic) parts of Japan. * = estimated value.

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Notes

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Genetics and biochemistry of in vitro-selected lysine mutants

G.W. Schaeffer

Lysine is an important and limiting amino acid for optimal nutritional quality of rice. Experiments were done to provide bridge technologies between biochemical selections and whole plant genetics and to characterize progeny from in vitro selections for altered lysine in established cultivars. Lysine mutants were recovered by growing anther culture-derived callus with inhibitors affecting the lysine pathway, namely inhibitory levels of lysine plus threonine and s-(aminoethyl) cysteine, an analog of lysine. Plants were regenerated from resistant cells. Progeny, crosses, F_2s, F_3s, F_4s, and backcrosses were analyzed for percentage lysine in endosperm proteins, chalkiness, and seed weight. Seed storage proteins were fractionated into solubility classes, amino acid levels were determined, and high lysine fractions were identified. The mutants conditioning modified lysine are recessive, and increased lysine was correlated with decreased seed size, although available segregants have normal seed size. The mutants have higher endosperm lysine in all protein solubility fractions, but the largest shift occurred in the globulin fraction. In vitro selections thus produce complex phenotypes with predictable lysine levels.

The nutritional quality of rice would be improved by higher protein and lysine percentages (Mertz et al 1964). Experiments were designed to test whether lysine pathway mutants that might be useful for basic studies or germplasm enhancement could be recovered from anther or tissue culture. The initial objectives were to recover mutants and to contribute to bridge technologies between biochemical techniques in the laboratory and the problems plant breeders face in cultivar development. Two useful bridge technologies in rice are anther culture and in vitro inhibitor selection. Cells were inhibited with millimolar (mM) lysine plus threonine (lys+thr). These two amino acids cooperatively inhibit the lysine pathway of wild type cells and permit the growth of mutant cells. The hypothesis was that cells that survive lys+thr selection pressure are insensitive to feedback inhibition or have alterations in other regulatory aspects that permit the deposition of higher levels of lysine and/or protein in the seed. Mutants conditioning levels of lysine in the seed were sought. Cells resistant to the

inhibitors were recovered, plants regenerated, and crosses made to identify and characterize the mutants.

Materials and methods

The procedures for mutant selection (Sano and Shiio 1970; Schaeffer and Sharpe 1981, 1983) and plant regeneration and progeny characterization (Schaeffer and Sharpe 1987, Schaeffer et al 1989) have been published, and only their most salient features are included here. Protocols included description of the mutants, the parents of crosses, and the progeny of crosses, as well as amino acid analyses of protein hydrolysates of single half seeds of mutants, parents, and progeny and hydrolysates of bulk samples from field-grown material. Endosperm proteins representing source or parental materials, mutants, and selfed progeny were fractionated into five solubility classes, and each fraction was analyzed for amino acid composition. Each amino acid is expressed as the percentage of total amino acids in acid hydrolysates.

Genetic sources

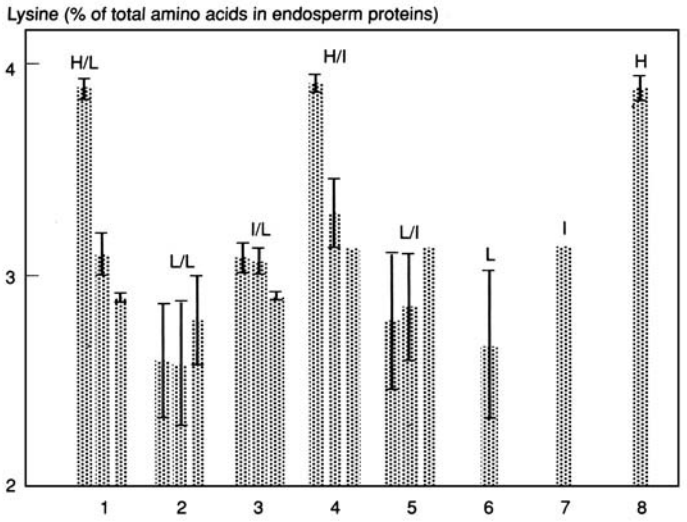
Biochemical selections, parents of crosses, progeny descriptions and characterizations, description of seed chalkiness, and methods of amino acid analyses were the same as those described by Schaeffer et al (1986, 1989). The mutant line was referred to as 4C, and the control cultivars were Calrose 76, the source of the original explants, and M101. Ml0l is a marker line homozygous for a recessive glabrous gene. Assam 5, of the subspecies near-indica, with an intermediate lysine level, was used as parental material in one cross (data set 5, Fig. 1). All the mutant lines were regenerated from anther culture-derived tissue cultures grown on inhibitory levels of lys+thr.

Protein fractionations

Single-seed analyses for lysine and protein levels were done with the endosperm half of dehulled seed. The embryo half was saved for germplasm development (Kumar and Khush 1987, Schaeffer et al 1989).

For protein fractionation, whole seeds were ground with a mortar and pestle and screened through a 40-mesh sieve. The ground samples were defatted twice with 10 volumes of acetone at room temperature for 2 h. The residue was collected on Whatman[1] #2 filter paper in a Buchner funnel and air-dried. Seed storage proteins were fractionated into solubility classes by procedures modified from that described by Luthe (1983). Proteases were inhibited with 1 mM phenylmethylsulfonylfluoride (PMSF), and all solvents were buffered with 10 mM Tris, pH 7.5. The defatted flour was extracted twice with each solvent at room temperature in the following order: water to extract albumin, 1.0M NaCl to extract globulins, 70% ethyl alcohol to extract

[1] Mention of a trademark or proprietary product does not constitute a guarantee or a warranty of the product by the USDA, and does not imply its approval to the exclusion of other products that may be suitable.



1. Percentage of lysine in endosperm proteins of cultivars, mutants, and F_1 seeds of crosses. H =high lysine, greater than 3.55%,; L = low lysine, less than 3.35%, and includes parental types; I = intermediate values. In each group pf 3, left column represents female, right column male, and middle column F_1 seed. Data sets 1–5 are crosses; 6 and 7 represent parental types; and 8 represents in vitro-selected mutant.

prolamins, and 0.5% sodium dodecylsulfate and 1% b-mercaptoethanol to extract glutelins. The extracts were filtered through Whatman #2 paper and processed as follows. Albumins were precipitated with 2 volumes of acetone at –20°C overnight and centrifuged, and the precipitate was solubilized in water and freeze-dried. Globulins were dialyzed against 1 mM PMSF overnight at 4°C and centrifuged. The globulin precipitate, salt-soluble but water-insoluble, was suspended in water and freeze-dried. The proteins in the water-soluble supernatant of this globulin fraction were precipitated and freeze-dried the same way the albumins were processed. The water-soluble globulin or residual salt-extractable albumin subfraction was abbreviated Rsealb. Prolamins were precipitated with cold acetone and processed as described above. Glutelins were dialyzed and processed the same way as the globulins except for Rsealb partitioning.

Rice grains were scored for chalkiness before analysis. A 1–10 scale was used for this rating, 1 representing clear vitreous seed, characteristic of fully adapted cultivars, and 10 representing completely opaque seed, often soft, shrunken, and crumbly (Schaeffer et al 1986). Chalky ratings from 2 to 9 represented intermediate values from visual estimates of white light transmission through seeds.

Protein determinations

Protein levels were expressed as the sum of amino acids in micromoles (µmol) recovered from acid hydrolysates and also by Pierce bicinchoninic acid protein assay reagents. Values for aspartic and glutamic acid after acid hydrolysis included the

corresponding amides. Cysteine was very low in most samples and was not used in some calculations.

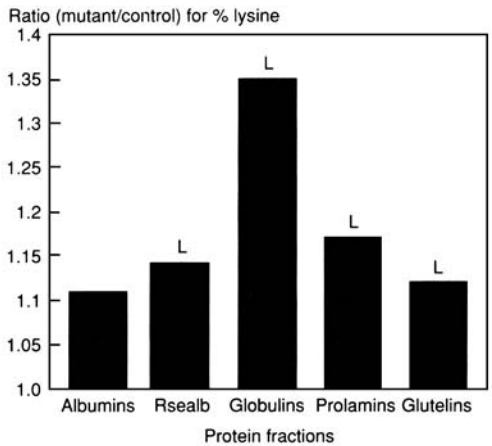
Statistical analyses

For the data shown in Table 1 and Figure 2, bulk samples were fractioned four times, and ratios for the mutant and control were calculated for each amino acid. The hypothesis that the mean mutant/control ratio equals one was tested using a *t*-test with a pooled estimate of the variance. Data in Figure 3 represent means from at least 12 plants each. Amino acid analyses were performed on composite half-seed samples consisting of 10 seeds each. The mean amino acid percentages were compared using analysis of variance and Fisher's protected least significant difference. Only some of the data are presented.

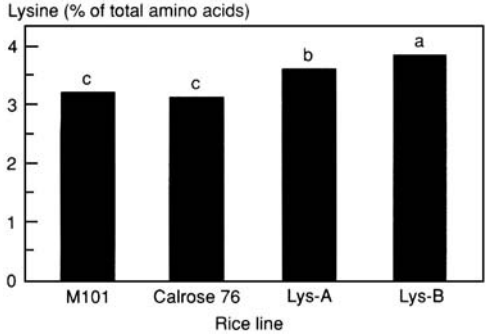
Table 1. Ratios of amino acids (mutant/control) in 5 solubility fractions of rice endosperm proteins.[a]

Fraction	Ratio				
	Aspartate	Threonine	Methionine	Isoleucine	Lysine
Albumins	1.012	1.03	0.885	0.952	1.107
Rsealb	0.994	1.454 L	0.581 S	1.275 L	1.148 L
Globulins	1.129 L	1.035	0.784 S	1.105	1.352 L
Prolamins	1.016	1.055	0.739 S	1.153	1.213 L
Glutelins	1.007	0.998	1.145 L	1.058	1.125 L

[a]Ratios based on percent of individual ammo acids among total amino acids in acid hydrolysates recovered from amino acid analyzer. Means calculated from 4 determinations Test of hypothesis mutant/control = 1 : L = mutant significantly larger than control (P=0.05), S = mutant significantly smaller than control (P=0.05).



2. Mutant/control ratios for lysine percentage in rice endosperm protein fractions. L = mutant significantly greater than control (P = 0.05).



3. Characteristics of field-grown lysine lines. Endosperm lysine percentage of total acid hydrolysates in rice cultivars (M101 and Calrose 76) and advanced lysine mutants derived from mutant/M101 (Lys-A and Lys-B) grown as space plants in field environments. Bars with different letters are significantly different at the 5% level.

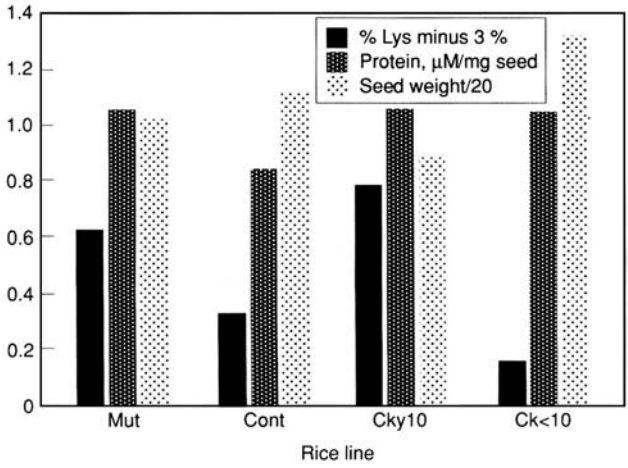
Results

The use of inhibitors in tissue culture media for the recovery of specific phenotypes is most successfully illustrated by the selection of cells resistant to herbicides. Other inhibitors, including analogs of amino acids or inhibitory levels of amino acids causing feedback inhibition, may also be used. For example, high (mM) concentrations of lys+thr cooperatively inhibit the aspartyl pathway for the synthesis of lysine, threonine, and methionine and hence inhibit and eliminate wild type cells in culture. Mutants that grow at mM lys+thr may be insensitive to feedback inhibition, and, when regenerated, produce phenotypes that may be useful for crop improvement. This paper describes the characterization of progeny from in vitro selection of rice cells with inhibitory levels of lys+thr.

Background generalizations

The first plants regenerated from in vitro inhibitor selection with lys+thr had only slightly higher seed lysine in proteins than did the starting cultivar Calrose 76. Frequently, recovered seeds with elevated lysine were chalky, with soft, crumbly endosperm, and were opaque to white light. A negative correlation of seed weight with lysine percentage in seed storage protein was demonstrated in the first F_2 population analyzed (Schaeffer and Sharpe 1987, Schaeffer et al 1989).

The generalized relationships between grain chalkiness, lysine percentage in grain protein, protein level, and seed weight are illustrated in Figure 4. The mutants had higher lysine and higher protein than the controls. Grain chalkiness is a common feature of rice grains from in vitro-derived plants, whether exposed to selection pressure or not (Schaeffer et al 1986). However, chalkiness was more intense in the high-lysine grains from tissue culture. Thus, grains representing F_2 progeny with a chalky rating of 10 had the most lysine, excellent protein, and reduced seed weight. Most F_2 plants with chalky ratings less than 8 produced grains with low lysine, normal



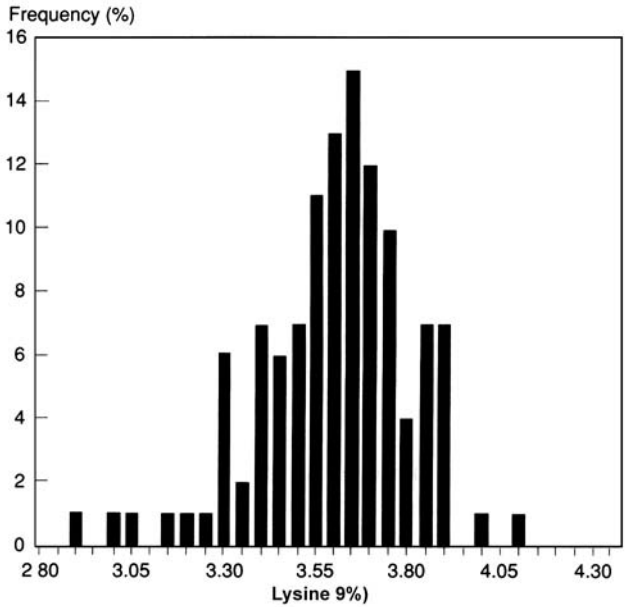
4. Relationships among seed weight, protein content, % lysine, and seed chalkiness in endosperm half-seeds of in vitro-selected mutant and nonselected control of rice. Absolute values were modified to bring parameters into the same scale: percent lysine in acid hydrolysates is expressed as % lysine minus 3%; protein is expressed as total µmol amino acids/mg seed; seed weight (wt) is expressed as mg/seed divided by 20. Typical standard error of the mean for 50 individuals is 0.48 for seed weight, 0.05% for lysine, and 0.03 for protein expressed as total µmol of amino acids.

protein, and larger seeds than the controls. Occasionally, heterosis was expressed in progeny from crosses between anther culture-derived, tissue-cultured mutants and the original starting parent, the source of the explant. Increased seed size in low chalky types was seen in the mutant/M101 cross.

Inheritance of elevated lysine

The gene(s) conditioning elevated endosperm lysine is (are) inherited as a recessive character (Schaeffer et al 1989). There was a good fit to the expected 3:1 ratio in the F_2 progeny. However, many lysine modifiers probably influence the final phenotype, and the 13:3 ratio probably represents a more precise fit. Histogram patterns for lysine percentage among F_2 seeds with lysine percentage greater than 3.54 occasionally suggest the recovery of high-lysine subclasses. These subclasses represent slight deviations from the strict 3:1 Mendelian segregation (Schaeffer et al 1986). Figure 5 illustrates the distribution in a selfed S_4 mutant line not yet fully homozygous.

Crosses among parental cultivars Calrose 76, M101, and Assam 5 with mutants having high, low, and intermediate lysine further demonstrate the recessive nature of gene(s) conditioning lysine level in rice. Results are illustrated in Figure 1. In the high × low cross (H/L), the F_1 was significantly higher in lysine percentage than was the low-lysine parent; nonetheless, the F_1s had lysine values much closer to the low parental value than to the mutant value. This is the expected pattern for the heterozygous condition. This is true for all crosses except I/L, in which the difference between the parents was small. Appropriate selections and selfings as well as mutant backcrosses



5. Histogram of % lysine in endosperm proteins of single seeds of S_4 mutant line. Mean % lysine for controls Calrose 76 and M101 was 2.87 and 3.22, respectively (Schaeffer et al 1989).

have produced lines with near normal seed set and near normal seed weight under field conditions. This is illustrated in Figure 3. The Lys-A and Lys-B lines had significantly greater endosperm lysine than the parental controls and had normal or greater than normal seed weight. Lys-A had normal fertility, and Lys-B had reduced fertility. Lines have been developed with normal seed weight, good fertility, and the potential for improved protein. Thus, germplasm exists for field trials and future selections of types adapted to specific environments. These lines have chalky endosperm and are not suitable for all commercial uses, particularly those requiring milling. However, vitreous types with good lysine have been recovered from subspecies crosses.

Characteristics of endosperm protein fractions

The amino acid composition of mutant proteins was significantly different from the control in several ways, including lysine content. The separation of endosperm proteins into solubility classes revealed that changes in amino acid composition occurred in all fractions. The most profound changes occurred in the salt (1% NaCl)-soluble globulin fractions and the globulin subfraction, Rsealb. Rsealbs are extracted with salt but stay in solution when dialyzed against water. The lysine percentage was higher in the mutant than in the controls in all solubility fractions, and significantly higher (P=0.05) in the Rsealb, globulin, prolamin, and glutelin fractions. Interestingly, methionine was significantly lower in the mutant in the Rsealb, globulin, and prolamin fractions, but significantly higher in the most important seed storage proteins, the glutelins. Aspar-

tate was significantly higher in the globulin fraction of the mutant, and threonine and isoleucine were significantly higher in the Rsealb fraction (Table 1). Thus, representatives of all the aspartate-lysine pathway amino acids were higher in the mutant than in the control in one or more of the endosperm protein fractions. Lysine was higher in all fractions, and the salt-soluble fractions were most profoundly changed. The relative lysine changes among the fractions is illustrated in Figure 2. The pattern suggests that the level or composition of more than one family of proteins was altered. Probably a broad spectrum of individual proteins is processed differently in the high-lysine mutant.

The quantity of lysine, expressed as milligrams per gram of seed, in different protein fractions gives a profile different from that of lysine expressed as percentage of total amino acids. The major portion of lysine is in the albumin and glutelin fractions. There is more than two times as much lysine in the mutant albumins (4C/C=2.08) than in the controls (Table 2). Both the increase in lysine percentage within the protein and the higher amount of protein contribute to this difference. Frequently, the mutant line has smaller seeds than the controls, and some increase in protein is normally associated with reduced seed size. Even so, advanced lines from crosses have been recovered with normal seed size, normal protein, and increased lysine.

From concept to phenotype

The high-lysine phenotype may be the result of several factors; a change in the metabolism by way of the lysine pathway, a change in RNA translation, a change in protein processing, or a combination of all three. Even though the phenotype is complex, there is a direct relationship between the inhibitor selection pressure with lys+thr and the high-lysine phenotypes recovered. Similar phenotypes have been recovered in four experiments, demonstrating the value and predictability of the

Table 2. Lysine distributions in fractions of rice whole-seed proteins isolated from mutant (4C) and control (C) lines.

Fraction		Protein (mg/100 g seed)[a]	Protein ratio (4C/C)	Total amino acids (mg/100 g seed)[b]	Lysine (mg/100 g seed)	Lysine (%)	Lysine ratio (4C/C)
Albumins	4C	1324	1.79	1533	87.1	5.68	2.08
Rsealb	4C	200	1.75	152	7.1	4.69	1.88
Globulins	4C	448	1.19	529	18.1	3.42	1.9
Prolamins	4C	257	1.08	538	3.3	0.62	1.75
Glutelins	4C	3938	1.44	5733	207.6	3.62	1.16
Albumins	C	738		824	41.9	5.09	–
Rsealb	C	114		86	3.8	4.44	–
Globulins	C	376		362	9.5	2.63	–
Prolamins	C	238		367	1.9	0.52	–
Glutelins	C	2738		4957	179.5	3.62	–

[a]Pierce assay. [b]Based on sum of amino acids, in micromoles, recovered from analyzer.

lys+thr selection procedures. These results validate the concept and establish a direct relationship between the selection protocols and the phenotypes recovered. Specific biochemical differences between the mutant and controls, and the genes associated with those differences are under investigation.

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Mutants for rice storage proteins

T. Kumamaru, M. Ogawa, and H. Satoh

Rice storage proteins of the starchy endosperm are localized in two types of protein bodies. Nutritional improvement of rice storage proteins needs a decrease in the indigestible protein body (PB-I) and/or an increase in the digestible protein body (PB-II). To obtain genetic materials to breed qualitatively and quantitatively improved rice storage proteins, we screened 3,000 mutant lines induced by treating rice fertilized egg cells with N-methyl-N-nitrosourea. We obtained 17 mutant lines, which were classified into 4 types: 13b-L, 10/13a-L, 10/16-H, and 57-H. 13b-L, 10/13a-L, and 10/16-H were characterized by a change in the content of 10-, 13-, and 16-kDa polypeptides, present in PB-I, while 57-H accumulated a 57-kDa glutelin precursor. 13b-L and 10/13a-L may be good genetic resources for improving the proteins in PB-I. The genes for 13b-L and 57-H, *esp-1* and *esp-2,* respectively, showed genetic behavior similar to that of the *wx* gene, while the genes for 10/13a-L and 10/16-H, *esp3* and *Esp-4,* respectively, were inherited maternally.

Rice is important in Asia as both a staple food and a source of protein. Attempts to improve rice storage proteins have been limited to increasing the total protein content (Beachell et al 1972, Kambayashi et al 1984, Osone and Takagi 1970) or increasing the lysine content (Shin et a1 1977). Advanced genetic studies on rice storage proteins have yet to be done. The purpose of our research is to collect genetic resources for rice storage proteins and to analyze their genetic behavior to establish a basis for genetic improvement, both qualitatively and quantitatively.

The major rice storage proteins in the starchy endosperm accumulate in two types of protein body (PB), PB-I and PB-II (Tanaka et al 1980), which account for about 20 and 60% of total rice protein, respectively (Ogawa et al 1987). Proteins present in PB-I and PB-II are hydrophobic, but they differ in molecular size (Krishnan and Okita 1986, Tanaka et al 1980, Wen and Luthe 1985) and biosynthesis mechanism (Tanaka and Ogawa 1985, Yamagata et al 1982, Yamagata and Tanaka 1986). The major proteins in PB-II are glutelin in nature and consist of two kinds of acidic and basic subunits with apparent molecular mass (MMr) of 40 and 20 kDa, respectively. They are synthesized as a 57-kDa precursor polypeptide on the membrane-bound polysomes

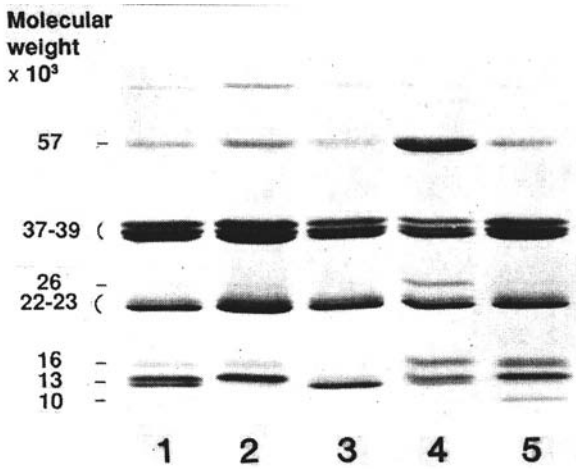
before being transported into the PB vacuole. In contrast, the proteins in PB-I are prolamin in nature and are composed of at least 4 components: 16-kDa, 13a (a larger component of 13 kDa), 13b (a smaller component of 13 kDa), and 10-kDa polypeptides (Ogawa et al 1987). They are also synthesized on membrane-bound polysomes but are deposited directly into the membrane-lumen to form PB-I. This biochemical differentiation of proteins in the two PBs implies that the genes for their biosynthesis, which involves accumulation into the individual PBs, must differ. Therefore, the genetic resources for rice storage proteins should be collected on the basis of individual polypeptides present in discrete PB types, and the genetic analysis of proteins present in PB-I and PB-II should be considered individually.

We screened storage protein mutants; we focused on proteins deposited in PB-I and PB-II, characterized them, and attempted to elucidate the genetic behavior of the mutants. We used mutants that Satoh and Omura (1979, 1981) induced by treating fertilized egg cells with N-methyl-N-nitrosourea (MNU), because they found various endosperm mutants (Satoh and Omura 1981). Thus, there is ample hope to successfully obtain genetic resources for storage proteins by using the MNU mutation method.

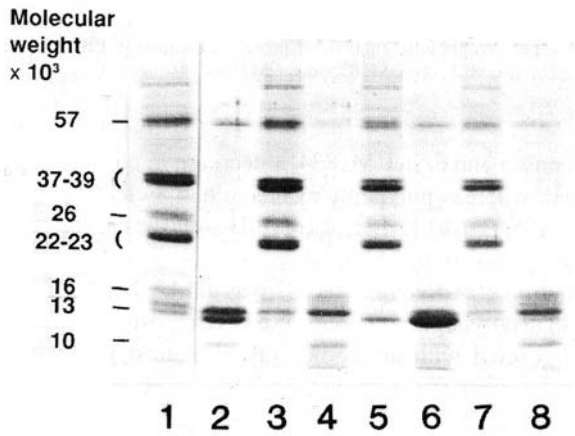
Screening and characterization of rice mutants by protein bodies

We obtained from about 3,000 mutant lines 17 mutants that showed marked differences in the concentrations of polypeptides separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) when compared with those of the original variety Kinmaze (Kumamaru et al 1988). The staining intensities of the individual polypeptide bands were assessed in relation to Kinmaze, and the mutants were classified into four types as shown in Figure 1. The first type (named 13b-L) was characterized by the low intensity 13b polypeptide band. The second type (10/13a-L) showed the low intensity 13a and 10-kDa bands. The third type (57-H) showed the high intensity 57-kDa band. In the fourth type (10/16-H) the high intensity 10- and 16-kDa bands were observed in addition to the low intensity 13b band. Three of the mutants—13b-L, 10/13a-L, and 10/16-H—are probably related to mutations of prolamin polypeptides in PB-I, while 57-H is probably related to a mutation of glutelin polypeptide in PB-II. We used the mutant lines CM21 (13b-L), CM1675 (10/13a-L), CM1787 (57-H), and CM1834 (10/16-H) in further experiments.

To clarify the differences in polypeptide composition of 13b-L, 10/13a-L, and 10/16-H, we attempted to isolate PB-I from each mutant by using a sucrose density gradient and pepsin digestion treatment (Ogawa et al 1989). Figure 2 shows the SDS-PAGE profiles of polypeptides in purified PB-I from Kinmaze and mutants. A sharp decrease in 13b was observed in CM21 and CM1834, while in CM1675 the 13a and 10-kDa polypeptides decreased markedly along with a remarkable increase in 13b. Furthermore, CM1834 showed high intensities of the 10- and 16-kDa polypeptides. Therefore, differences in polypeptide composition between the mutants and Kinmaze, observed by SDS-PAGE of the whole grain (Fig. 1), were confirmed to be derived from changes in the polypeptide composition present in PB-I. Further two-dimensional gel

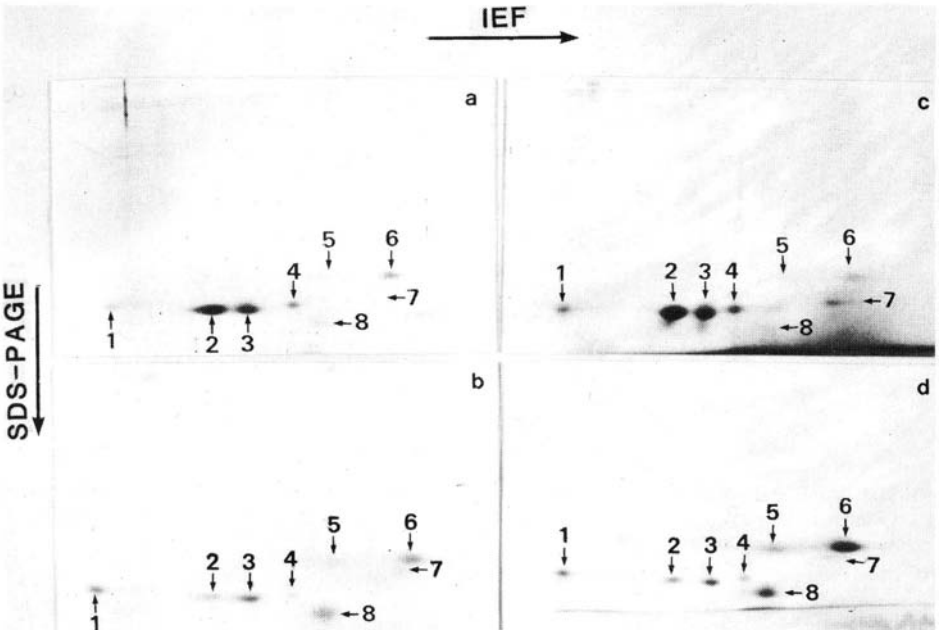


1. SDS-PAGE analysis of salt-insoluble proteins extracted from starchy endosperm of each mutant type. 1 = Kinmaze, 2 = CM21 (13b-L), 3 = CM1675 (10/13a-L), 4 = CM1787 (57-H), 5 = CM1834 (10/16-H).



2. SDS-PAGE analysis of proteins contained in PB-I purified from starchy endosperm. 1 = Kinmaze total protein, 2 = Kinmaze PB-I, 3 = CM21 total protein, 4 = CM21 PB-I, 5 = CM1675 total protein, 6 = CM1675 PB-I, 7 = CM1834 total protein, 8 = CM1834 PB-I.

electrophoretic analysis of proteins from PB-I clearly showed the differences in polypeptide composition in SDS-PAGE (Fig. 3). The proteins of 13 and 16 kDa consist, respectively, of 4 (nos. 1, 2, 3, and 4) and 2 polypeptides (nos. 5 and 6), while the 10-kDa polypeptide has only polypeptide no. 8. Differences in polypeptide composition observed in CM21 were correlated with decreases in polypeptides nos. 2, 3, and 4. On the other hand, polypeptides nos. 2, 3, and 4 in CM1675 showed stronger intensities than in Kinmaze, while polypeptide no. 8 appeared faint, along with weak intensities



3. Two-dimensional gel electrophoretic analysis of protein contained in PB-I purified from starchy endosperm. a = Kinmaze, b = CM21, c = CM1675, d = CM1834.

for polypeptides nos. 5 and 6. In CMl834, a decrease in polypeptides nos. 2, 3, and 4 was also observed, whereas polypeptides nos. 6 and 8 clearly increased. Therefore, these mutations–13b-L, 10/13a-L, and 10/16-H–can be characterized by changes in PB-I polypeptide composition.

The 57-H mutation observed in CM1787 was unlike that in Kinmaze in that it had an increased 57-kDa polypeptide content. We have reported that the 57-kDa polypeptide in CM1787 reacted with antibodies raised against purified glutelin subunits (Ogawa et al 1991); we also found that the polypeptide is deposited in another PB distinct from PB-I and PB-II. Therefore, the 57-H mutation has a remarkable increase in the glutelin precursor polypeptide.

To determine whether or not the altered polypeptide compositions caused changes in the protein contents of PB-I and PB-II, the protein contents extracted from rice starchy endosperm by several kinds of solvents were determined (Table 1). The albumin-globulin content of mutants was the same level as that of Kinmaze. The prolamin content of CM1675 was about twice that of Kinmaze, and CM1787 had about half. There was no remarkable reduction of prolamin in CM21 and CM1834, although a decrease in the 13b polypeptide was observed in SDS-PAGE. Therefore, the increase of prolamin in CM 1675 was the result of an increase in the 13b polypeptide, while that in CM21 and CM 1834 may have been due to an increase in other kinds of prolamin in PB-I, rather than to a decrease in the 13b polypeptide. In the case of the glutelin

Table 1. Content of proteins extracted from the starchy endosperm of mutants.

Protein fraction	Protein content (mg/100 mg polished grain)				
	Kinmaze	CM21	CM1675	CM1787	CM1834
Albumin-globulin	1.16	1.20	1.01	1.34	1.25
Prolamin	0.57	0.70	0.95	0.32	0.56
Glutelin	4.74	7.37	4.16	3.47	5.81
Total	6.47	9.27	6.12	5.13	7.62

Table 2. Contents of pepsin-indigestible and -digestible proteins in rice starchy endosperm of mutants.

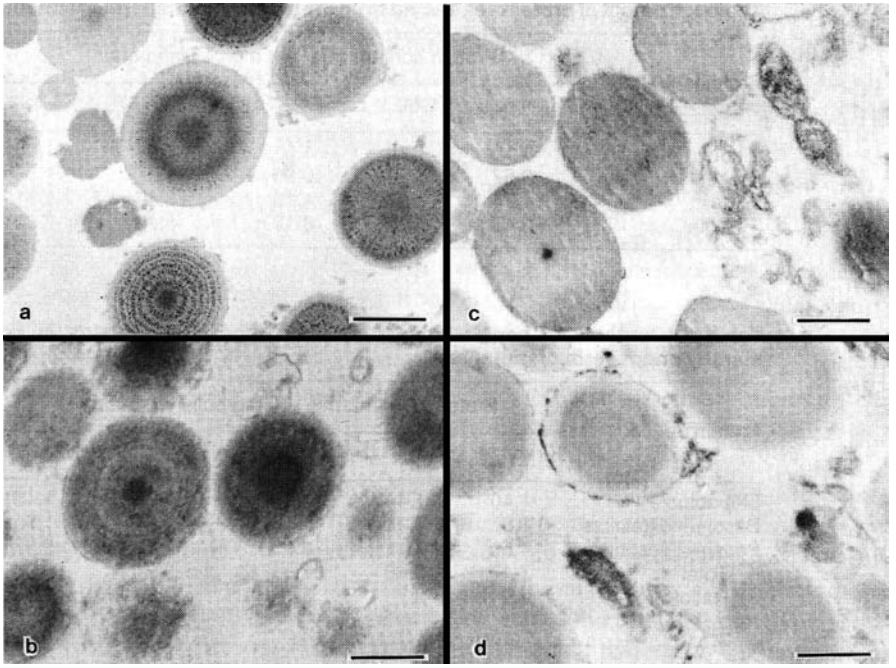
Protein fraction	Protein content (mg/100 mg polished grain)				
	Kinmaze	CM21	CM1675	CM1787	CM1834
Salt-soluble	1.40	1.44	1.38	1.35	1.45
Pepsin-indigestible	1.57	1.91	1.50	1.54	1.76
Pepsin-digestible[a]	4.50	7.11	4.63	4.02	5.87
Total	7.47	10.46	7.51	6.91	9.08
Ratio[b] of PB-II/PB-I	2.9	3.7	3.1	2.6	3.3

[a]Content was determined by subtracting sum of pepsin-indigestibles and salt-soluble proteins from total proteins. [b]Value was calculated from proportion of content of pepsin-indigestible proteins to that of pepsin-digestible proteins.

content of the mutants, CM21 and CM1834 showed higher levels than that of Kinmaze. On the other hand, the glutelin content of CM1675 was similar to that of Kinmaze, while that of CM1787 was remarkably reduced. Therefore, CM21 and CM1834 may be regarded as high-glutelin type mutants.

The protein content in PB-I and PB-II in mutants was also determined by pepsin digestion (Ogawa et al 1989). The pepsin-indigestible and -digestible protein contents can be considered, respectively, as the PB-I and PB-II protein contents. The protein contents of PB-I and PB-II varied consistently in CM21 and CM1834 compared with those of Kinmaze (Table 2). In contrast, in CM1675 and CM1787, they were of the same concentration as in Kinmaze. We therefore conclude that CM21 and CM1834 are mutants in which the PB-I and PB-II protein contents increased concomitantly with the different polypeptide compositions of PB-I. CM1675 has a simplified PB-I polypeptide composition, in spite of the similarity of its PB-I and PB-II protein contents to those of Kinmaze. Finally, CM1787 is a mutant in which the PB-I and PB-II polypeptide contents are remarkably reduced along with the marked increase in 57-kDa glutelin polypeptide.

To determine whether or not changes in polypeptide composition cause morphological changes in PB-I, electron microscopic observations were made of isolated PBs from mutants. CM21 and CM1834 had lamellar structures similar to that of Kinmaze



4. Transmission electron micrographs of purified PB-I. Bar = 1 µm. a = Kinmaze, b = CM21, c = CM1675, d = CM1834.

(Fig. 4). The electron densities of the inner structure were relatively higher in CM21 and CM1834 than in Kinmaze and CM1675. In contrast, PBs isolated from CM1675 did not show any typical lamellar structure. The characteristic PB structures in CM21 and CMl834 suggest that the low content of 13b polypeptide compared with those of 16-kDa, 13a, and 10-kDa polypeptides causes the observed morphological changes. In the case of CM1787, however, no PB-I with the typical lamellar structure was observed in the endosperm, although specific polypeptides in PB-I were present (Ogawa et al 1991). Our recent work suggests that PB-I in CM1787 differs in shape and size from those in other mutants and in Kinmaze (unpubl. data).

Genetic analysis of four mutants for protein body

Few studies have been made on the major gene for rice storage protein. This section deals with the genetic analysis of each polypeptide in four mutant lines.

Genetic behavior

The genetic behavior of four mutants was analyzed by crossing with the original variety (Kumamaru et al 1987, 1990). Table 3 shows the results of F_1 and F_2 seed analysis obtained from the reciprocal crosses of Kinmaze and mutant lines CM21 and CM1787.

Table 3. Segregation of normal and mutant types in F_2 seeds of crosses between Kinmaze and mutants.

Cross	Phenotype of F_1 seeds	Segregation in F_2 seeds			χ^2 (3:1)
		Normal	Mutant	Total	
Kinmaze/CM21	Normal	163	48	211	0.57
Kinmaze/CM1787	Normal	144	56	200	0.96

Table 4. Phenotype of F_1 and F_2 seeds, and segregation of normal and mutant types in F_2 plants as seen in F_3 seeds of reciprocal crosses between Kinmaze and mutants.

Cross (female/male)	Phenotype of F_1 seeds	Phenotype of F_2 seeds	Segregation in F_2 plants			c^2 (3:1)
			Normal	Mutant	Total	
Kinmaze/CM1675	Normal	Normal	118	43	161	0.25
CM1675/Kinmaze	10/13a-L	Normal	–	–	–	–
Kinmaze/CM1834	Normal	10/16-H	39	120	159	0.02
CMl834/Kinmaze	10/16-H	10/16-H	–	–	–	–

The F_1 seeds of all combinations showed the normal Kinmaze type. The segregation of normal and mutant types in the F_2 seeds of crosses between Kinmaze and the 2 mutants showed a good fit to a 3:l ratio. Therefore 13b-L and 57-H in CM21 and CM1787 are each controlled by a single recessive gene.

The genetic behavior of CM1675 and CM1834 was quite different. Table 4 shows the results of the analysis of F_1, F_2, and F_3 seeds obtained from reciprocal crosses between Kinmaze and mutant lines CM1675 and CM1834. The F_1 seeds of all combinations showed the maternal parent type. The F_2 seeds of Kinmaze/CM1675 showed the normal type. F_2 plants were cultivated, and the protein of a group of a single F_3 seed per F_2 plant was analyzed. Normal and 10/13a-L types were segregated in the F_3 seeds, and the segregation showed a good fit to the 3:1 ratio. In Kinmaze/CM1834, the F_2 seeds showed the 10/16-H type, and the segregation of normal and 10/16-H types in F_2 plants investigated by the F_3 seeds was a good fit to the 1:3 ratio. 10/13a-L in CM1675 and 10/16-H in CM1834 thus follow maternal inheritance and are controlled by a single recessive and a single dominant gene, respectively.

From the results of the allelism test (data not shown), the genes of 13b-L, 57-H, 10/13a-L, and 10/16-H were designated *esp-1, esp-2, esp-3,* and *Esp-4,* respectively.

Chromosome location

To determine the chromosomes on which *esp-1, esp-2,* and *esp-3* are located, the mutant lines were crossed with trisomic series derived from Kinmaze, and the resultant F_2 and F_3, seeds were analyzed (Kumamaru et al 1987, 1990). Genes *esp-1, esp-2,* and *esp-3* showed trisomic segregation in crosses with trisomic types F, G, and G, respectively (Table 5). This analysis suggests that *esp-1, esp-2,* and *esp-3* are located on the extra chromosomes of trisomic types F, G, and G, respectively. Since the extra

Table 5. Segregation of normal and mutant types in F_2 of trisomic F_1 plants derived from the crosses between trisomics and mutant lines.

Type of trisomic (chromosome)	Gene	Segregation in F_2			c^{2a}(3:1)
		Normal	Mutant	Total	
F (7)	*esp-1*	190	16	206[b]	32.63***
G (11)	*esp-2*	186	12	200[b]	34.56***
G (11)	*esp-3*	174	11	185[c]	35.82***

[a] *** = Significant at the 0.1% level. [b]Number of F_2 seeds. [c]Number of F_2 plants investigated through F_3 seeds.

chromosomes of types F and G correspond to chromosomes 7 and 11, respectively (Iwata and Omura 1971, 1975), *esp-1*, *esp-2*, and *esp-3* must be located on chromosomes 7, 11, and 11, respectively. (Chromosome numbers correspond to the new unified chromosome numbering system.)

Perspective and conclusion

We found that the mutants are also characterized by a decrease and/or increase in the content of prolamin and glutelin. However, it is not clear whether or not the genes for controlling the content of individual polypeptides are correlated with genes *esp-1*, *esp-2*, *esp-3*, and *Esp-4*. Therefore, identification of the genes controlling the content of individual polypeptides in the fraction of prolamin and glutelin should be carried out to obtain useful genetic resources for modifying the contents of prolamin and glutelin.

Indigestibility of PB-I causes a decrease in the overall nutritional value of rice. Changes in polypeptide composition of PB-I, as seen in CM1675, affect the morphological features of PB-I and the solvent solubility of prolamin. This suggests that a simplified polypeptide composition in PB-I would lead to increased digestibility. Therefore, the physicochemical properties of PB-I could possibly be altered by using these mutations.

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Genes for late heading and their interaction in the background of Taichung 65

Kuo-Hai Tsai

Three independent recessive genes for late heading—*ef-2*(t), *ef-3*(t), and *ef-4*(t)—have been identified in crosses with Taichung 65 (T65), although their chromosomal locations remain unknown. Gene *ef-2*^{x}(t) (formerly called *lf-1*) was identified in an induced late-heading line of T65. It delays heading for about 15 d and confers some photoperiod sensitivity on its carrier. Several late-heading genes found in lines derived from offtypes occurring in the progenies of crosses of isogenic lines of T65 were found to be allelic to *ef-2*(t). Gene *ef-3*(t) (formerly *lf-2*) was identified in Philippine variety Inakupa. It also delays heading for 10 d. When it was combined with *ef-2*(t), the F_1 was like T65 but the F_2 plants homozygous for both genes showed an extremely late heading time. When either *ef-2*(t) or *ef-3*(t) was combined with *Ef-2* for early heading, their effects were counterbalanced, and the heading time of T65 was expressed. Gene *ef-4*(t) was identified in the Chinese early-heading variety Nantsao 33. Its effect was similar to that of *ef-3*(t), and when combined with *ef-2*(t) or ef-*3*(t), heading was strongly delayed.

During the course of work over many years on the analysis of genes for early heading by the use of isogenic lines of Taichung 65 (T65), genes for late heading were also found, viz., *ef-2*(t), *ef-3*(t), and *ef-1*4(t) (Tsai 1986a,b,c; 1987). Although detection of the first two genes was reported, the gene symbols used were old ones, and the data presented were not conclusive. In this paper, information on these two genes is briefly presented, with revised gene symbols and some new data, and the discovery of another gene, *ef-4*(t), is reported. The interactions of these genes are also described.

Materials and methods

Three late-heading lines obtained after one to three backcrosses with T65 were the main materials in this study (Table 1). T65(1)lf-1 was selected from an M_3 progeny of X-rayed (40 krad) seed of T65 and was found to carry mutant gene *ef-2*^{x}(t) which was previously called *lf* or *lf-1* (Tsai 1986 a,b,c). T65(3)221L and an early-heading line, T65(5)221E, were derived from the Philippine japonica variety Acc. 221 (Inakupa).

Table 1. Lines carrying late-heading and early-heading genes and their days to heading.

Line[a]	Gene	Change in days to heading[b]		Gene donor
		Winter	Summer	
T65 (control)	*ef-1*	0(=128) a	0(=81) a	
T65(1)lf-1	*ef-2*^{x}(t)	+11 c	+19 c	X-ray induced mutant
T65(3)221L	*ef-3* (t)	+ 5 b	+ 9 b	Acc. 221 (Inakupa)
T65(2)TK45L	*ef-4* (t)	+ 3 b	+ 9 b	Nantsao 33
T65(20)E[a]	*Ef-1*^{a}	– 9	– 14	Tatung-tsailai
T65(5)221E	*Ef-1*^{221}	– 8	– 11	Acc. 221 (Inakupa)

[a]Figures in parentheses show number of backcrosses. [b]Relative to T65, for which values are 128 d in winter and 81 d in summer. Within a column, values followed by a common letter are not different at the 1% level of significance.

T65(3)22IL carries the late-heading gene *ef-3*(t), which was previously called *lf-2* (Tsai 1986c, 1987). T65(2)TK45L was derived from the Chinese indica variety Nantsao 33, which heads about 1 mo earlier than T65. It seems to carry the late-heading gene *ef-4*(t), as reported in this paper. These late-heading lines were intercrossed to examine the mode of inheritance and interaction of their genes.

The experiments were carried out at Taichung (24° N), seeds being started in late January to early February for the first (winter) crop and in early July for the second (summer) crop. Single-plant hills were spaced at 25 cm in both directions, and a standard dosage of fertilizer (8-6-5 g NPK/m^2) was applied to the experimental field in both cropping seasons. Heading time was recorded at the emergence of the first panicle. Plant height was recorded as the total length of culm and panicle. The mean value for about 25 plants of a line was taken as the line mean. All F_1 panicles were bagged to prevent outcrossing.

Results and discussion

Genetic analysis for heading time was carried out on T65.

Detection of genes

Three recessive late-heading genes—*ef-2*(t), *ef-3*(t), and *ef-4*(t)—were identified.

ef-2^{x}(t). The late-heading mutant line T65(0)lf-1 was previously found to have a recessive gene, *lf-1* (Tsai 1986a,b). When backcrossed with T65, the heading-delaying effect of the gene was mitigated to some extent. The gene carried by T65(1)lf-1 is now renamed *ef-2*^{x}(t), and its dominant allele in T65 is *Ef-2*. When late-heading lines carrying *ef-2*^{x}(t) were crossed with the early-heading line T65(20)E^{a}, which has *Ef-1*^{a}, the F_1 plants were early heading, and the F_2 segregated into 9 early:6 T65-like:1 late type (Tsai 1986a,b). This shows that *ef-2*(t) and *Ef-1* are independent, and plants having *Ef-1* and *ef-2*(t)/*ef-2* (t) express a T65-like heading time as the result of the counterbalancing effect of the early-heading and late-heading genes.

Table 2. Late-heading alleles found at the *ef-2* (t) locus and their effects on days to heading and plant height (mean for 1982–89).[a]

Allele	Source	Change in days to heading[b]		Change in plant height[c] (cm)	
		Winter	Summer	Winter	Summer
Ef-2	T65	0 a	0 a	0 a	0 a
ef-2^{x}	T65(1)lf-1	+10 b	+17 b	–1a	0 a
ef-2^{L-1}	AB60L-1[d]	+15 c	+21 c	–10 b	–1a
ef-P^{L-2}	AB60L-2[d]	+14 c	+26 d	–12 c	–8 b
ef-2^{L-3}	AB60L-3[d]	+14 c	+21 c	–20 d	–16 d
ef-2^{L-4}	AB60L-4[d]	+15 c	+20 c	–26 c	–14 c
ef-2^{w}	9-4N-2L[d]	+13 c	+25 d	–10 b	–8 b

[a]Within a column, values followed by a common letter are not different at the 1% level of significance. [b]Relative to T65 (winter = 128 d, summer = 81 d). [c]Relative to T65 (winter = 124 cm, summer = 109 cm).[d]Nearly fixed line derived from an offtype found in the progeny of crosses between isogenic lines of T65 (Tsai 1990).

On the other hand, several nearly fixed late-heading lines (AB60L-1, etc.) were obtained in the progeny of offtypes showing early heading or weak growth, which occurred in crosses between certain isogenic lines of T65 (Tsai 1989, 1990). Unexpectedly, the genes for late heading carried by these offtype-derived lines were found to be allelic to *ef-2*^{x}(t). Tsai (1990) attributed the occurrence of these offtypes to moves of a transposon. On this hypothesis, the *Ef-2* locus may be regarded as a recipient site of the transposon. Lines having these *ef-2*(t) alleles slightly but significantly differed from one another in heading time and plant height (Table 2). All these *ef-2*(t) alleles, in homozygotes, appeared to confer a certain degree of photoperiod sensitivity on their carriers (Tsai 1986c).

ef-3(t). The Philippine japonica variety Acc. 221 (Inakupa) heads about 2 wk later than T65 and carries genes *Rc* (red pericarp), *gl* (glabrous hull), and *wx* (glutinous endosperm). Its cross with T65 showed that it carries a dominant early-heading gene at the *Ef-1* locus and a recessive gene for late heading (Tsai 1985, 1986c). Homozygotes for this gene headed 5–9 d later than T65. The responsible gene is designated *ef-3*(t) in this paper, and its dominant allele in T65, *Ef-3.* After three backcrosses with T65, a semi-isogenic line with this gene, T65(3)221L, was obtained. It showed the same heading time and other characters as observed previously in T65(1)221L, a line obtained by one backcrossing with T65 (Tsai 1986c). The heading-delaying effect of *ef-3*(t) was counterbalanced by the heading-advancing effect of *Ef-1*^{a} when the two were combined (Tsai 1986c). The *ef-3*(t) locus is independent of *Ef-1* and *ef-2*(t).

ef-4(t). The Chinese indica variety Nantsao 33 heads 30 d earlier than T65 in both the winter and summer seasons. Its early-heading gene is under observation. From a backcross with T65, line T65(2)TK45L that heads 3-9 d later than T65 was obtained. When it was crossed with T65, the F_1 plants headed about 5 d earlier than T65, and the F_2 showed a distribution in heading time suggesting a segregation into 3 early: 1 late. The symbol *ef-4*(t) is assigned to the late-heading gene this line carries, although its effect is less pronounced than that of *ef-2*(t).

Crosses between late-heading lines

When T65(1)lf-1 with *ef-2*^{x}(t) and T65(3)221L with *ef-3*(t) were crossed, the F_1 plants showed almost the same heading time as that of T65, and the F_2 segregated into 3 early (55 p1ants):l late (15 plants), each group having a wide range (Table 3). This was because the heading-delaying effect of *ef-2*^{x} (t) was much more pronounced than that of *ef-3*(t), particularly in the 1989 summer season in which the F_2 populations were tested. The heading dates of the F_2 plants varied from T65-like to a very-late type, and may be regarded as representing a 9:3:3:1 segregation, which was earlier observed in the F_2 between T65(0)lf-1 and T65(1)221L (Tsai 1986c).

When T65(3)221L with *ef-3*(t) and T65(2)TK45L with *ef-4*(t) were crossed, the F_1 plants headed 5 d earlier than T65, and the F_2 gave a wide range of heading date from T65-like to a very-late type with a gap of frequency separating the plants into early- and late-heading groups (Table 3). Probably, *ef-4*(t) has a heading-advancing effect in heterozygotes (*Ef-4/ef-4* (t)) as shown by the early heading of F_1 plants. In addition, late-heading plants generally have a larger variance in heading time than earlier heading ones (Tsai 1990), while the lines used in the present experiment were derived

Table 3. Distribution of heading dates in parental lines and their F_2 populations, 1989.

Parent or cross	Gene	Oct										Nov				Mean ±s [a] (Oct)
		4	7	10	13	16	19	22	25	28	31	3	6	9	12	
T65(1)lf-1	(*ef-2*(t))								2	7	7	3				29.6 ± 2.4
T65(3)221L	(*ef-3*(t))			2	5	6	5									15.3 ± 2.5
T65(2)TK45L	(*ef-4*(t))				7	3										13.9 ± 1.5
(3)221L/(1)lf-1		5	14	27	8	1				4	3	5	2		1	14.3 ± 10.1
				(55)								(15)				
(3)221L/(2)TK45L		5	9	12	8	5	3		4	2	7	3	5	1	1	18.2 ± 11.5
(1)lf-1/(2)TK45L			10	26	43	15	5	2	4	7	6	6	2		1	16.3 ± 8.2
T65	(*ef-1*)	7	18	7	3											7.1 ± 2.5

[a] Computed from original data in which heading was recorded daily.

Table 4. Interaction of late-heading genes in homozygous combinations.

Gene combination	Change in days to heading [a]	
	Winter	Summer
ef-1 Ef-2 Ef-3 Ef-4 (=T65)	0	0
ef-1 ef-2^{x}(t) *Ef-3 Ef-4* (=T65(1)lf-1)	+13	+20
ef-1 Ef-2 ef-3(t) *Ef-4* (=T65(3)221L)	+9	+11
ef-1 Ef-2 Ef-3 ef-4(t) (=T65(2)TK45L)	+10	+13
ef-1 ef-2^{x}(t) *ef-3*(t) *Ef-4*	No heading	+32
ef-1 Ef-2 ef-3(t) *ef-4*(t)	No heading	+28

[a] Relative to T65 (winter = 125 d, summer = 84 d). 1989 data for winter crop; 1987-89 mean for summer crop.

from a few backcrosses with T65 and were not isogenic. It is difficult to critically elucidate the pattern of F_2 segregation observed, yet the data may be considered as representing a 9:3:3:1 segregation.

When T65(1)lf-1 with *ef-2*^{x}(t) and T65(2)TK45L with *ef-4*(t) were crossed, the F_1 plants headed 8 d earlier than T65, and the F_2 also showed a wide range of heading date from T65-like to a very-late type (Table 3). The pattern of variation in heading date of F_2 plants may also represent segregation into 9 T65-like:3 TK-like:3 1f-like:1 very-late. Thus, locus *ef-4*(t) may be considered independent of both *ef-2*(t) and *ef-3*(t).

To examine the interactions of these recessive genes for late heading, lines homozygous for two of them were observed (Table 4). Generally, when two late heading genes were combined, their effects were greatly reinforced. The double homozygotes for *ef-2*^{x}(t) and *ef-3*(t), as well as those for *ef-3*(t) and *ef-4*(t), headed about 1 mo later than T65 in the summer and did not produce panicles in the winter. Gene *ef-2*(t) confers some photoperiod sensitivity, but *ef-3*(t) and *ef-4*(t) do not seem to do so, yet it is unknown whether or not their combinations bring about some sensitivity.

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Notes

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Photoperiod-conditioned male sterility and its inheritance in rice

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When variety Nongken 58s (NK58s), a progeny of Nongken 58 (NK58), was exposed to various photoperiods in the Koitotron S-153W growth chamber after panicle initiation, the percentage of abortive pollen grains increased with daylength, reaching 94% at 14 h/d. NK58 showed no reaction. The photoperiod-sensitive stage proved to be chiefly that of the stamen and pistil primordia, and less evidently that of the panicle branch primordia and meiosis of the microsporocytes. Cytological studies showed that during microsporogenesis of NK58s the meiotic divisions were accompanied by aberrations; yet pollen abortion happened mainly at the uninucleate stage, resulting in empty pollen grains of irregular shape. The inheritance of this male sterility was studied in several crosses, with the F_1 usually being fertile. The F_2 plants segregated extensively and continuously. It appears that a single gene controls photoperiod sensitivity, and a set of genes regulates the metabolism of the uninucleate microspores, the effects of which are cumulative.

In 1973 at the Mianyang Seed Farm, Hubei, Shi Mingsong discovered in a population of late japonica rice (*Oryza sativa* L. ssp. Keng Ting) variety Nongken 58 (NK58), a single plant that was male sterile but had late tillers bearing a few seeds. Its progeny gave rise to both fertile and male sterile segregating lines. After 20 generations, Shi succeeded in selecting Nongken 58s (NK58s), which was named Hubei photoperiod-sensitive genetic male-sterile rice (Shi 1981, 1985). NK58s differed from NK58 in its slightly earlier heading but reacted to short photoperiod in the way NK58 did by accelerating panicle initiation. NK58s behaved as a mutant strain that is male sterile and depends on daylength. If heading was before the first week of September, the pollen grains were essentially abortive. After that the pollen grains became fertile and the seed set partially recovered (Shi et al 1987). This interesting "photoperiod-conditioned male sterility" has been transferred to many cultivars by crossing in attempts to produce hybrid seed. Therefore, the inheritance of this novel category of male sterility has been studied with great interest.

Materials and methods

Seeds of NK58s and NK58 were provided by Zhu Yingguo and Yao Hongchao. Isogenic lines Lm^e and Lm^u were provided by Kikuchi Fumio, Institute of Agriculture and Forestry, Tsukuba, Japan. Seeds of NK58s/02428 (F_1) and 005s/Zhe Geng (F_2) came from the Institute of Agricultural Research, Dongxihu, Wuhan.

Experiments with different photoperiods were conducted in the Koitotron S-153W growth chamber at the China National Rice Research Institute, Hangzhou. NK58s and NK58 plants were exposed to a photoperiod of 10 h/d beginning at the 6-leaf stage for 12 d until the initiation of branchlet primordia. Then the plants were treated with 13.0, 13.5, 13.75, or 14.0 h of light/d until heading to examine the effect on pollen sterility. In one series of experiments, NK58s plants were transferred from 13 to 14 h/d and vice versa to determine the stage that is sensitive to short day. Temperatures during the light period and the dark period were distinct and were maintained constant for each series of experiments. Pollen abortion was examined daily in anthers sampled from the middle part of the panicle. The anthers were squashed on a slide, stained with I-KI solution, examined, and measured under the light microscope. The absence of iodine reaction on starch was taken as the indicator of abortion. Cytological studies were carried out on 10-µm microtome sections of anthers stained with haematoxylin.

Results and discussion

Pollen development of NK58s under natural conditions and in growth chambers with different daylength was studied. Inheritance of male sterility was investigated in progenies of several crosses.

Pollen abortion of NK58s under field conditions

NK58s plants that were sown in May began heading at the end of August. When anthesis occurred before 5 September, 70–90% of the pollen grains proved to be abortive. After this, the percentage of sterile pollen became lower until 20 September, when the pollen grains were essentially fertile. However, the degree of sterility fluctuated from plant to plant and from day to day (Table l), perhaps partly because of temperature fluctuation; but the transition in fertility was gradual and not synchronous in all plants.

Usually three types of pollen grains are classified as sterile: the typical abortive pollen with irregular shape and the abortive pollen with spherical shape are both empty cells; cells that are faintly or only partially stained are said to have “abortive staining.” Photoperiod-conditioned sterile pollen grains are primarily abortive with irregular shape.

Some breeders have suggested that NK58s and strains with similar transition of fertility could be used as both the male sterile line and the maintainer line for hybrid seed production. Therefore, the mode and the mechanism of such transition need further study.

Table 1. Daily changes in pollen fertility in NK58s under field conditions, Wuhan, China.

Heading date	Abortive pollen with irregular shape (%)		Fertile pollen (%)	
	Mean	Range	Mean	Range
31 Aug	96.8	–	2.8	–
1 Sep	83.2	58.8–99.8	15.6	0.2–37.9
2	95.3	84.4–99.4	3.7	0.5–12.7
3	83.3	76.1–90.5	14.9	7.8–22.0
4	76.6	48.6–96.1	18.3	6.6–44.6
5	43.7	9.2–64.7	53.2	32.9–83.9
6	27.6	10.4–56.4	69.0	39.3–85.0
7	35.7	19.4–62.4	59.6	30.4–85.8
8	50.2	21.1–84.1	46.6	14.4–70.8
9	33.8	15.7–69.4	63.2	29.1–83.1
10	32.5	24.7–63.5	50.3	30.1–75.2
11	65.8	43.7–94.4	30.2	2.2–52.4
12	46.3	16.1–96.2	37.3	1.3–82.8
13	47.2	15.2–77.8	40.6	12.2–80.1
20	14.3	–	84.1	–
21	12.4	–	84.6	–

If the transition of fertility were dependent solely on daylength, such strains would be restricted in season and latitude. Recent research has posed the question of temperature effects on sterility. Therefore, experiments in controlled environment growth chambers are necessary.

Effect of photoperiod on pollen abortion in NK58s

NK58s plants grown in chambers with 13, 13.5, and 14 h of light/d after panicle initiation produced 21.4–86.1% abortive pollen with irregular shape and 2.2–6.3% spherical abortive pollen. The percentage of fertile pollen and the seed set decreased drastically at 14 h/d (Table 2,3; Fig. 1). In identical treatments, the pollen fertility and seed set of NK58 did not react to photoperiod. The percentage of pollen with “abortive staining” increased in NK58s at 13 h/d. The microspores may have been underdeveloped and binucleate, which arrests starch accumulation. Whether such pollen is able to germinate and grow in the style tissues is being studied.

The critical daylength for NK58s may be that of the earliest date of fertility transition, when pollen grains become fertile. Or it may be the daylength necessary for normal microsporogenesis at its critical stage. The transition is gradual and asynchronous, and the effect of natural daylength under field conditions differs from the effect of fixed photoperiod in experiments with growth chambers. A physiological-biochemical-biophysical approach may be needed to describe photoperiod-conditioned male sterility.

Table 2. Effect of photoperiod length on percentage of pollen types (growth chamber at 30 °C in light period, 22 °C in dark period).

Pollen type	Pollen type (%) at given photoperiod			
	13 h	13.5 h	14 h	Natural daylength in Hangzhou
		NK58s		
Abortive, irregular	21.4	63.9	86.1	90.4
Abortive, spherical	6.3	2.2	2.8	1.6
Abortive staining	19.9	6.7	4.9	6.7
Fertile	52.4	27.2	6.2	1.3
		NK58		
Abortive, irregular	7.3	7.4	7.6	5.9
Abortive, spherical	1.5	1.8	2.3	1.3
Abortive staining	3.9	10.7	10.4	6.7
Fertile	87.3	80.1	79.7	86.1

Table 3. Pollen abortion and spikelet fertility in treatments of different photoperiod (growth chamber at 25 °C in light period, 20 °C in dark period).

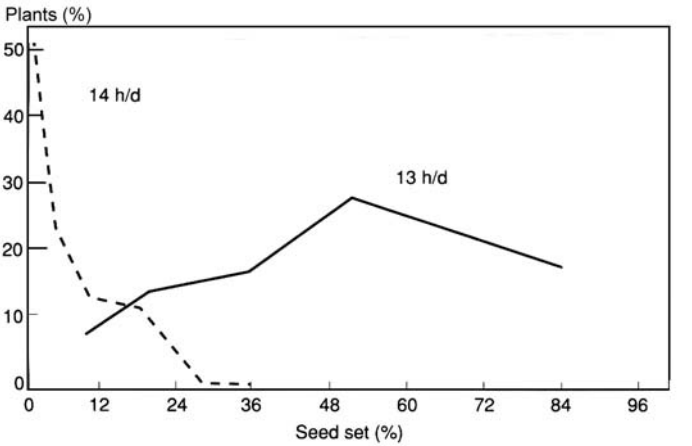
Item	Percentage at given photoperiod			
	13 h	13.5 h	13.75 h	14 h
		NK58s		
Pollen abortion	48.4	79.9	83.9	82.3
Spikelet fertility	53.6	6.3	2.9	6.2
		NK58		
Pollen abortion	20.4	23.1	24.5	22.9
Spikelet fertility	86.7	74.0	69.8	74.3

Photoperiod-sensitive stage in NK58s

NK58s plants were transferred from chambers at 14 h/d to chambers at 13 h/d at different stages of organogenesis. Table 4 shows that the percentage of abortive pollen with irregular shape was highest when the stamen and pistil primordia stage was subjected to 14 h of light/d. The panicle branch primordia, panicle branchlet primordia, and meiosis stages were also somewhat sensitive. Since the spikelets in the rice panicle differentiate asynchronously, photoperiod sensitivity was determined by judging the stage of differentiation of the majority of the spikelets. During other stages, sensitivity to photoperiod was very low (Table 4).

From cytological studies (see next section), pollen abortion is known to occur mainly at the uninucleate microspore stage. Photoperiodic signals in the sporogenous tissue must have induced metabolic changes, which lead eventually to abortion of the microspores. Thus, expression of the male sterility gene or failure of expression of the fertility gene is under the influence of multiple factors.

Plants (%)



1. Distribution of NK58s plants with different degrees of fertility under 2 photoperiods.

Table 4. Abortive pollen grains with irregular shape in NK58s treated with 14 h photoperiod at different stages of organogenesis and microsporogenesis (growth chamber at 30 °C in light period, 25 °C in dark period).

Stage at transfer from 13 h/d to 14 h/d and back to 13 h/d	Abortive pollen with irregular shape (%)
Panicle branch primordia	14.3
Panicle branchlet primordia	20.1
Spikelet primordia	0.6
Stamen and pistil primordia	61.6
Pollen mother cells (PMC)	1.6
Meiotic divisions of PMC	21.8
Growth of pollen grains	0.9

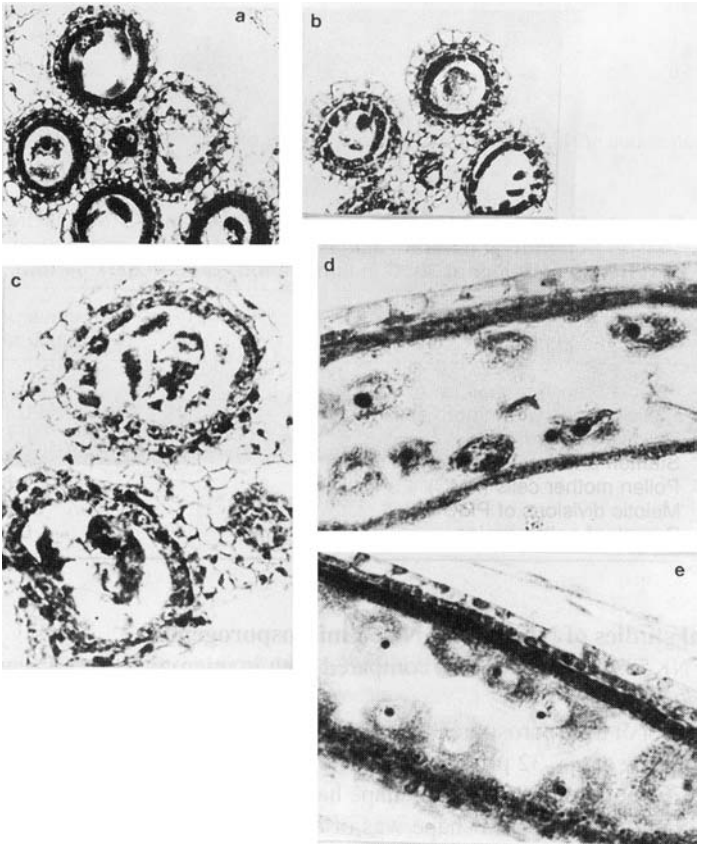
Cytological studies of NK58s and NK58 microsporogenesis

Anthers of NK58s and NK58 were compared both in microtome sections and by the acetocarmine method.

The diameter of the microspore is about 11 µm at the early uninucleate stage, 25 µm at the uninucleate stage, 32 µm at the binucleate stage, and 38 µm at the trinucleate stage. Abortive pollen with irregular shape had a diameter of 25-36 µm (av 32 µm). Abortive pollen with spherical shape was of a similar size, but pollen with abortive staining was larger: 29-41 µm in diameter (av 35 µm). The first type is produced at the uninucleate stage when the nucleus and then the cytoplasm disintegrate ultimately. The second type is produced somewhat later, at the uninucleate stage or at the beginning of the binucleate stage. The third type is produced at the binucleate stage when the

Table 5. Frequency of aberrations in NK58s and NK58 microsporogenesis (growth chamber at 30 °C in light period, 22 °C in dark period).

Light period (h/d)	Total cells examined (no.)	Aberrations (%) at microsporogenesis				
		Meiosis	Early uninucleate	Uninucleate	Binucleate	Trinucleate
			NK58s			
14	2286	27.3	54.7	86.0	86.7	91.4
13.5	4725	22.5	12.4	35.5	79.3	84.8
			NK58			
14	5292	6.1	1.6	17.4	11.8	6.1
13.5	4663	3.8	1.0	8.4	9.4	7.4

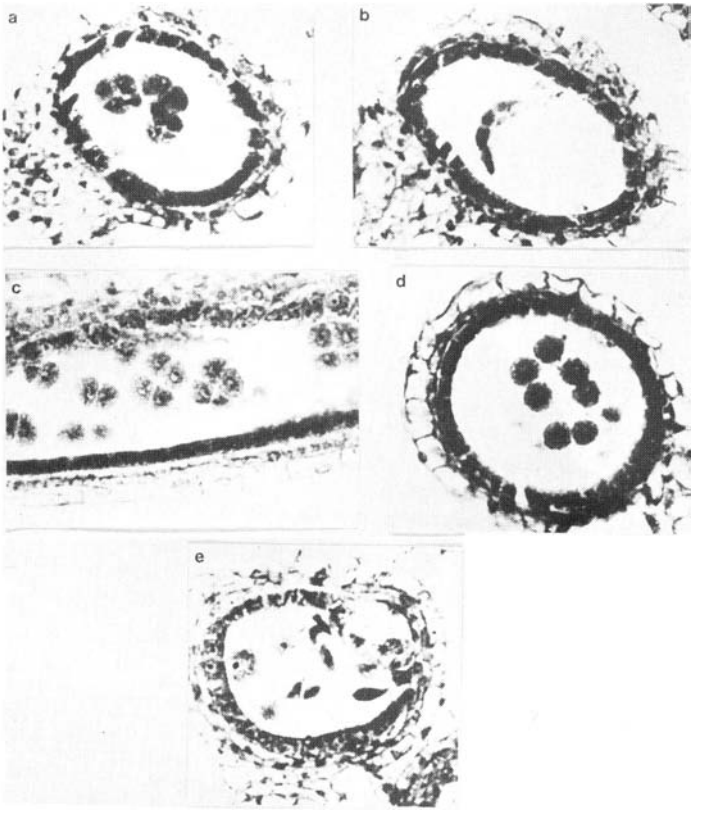


2. a) NK58s prophase I cells with disintegrating nuclei and cytoplasm (×400). b) NK58s pollen mother cells adherent to each other (x400).c) NK58s pollen mother cells with disintegrating nuclei and cytoplasm (×660). d) NK58s pollen mother cells with disintegrating nuclei and cytoplasm (×660). e) Normally developing NK58 pollen mother cells (×660).

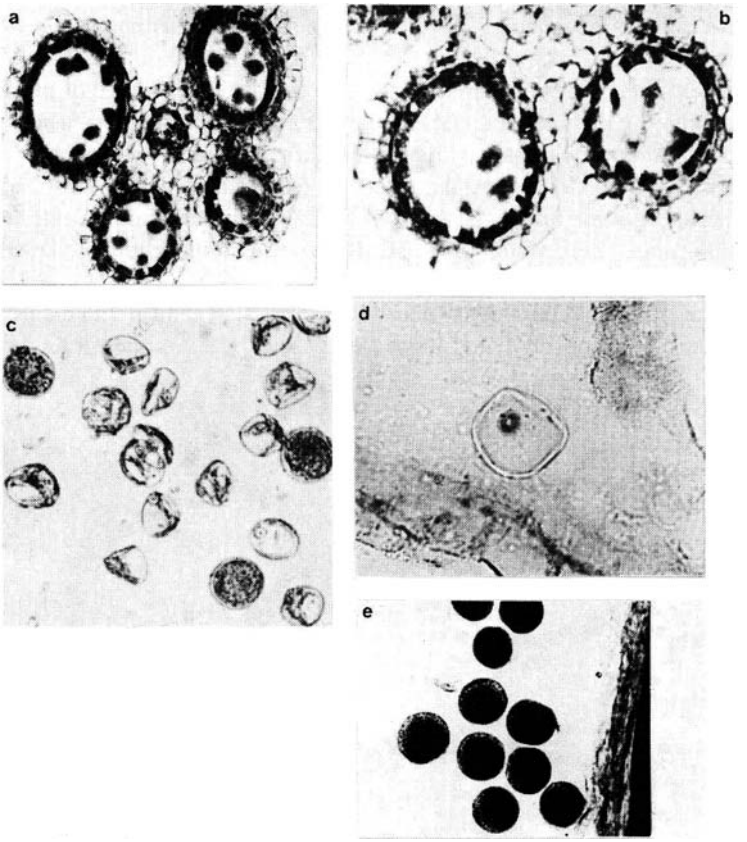
generative nucleus degenerates, with the vegetative nucleus partially retaining its function.

In NK58s, aberrations appeared as early as the pachytene stage of prophase I in meiosis, including disintegration of the nucleus and cytoplasm, and fusion of pollen mother cells. Abnormal tetrads with different sizes and arrangements were formed.

At 14 h of light/d, NK58s produced only 8.6% fertile pollen grains. Most of the pollen aborted after meiotic divisions; thus 54.7% of the early uninucleate and 86.2% of the uninucleate microspores were abortive. At 13.5 h of light/d, the percentage of aborted pollen was lower, but at the mature stage fertile pollen decreased to 15.2%. Evidently, at 13.5 h of light/d, pollen abortion occurred later and in smaller proportions. NK58, on the other hand, produced only 6.1 and 7.4% abortive pollen grains in the same treatments (Table 5; Fig. 2,3,4).



3. a) NK58s tetrad in tripod arrangement (×660). b) NK58s tetrad in linear arrangement (×660). c) Normal NK58 tetrads (×660). d) Normal early uninucleate NK58 pollen (×660). e) Abnormal early uninucleate NK58s pollen (×660).



4. a) Asynchronous development of NK58s microspores (x660). b) Abnormal early uninucleate NK58s pollen (x660). c) Typical abortive NK58s pollen with irregular shape and 3 pollen grains with "abortive staining" (x400). d) Abortive NK58s pollen with spherical shape (x660). e) Fertile NK58 pollen grains (x400).

Inheritance of photoperiod-conditioned male sterility in crosses

Inheritance of photoperiod-conditioned male sterility was studied in several crosses between NK58s or its derivative and other japonica strains. The F_1 progenies were essentially fertile. For example, the seed set rate of female parent 005s was 7.0 ± 4.9% and that of male parent Zhe Geng was 79.3 ± 4.9%, while their F_1 had a seed set rate of 66.6 ± 4.1 %.

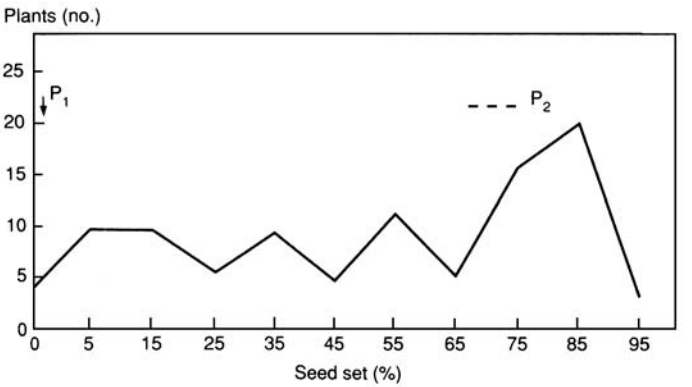
The F_2 populations segregated extensively in all crosses studied. If the segregants were classified according to seed set, the distribution curve was continuous with two or more peaks (Table 6, 7; Fig. 5). If those plants with seed set rate over 60% were classified as fertile, and those with seed set rate lower than 30% as highly sterile, there would be a group of semisterile plants of variable size. Various models of genetic

Table 6. Segregation for spikelet fertility in F_2 population under field conditions. Wuhan (30°27' N), China, 1989.

Cross	Plants counted (no.)	Sterile plants (0-30%) seed set (no.)	Semisterile plants (30-60%) seed set (no.)	Fertile plants (60-100%) seed set (no.)	Ratio of steriles to non-steriles in F_2	Male sterile plants (%)
NK58s/02428	152	10	22	120	1:14.2	6.6
NK58s/Lm^u	159	10	14	135	1:14.9	6.3
NK58s/Lm^e	250	7	17	226	1:34.7	2.8
005s/Zhe Geng	111	32	29	50	1:2.46	28.8

Table 7. Classification of the Nongken 58s/02428 F_2 plants by seed set (%).

Median value of group	Plants (no.)	Median value of group	Plants (no.)
0.5	5	55	13
5	10	65	7
15	10	75	17
25	7	85	21
35	10	95	5
45	6		



5. Distribution of Nongken 58s/02428 F_2 plants by seed set.

interpretation have been proposed with one, two, or more pairs of genes involved. It seems that photoperiod-conditioned male sterility is inherited as a quantitative-qualitative character. For the present, we assume that it is controlled by a single gene for photoperiod responsiveness and a set of genes regulating the metabolism of uninucleate microspores, the effects of which are cumulative.

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Thermosensitive genetic male sterility induced by irradiation

K. Maruyama, H. Araki, and H. Kato

A thermosensitive genetic male sterile line of rice, H89-1, was induced by irradiation. It was male sterile under high temperature but became fertile under low temperature. It responded to temperature above 30ºC. High temperature for 1 h/d was enough to reduce the seed set ratio. The thermosensitive stage was about 3 wk before heading. This thermosensitive genetic male sterility was controlled by a recessive gene, and will be of use in F_1 seed production.

Photoperiod-sensitive genetic male sterility in rice was first reported by Shi Mingsong (1985). It is used instead of cytoplasmic male sterility for hybrid rice seed production in China. Here, we report on thermosensitive genetic male sterility developed in Japan.

Induction of a thermosensitive genetic male sterile (TGMS) line

A Japanese improved variety, Reimei, was irradiated with gamma rays at 20 krad, and 2,500 lines of the M_2 were planted in 1987. We selected 281 lines in which sterile plants were segregated, and replanted a sterile plant of each line in the greenhouse. Among them were four fertile plants. After preliminary tests in the greenhouse and in the field, we selected one of the four lines as a thermosensitive line, naming it H89-1. The process of selection and the agronomic traits of H89-1 are shown in Tables 1 and 2.

We set up four temperature levels (34-27°C, 31-24°C, 28-21°C, and 25-18°C) and three daylength levels (15 h light [=15L]-9 h dark [=9D], 13.5L-10.5D, and 12L-12D) in the phytotron. H89-1 exhibited zero seed set under 34-27 °C and 31-24 °C but was partially fertile under 28-21 °C and completely fertile under 25-18 °C. It apparently responded to temperature only because the results under different daylengths were similar. The percentage of fertile pollen corresponded to the seed set ratio. Anthers of sterile plants were shorter than those of fertile plants. Stigma length increased when the plants were sterile, but elongation was correlated with temperature (Table 3).

Table 1. Breeding procedure of the thermosensitive genetic male sterile (TGMS) line H89-1.

Year	Generation	Selection
1983	M_1	Irradiation of Reimei with g-ray at 20 krad; 1 panicle harvested per plant
1984-86		Stored
1987	M_2	2,500 lines, 10 plants each; 281 sterile lines selected, retransplanted in greenhouse; 4 fertile lines selected out of 281
1988	M_3	4 lines in greenhouse
	M_4	4 lines in field; 1 line selected, tested for thermosensitive genetic male sterility in phytotron
	M_5	1 line in greenhouse, named H89-1; tested for thermosensitive genetic male sterility in phytotron
1989	M_6	1 line in field, tested for thermosensitive genetic male sterility in phytotron

Table 2. Agronomic characteristics of the TGMS line H89-1.

Line	Transplanting	Heading	Culm length (cm)	Panicle length (cm)	Panicles (no./plant)	Fertility
H89-1	15 May	26 Jul	68	19	11	Fertile
Reimei	15 May	27 Jul	69	20	12	Fertile
H89-1	8 Jun	20 Aug	73	24	9	Semifertile
Reimei	8 Jun	17 Aug	76	24	14	Fertile
H89-1	26 Jun	27 Aug	75	22	14	Sterile
Reimei	26 Jun	27 Aug	78	23	17	Fertile

Inheritance of thermosensitive genetic male sterility

We observed two sterile plants out of eight in the M_2 of this TGMS line. This suggested control by a single recessive gene. We tested the F_1 and F_2 of nine crosses and the F_3 lines of one cross. The seed fertility of all F_1s was normal, and their pollen grains were normal under the microscope (Table 4). Chi-square tests for 3 fertile plants: 1 sterile plant did not fit well in 2 crosses. But the segregation in the F_3 lines fit well the ratio of 1:2:1 (Table 5). A decrease in the germination rate of H89-1 was sometimes observed. This may be the reason for the segregation skew in the F_2 population. We therefore concluded that thermosensitive genetic male sterility of H89-1 was controlled by one recessive gene, the locus of which is not yet known.

Table 3. Seed set percentage and floral characteristics of H89-1 under various temperatures and daylengths.[a]

Day-night temperature (°C)	Daylength (h)	Seed set (%)	Fertile pollen (%)	Anther length (mm)	Stigma length (mm)
		H89-1			
25-18	15.0	92.6	94	2.0	0.9
	12.0	70.4	73	1.7	1.0
28-21	15.0	14.3	17	1.8	1.1
	13.5	9.6	35	1.8	1.1
	12.0	0.9	11	1.6	1.1
31-24	15.0	0.0	0	1.7	1.3
	13.5	0.3	0	1.8	1.2
	12.0	0.2	0	1.8	1.1
34-27	15.0	0.0	0	1.8	1.3
		Reimei			
31-24	15.0	97.3	99	2.3	1.1
	13.5	97.3	95	2.0	1.1
	12.0	90.0	94	1.9	1.1

[a]Experiments were done in a temperature-controlled growth chamber with natural sunlight in 1988-90. Daylength was adjusted by illumination.

Table 4. Fertility of F_1 and segregation of TGMS type in the F_2.

Cross	F_1		F_2			
	Seed set (%)	Fertile pollen (%)	Plants used (no.)	Normal plants (no.)	TGMS-type plants (no.)	x^2 (3:1)[a]
H89-1/Reimei	95.3	94.8	115	95	20	3.55
H89-1/Mangetsu Mochi	89.5	95.0	228	182	46	2.83
H89-1/Murasakiine	95.6	96.1	226	172	54	0.14
H89-1/89SL-621	99.2	94.6	229	191	38	8.63*
H89-1/FI123	93.9	98.4	229	167	62	0.52
H89-1/FI128	95.1	93.8	230	192	38	8.81*
H89-1/FI201	82.3	93.3	115	95	20	3.55
H89-1/FI237	95.9	98.0	229	195	34	12.50*
Nekken 2/H89-1	95.4	94.1	227	180	47	2.23

[a]* = probability < 5%.

Table 5. Segregation of normal type and TGMS type in F_2 genotype from F_3 lines of Nekken 2/H89-1.[a]

Item	Lines tested (no.)	Segregating lines (no.)	Normal lines (no.)	TGMS lines (no.)	x^2 (2:1:1)
Expected value	98	49	24.5	24.5	
Observed value	98	52	27	19	1.67 (0.5>P>0.25)

[a]Each line consisted of 23 F_3 plants.

Physiological characteristics of TGMS line

An experiment was done to determine the influence of maximum or minimum temperature on pollen fertility. H89-1 was partially fertile under 28 °C and sterile under 31 °C (Table 6). This result means that H89-1 responds to maximum temperature. Another experiment was done to learn the required duration of high temperature. Even 1 h of high temperature could influence pollen fertility and affect seed fertility (Table 7). To determine the thermosensitive stage, H89-1 was grown at high temperature (31-24°C), but with 3 d of low temperature (25-18°C) inserted before heading. When the low temperature treatment was at 22 and 26 d before heading, the panicles became partially fertile (Table 8).

Table 6. Fertility of H89-1 under constant temperature.[a]

Temperature (°C)	Seed set (%)	Fertile pollen (%)
	H89-1	
31	0.0	0.0
28	3.2	11.9
26	89.7	92.5
24	88.9	95.9
21	82.7	92.9
	Reimei (control)	
	78.6-95.8 (min-max)	91.1-99.8 (min-max)

[a]Experiments were done in 1989 in temperature-controlled growth chamber with natural sunlight. Daylength was adjusted to 15 h from 0430 to 1930.

Table 7. Fertility of H89-1 under various durations of daily high temperature.[a]

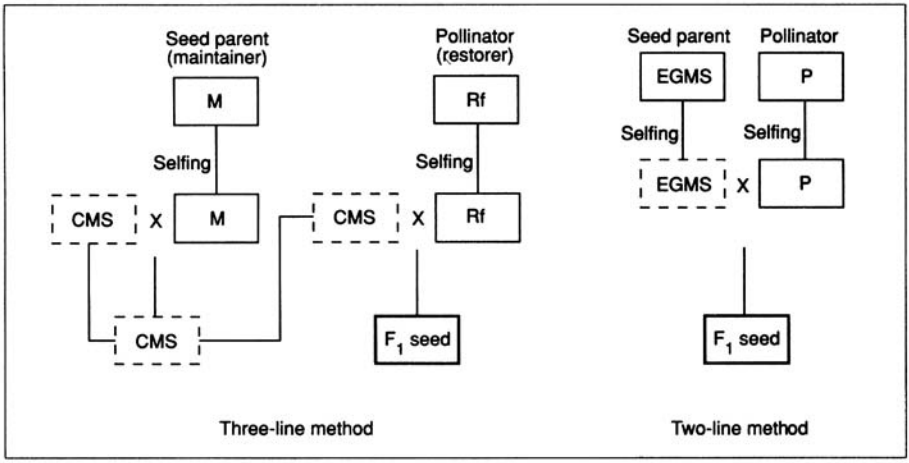
Duration (h) of high temperature		Line or variety	Seed set[b] (%)	Fertile pollen (%)
30 °C	25 °C			
12	12	H89-1	28.2*	39.2
		Reimei	94.2	96.9
6	18	H89-1	15.1*	23.5
		Reimei	95.0	95.4
3	21	H89-1	34.2*	9.6
		Reimei	91.7	96.9
1	23	H89-1	70.7*	38.2
		Reimei	87.9	98.0
0	24	H89-1	84.5	82.2
		Reimei	89.6	97.7

[a]Experiments were done in 1989 in temperature-controlled growth chamber with natural sunlight. Daylength was adjusted to 13.5 h from 0500 to 1830. [b]Differences in seed set percentage between H89-1 and Reimei significant at the 5% level.

Table 8. Fertility of H89-1 grown under high temperature (31-24 °C) and given 3 d of low temperature (25-18 °C).[a]

Item	H89-1 sown on					Reimei sown on 19 Dec
	5 Dec	12 Dec	19 Dec	26 Dec	2 Jun	
Panicle length (mm) at low temperature treatment	150.4	7.8	1.0	0.5	0.2	1.4
Days from treatment date to heading date	6	16	22	25	26	23
Seed set (%)	0.0	0.0	0.0	16.0	11.6	39.0[b]
Fertile pollen (%)	0.0	0.0	50.2	35.5	0.0	94.2

[a] Experiments were done in 1988 and 1989 in temperature controlled growth chamber with natural sunlight. Daylength was adjusted to 15 h from 0430 to 1930. [b] Rice plants damaged by acarid.



1. Comparison of F_1 seed production systems. Three-line method: F_1 seed production system by cytoplasmic male sterility (CMS) and restorer gene (Rf). Two-line method. F_1 seed production system by environment-sensitive genetic male sterility (EGMS).

Utilization of thermosensitive genetic male sterility

F_1 seed-producing systems by cytoplasmic male sterility and environment-sensitive genetic male sterility are shown in Figure 1. The advantages of environment-sensitive genetic male sterility (such as photoperiod-sensitive genetic male sterility or thermo-sensitive genetic male sterility) over cytoplasmic male sterility in F_1 breeding and seed production are

- easier multiplication of the environment-sensitive genetic male sterile line itself,
- no need to establish a cytoplasmic male sterile line by backcrossing, and
- possibility of practicing breeding irrespective of restorer genes.

There is one more benefit with environment-sensitive genetic male sterility: cytoplasmic male sterility of gametophytic varieties like BT reduces pollen number to half and promotes cold damage. The F_1 produced by TGMS lines under recessive genetic control will not be sensitive to cold weather.

Thermosensitive genetic male sterility can be used more easily in tropical and subtropical areas. Seed for TGMS rice can be multiplied in cool mountainous areas or suitable cropping seasons. Photoperiod-sensitive genetic male sterility is adaptable for temperate and subtropical areas, where the difference in daylength among seasons is apparent.

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Discussion

Session 4: Genetics of morphological and physiological traits

Q—*Khush:* You have shown linkage of a grain mutant with *lhs-1* or hull sterile. We have shown that *lhs-1* and *ops* (open palea sterile) are allelic. However, *ops* has priority and *lhs-1* is redundant. Why are you still using *lhs-1*?

A—*Takeda: ops* had priority, but because we had two gene names independently, we discussed establishing a new gene symbolization system and agreed to use *lhs* for the character.

Q—*Janoria:* Does the large-grain gene pleiotropically reduce spikelet number, leading to fewer grains, or is reduced grain number due to spikelet degeneration?

A—*Takeda:* The *UC-f* gene pleiotropically reduces the number of secondary rachis branches significantly. Thus it decreases the number of grains on the panicle.

Q—*Reddy:* In maize, the recessive opaque-2 enhances production up to 100% by lowering the zein content. Is it possible in rice to manipulate a specific fraction of the protein for efficient gene transfer?

A—*Schaeffer:* I don't have an easy answer. Not all solubility fractions are changed equally, and there is one globulin protein that is low in lysine in the control and is quantitatively reduced in the mutant. However, the fraction is probably too small to affect the total lysine percentage in the endosperm. We have found changes in the mutant in in vitro translations, but we have not identified these changes.

Q—*Oka:* What variety did you use in your mutation experiment?

A—*Schaeffer:* The source of the anther-derived callus was Calrose-76.

Q—*Tanaka:* The clearest change appeared in the globulin fraction. Have you ever checked what polypeptide is most responsive to the change? One effective way to know the high Lys polypeptides may be radio-labeled lysine administration experiments.

A—*Schaeffer:* Few quantitative changes are visible on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and we have searched for specific bands. Some differences seem to be associated with the mutants, but they are diffuse or very large. Hence we think the mutants may be processing mutants. We have done ^{3}H-lysine incorporation into 14-d-old seed (14 d postanthesis) and see 1 large protein that is much higher in concentration in the mutant than in the control. Also, ^{3}H-lysine labeling in tissue culture (cells in suspension) showed rapid incorporation into protein related to stress.

Q—*Boonjarwat:* Since you mentioned that the increase in lysine percentage in the mutants is not specific to just one protein, could you comment on that effect? If it is because of protein processing, have you found any "new" protein in the high-lysine mutant by two-dimensional gel?

A—*Schaeffer:* The increased mutant control ratio shows that no single protein family is responsible for the high-lysine phenotype, and therefore it appears that lysine is attached to many proteins and is cleared or processed differently in the mutant than in the control. Tritiated labeling shows a large protein much more prevalent in the mutant, which does not resolve in SDS gels. From the study of cell suspensions it appears that some proteins such as chitinases are constitutively synthesized in the mutant but are barely visible in the controls.

Q—*Brar:* The high-lysine mutants or variants are very interesting. Is it a mutation in the structural gene or in the regulatory asparatokinase? Do you think such amino acid-overproducing mutants resulting from feedback inhibition are true breeding?

A—*Schaeffer:* The lines that were grown in the field (F_5s) are true breeding. Some earlier lines were unstable, others stable. Dr. Matthews has isolated and partially sequenced aspartyl kinase, and we shall examine progeny for variations by Southern hybridization with probes for this enzyme. We should have an answer in the near future.

C—*Wu:* In your paper, Esp was used for both 13-b-1 and 5.7-H. It is better to use a different symbol for different kinds of storage protein, e.g., *glu* for glutelin, *pro* for prolamin, etc.

Q—*Chang:* Do the *ef-2* and *ef-3* genes affect the basic vegetative phase or the optimum photoperiod?

A—*Tsai:* The line carrying the late-heading gene *ef-2(t)* showed some photoperiod sensitivity, but the line having *ef-3(t)* did not. Their basic vegetative phase and photoperiod sensitivity phase will be evaluated.

Q—*Chaudhuri:* You mentioned some transposon activity at your *ef* loci. Will you please elaborate?

A—*Tsai:* Some late-heading lines in this study were obtained from mutant progeny, and offtypes showing earliness in heading or weakness in growth occurred in the progeny of crosses between certain isogenic lines of Taichung 65. But it was not expected that the genes for late heading carried by those offtype-derived lines would be allelic to the recessive mutated gene, $ef\text{-}2_X$. As a plausible hypothesis, it was assumed that a transposon would be concerned with the *ef-2(t)* locus. I will try to examine the presence of a transposon by molecular techniques.

Q—*Shao Qiguan:* How were 14 photoperiod treatment groups carried through different development stages, and how was temperature controlled?

A—*Wang Xiangmin:* Temperatures within the "Koitotron S-153W" growth chambers were maintained by a computer and remained constant for the period of my experiments at 25°C (light period) and 20°C (dark period). Second, seedlings were treated with 14-, 13.5, and 13-h photoperiods after panicle initiation by short day (10 h/d) induction on day 12, beginning at the 6-leaf stage. Except in the series of experiments for determining the photoperiod-sensitive stage, all plants were treated with different daylength in growth chambers for the whole period of panicle development until heading.

Q—*Virmani:* IRRI is thankful to Japan for sharing the thermosensitive genetic male sterile (TGMS) line for evaluation in the tropics. We have observed some pollen-free anthers in the TGMS mutant H89-1. Have you observed similar behavior in Japan?

A—*Maruyama:* Yes, many times we have observed completely pollen-free anthers. And sometimes we have observed anthers in which one-half (along the longer axis) was empty and the other half was filled with normal pollen grains. This indicates that the reaction to high temperature is a very sensitive one.

Q—*Yuan:* How many days are needed to cause pollen to become sterile under high temperature conditions?

A—*Maruyama:* From our experiment to decide the critical stage, 3 d of cool temperature is enough to restore fertility. So, if 3 d of high temperature is applied at the critical stage, it will cause male sterility. But I am not sure how many days of high temperature are needed to cause complete sterility.

SESSION 5

Genetics of Disease and Insect Resistance

Transfer of blast and bacterial blight resistance from *Oryza minuta* to *O. sativa*

A.D. Amante-Bordeos, R.J. Nelson, N.P. Oliva, R.D. Dalmacio, H. Leung, and L.A. Sitch

Oryza minuta L. is a tetraploid wild rice with resistance to blast (BI caused by *Pyricularia oryzae*) and bacterial blight (BB caused by *Xanthomonas oryzae* pv. *oryzae*). Triploid interspecific hybrids (2n=36) were produced between *O. sativa* cultivar lR31917-45-3-2 (2n=24) and *O. minuta* Acc. 101141 (2n=48) using embryo rescue. By backcrossing and further embryo rescue, we obtained BC_1, BC_2, and BC_3 progenies. The chromosome numbers ranged from 44 to 47 in the BC_1, and from 24 to 37 in the BC_2. All F_1 hybrids and BC_1 progeny were resistant to both BI and BB, with the exception of one BC_1 plant that was moderately susceptible to BI. Of 16 BC_2 progeny tested, 13 were resistant to BI. One BI-resistant plant, WHD IS 75-1, had 24 chromosomes. The reaction to BI among the selfed progeny of WHD IS 75-1 ranged from resistant to fully susceptible, with some progeny showing intermediate reactions. Classifying the BC_2F_2 into two classes, resistant or susceptible to BI, gave a segregation ratio consistent with the action of a single dominant gene. Ten of the 21 BC_2 progeny tested were resistant to races 2, 3, and 6 of the BB pathogen. One resistant plant, WHD IS 78-1, had 24 chromosomes. Among the BC_2F_2 progeny of WHD IS 78-1, there were recombinant phenotypes for disease reaction to races 2, 3, and 6 of the BB pathogen, indicating the presence of race-specific resistance genes. Further tests are needed to determine the genetics of resistance to BI in the line derived from WHD IS 75-1 and to BB in the line derived from WHD IS 78-1.

The wild *Oryza* species offer a rich source of variability for rice improvement. They possess a number of important traits, most notably resistance to insect pests (Heinrichs et a1 1985) and diseases (Sitch 1990). Until recently, alien gene transfer in rice has received limited attention. A dominant gene for resistance to grassy stunt virus was transferred from *O. nivara* to *O. sativa* (Khush 1977). More recently, brown planthopper (BPH) resistance was incorporated from *O. officinalis* into cultivated rice (Jena and Khush 1986, 1989).

O. minuta, an allotetraploid species native to Asia, is a potential source of resistance to two important rice diseases: rice blast (Bl) caused by *Pyricularia oryzae* and bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo). It is also

resistant to BPH and whitebacked planthopper (Heinrichs et al 1985). With the use of embryo rescue and other tools that allow the transfer of genes from wild species, it is feasible to diversify the genetic base of resistance in cultivated rice.

O. minuta has a genome composition of BBCC, distinct from that of *O. sativa* (AA genome). Hybrid sterility and a low frequency of chromosome pairing, and hence limited recombination, are strong barriers to gene transfer between the two species (Sitch et al 1989). The transfer of genes between such distantly related species commonly involves the production of monosomic alien addition lines (MAALs)—lines with the full chromosome complement of *O. sativa* plus a single alien chromosome. The desirable gene must then be incorporated into the *O. sativa* genome, and the alien chromatin content further reduced by the induction of translocations.

This paper discusses the production of *O. sativa/O. minuta* F_1 hybrids and their backcross (BC) derivatives, the reactions of both parents and their interspecific derivatives to Bl and BB, and the identification of euploid progeny with resistance to both diseases.

Materials and methods

O. minuta (International Rice Germplasm Center Acc. No. 101141) was hybridized to the *O. sativa* elite line IR31917-45-3-2 using the latter as the female parent. IR31917-45-3-2 is susceptible to B1 and is moderately susceptible to susceptible to Philippine races 2, 3, 4, and 6 of Xoo. It apparently carries the *Xa-4* gene, which confers resistance to races 1 and 5 of Xoo.

Successive backcrosses to IR31917-45-3-2 were made to recover the *O. sativa* genome and produce lines with limited *O. minuta* genetic material. To prevent premature abscision, pollinated panicles were sprayed once daily with a solution of 75 ppm gibberellic acid + 75 ppm naphthalene acetic acid. The F_1, BC_1, and BC_2 seeds began to degenerate at approximately 14 d after pollination. Embryo rescue was carried out on 14-d-old seeds to ensure survival of the progeny. Embryos were cultured on 1/4-strength Murashige and Skoog's medium (Jena and Khush 1984, Murashige and Skoog 1962). To overcome hybrid sterility, chromosome doubling was attempted through colchicine treatment of axillary buds of the F_1 hybrids; partially fertile derivatives were obtained.

Chromosome numbers of the hybrids and BC progeny were determined from mitotic or meiotic analyses. To determine the extent of recombination, the frequency of chromosome pairing was examined in 4 F_1 hybrids at pachytene, 9 F_1 hybrids at diakinesis, and 18 F_1 hybrids at metaphase I. The morphology of the hybrids and BC derivatives was examined, and their pollen and spikelet fertilities were determined.

The parents and derived progeny were tested for reactions to the B1 and BB pathogens. Clones of *O. minuta,* the F_1 hybrids, and the BC_1 and BC_2 progenies were obtained from nodal cuttings or from tillers. For evaluating B1 resistance, clones at the 3- to 4-leaf stage and 21-d-old seedlings of IR31917-45-3-2 were spray-inoculated with a spore suspension of B1 isolate PO6-6 (1×10^5 spores/ml) or exposed to natural

inocula in the International Rice Blast Nursery (IRBN). The disease reaction was evaluated 6 d after inoculation (Leung et al 1988).

For evaluation of BB resistance, IR31917-45-3-2 was inoculated approximately 50 d after sowing, while clones of *O. minuta* and the derived progeny were inoculated when several tillers were available. Representative isolates of the six Philippine Xoo races were used: PXO61 (race 1), PXO 86 (race 2), PXO 79 (race 3), PXO 71 (race 4), PXO 112 (race 5), and PXO 99 (race 6). A bacterial cell suspension (approximately 1×10^9 cells/ml) was applied using the clipping method. Lesion length was measured 14 d after inoculation and reaction type was determined (Machmud 1978).

Results

Interspecific hybrids between *O. sativa* IR31917-45-3-2 and *O. minuta* Acc. 101141 were obtained, and BC_1, BC_2, and BC_3 progenies were produced by successive backcrossing to the *O. sativa* parent. Euploid progeny with resistance to Bl or BB have been identified from among the BC derivatives.

Production, morphology, and fertility of F_1 hybrids and backcross derivatives

Production of the F_1 hybrids and BC progeny and the characterization of these interspecific derivatives in terms of chromosome number, morphology, and fertility are discussed below.

F_1 hybrids. The cross IR31917-45-3-2/*O. minuta* Acc. 101141 gave only 4% seed set from the 2,082 spikelets pollinated. Eighteen F_1 hybrids were obtained after embryo rescue.

All F_1 hybrids were triploid (ABC genome), with 36 chromosomes. Table 1 shows the mean chromosome pairing frequencies at pachytene, diakinesis, and metaphase I. A low frequency of trivalents, presumably representing associations of the A, B, and C genome homoeologous chromosomes, was observed at all three stages. As meiosis progressed, the level of pairing was reduced. The low level of chromosome pairing

Table 1. Means and ranges (in parentheses) of chromosome pairing in *O. sativa/O. minuta* hybrids at pachytene, diakinesis, and metaphase I.

Stage of meiosis	Chromosome pairing (means)					Cells examined (no.)	Chiasma frequency per cell
	I	II (rod)	II (ring)	III	IV		
Pachytene	28.18 (16–36)	3.80[a] (0-10)		0.08 (0–1)	0	40	3.93[b] (2.70–5.20)
Diakinesis	30.97 (19–36)	1.29 (0-5)	0.86 (0-6)	0.24 (0-3)	0.0001 (0-1)	313	3.49 (1.19-4.61)
Metaphase I	31.23 (17–36)	2.08 (0-9)	0.17 (0-4)	0.08 (0-3)	0.002 (0-2)	1330	2.59 (0.74–4.53)

[a]No distinction was made between rod and ring bivalents. [b]Mean chiasma frequency per cell calculated on the basis of 1 chiasma per bivalent.

suggests that a limited amount of gene transfer from *O. minuta* to *O. sativa* may occur through recombination.

The F_1 hybrids more closely resembled the *O. minuta* parent than the *O. sativa* parent, indicating dominance of the wild traits. The hybrids were vigorous, tillered profusely, were perennial, and were completely male sterile. Only 7 seeds were obtained from the 38,000 spikelets pollinated (0.02% seed set). Colchicine treatment of axillary buds resulted in the production of five partially fertile plants. From these plants, a 0.12% seed set was obtained from backcrossing 9,300 spikelets.

BC_1 progeny. Two BC_1 plants (2n=46) were obtained by backcrossing the original F_1 hybrids, and 4 BC_1 (2n=44-47 for the 3 whose chromosome numbers were determined) were obtained by backcrossing the partially fertile colchicine-treated hybrids, The BC_1 plants differed slightly in morphology, suggesting that they had lost different chromosomes. All BC_1 plants were completely male sterile.

BC_2 progeny. Two hundred embryos were rescued after pollination of 35,000 BC_1 spikelets, and 23 BC_2 plants were obtained. Two BC_2 plants had 24 chromosomes. Eighteen BC_2 plants had 32-37 chromosomes, carrying 8-13 *O. minuta* chromosomes. The chromosome number of three BC_2 plants was not determined.

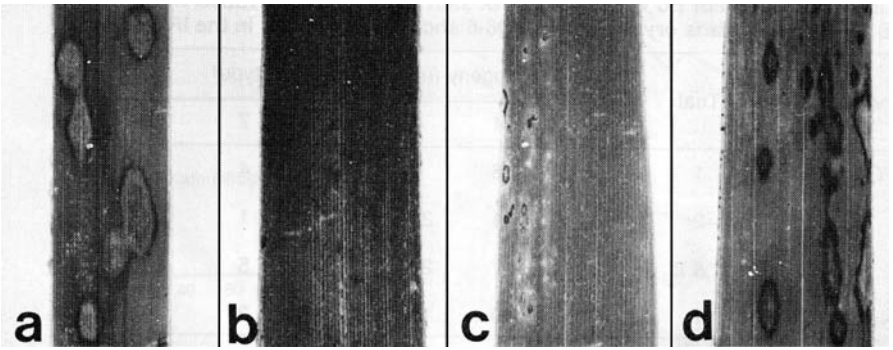
The BC_2 plants were morphologically diverse. Traits absent in either parent or in earlier generations, such as purple basal leaf sheath and apiculus, were observed among the BC_2 plants, presumably resulting from new gene combinations. The BC_2 plants with 32-37 chromosomes were completely male sterile and gave low seed sets (0–4.4%) upon backcrossing.

Two BC_2 plants had 24 chromosomes and were of special interest. The plant designated WHD IS 78-1 was resistant to BB (discussed later). It was morphologically distinct from IR31917-45-3-2, having a purple leaf sheath, apiculus, and stigma, and seeds with black hulls. It also had low fertility (21.8% pollen fertility and 16.9% spikelet fertility) and gave only 22.8% seed set upon backcrossing. This suggests that a considerable amount of *O. minuta* genetic material had been introgressed into this plant. The BC_2F_2 progeny of WHD IS 78-1 segregated for several traits, including plant height (tall vs dwarf), and color (purple vs green) of awns, leaf sheath, apiculus, and stigma. Spikelet fertility varied from 0.7 to 79.0%.

The other euploid BC_2 plant, WHD IS 75-1, was resistant to Bl (discussed later). It was morphologically similar to IR31917-45-3-2. It also had a higher fertility (60.8% pollen fertility, 73.8% spikelet fertility) than WHD IS 78-1. The morphological similarity of WHD IS 75-1 and its BC_2F_2 progeny to IR31917-45-3-2 suggests that only a limited amount of *O. minuta* genetic material was introgressed.

Evaluation for blast resistance

Figure 1 shows the type of Bl lesions observed on IR31917-45-3-2, *O. minuta* Acc. 101141, and the F_1 hybrids. IR31917-45-3-2 was consistently susceptible to Bl when artificially inoculated with PO6-6, a highly virulent strain of *P. oryzae,* and when exposed to natural inocula in the IRBN (Fig. 1a). In contrast, *O. minuta* Acc. 101141 was highly resistant. Lesions were either absent or very small, indicating a hypersensitive response to infection (Fig. 1b).



1. Blast lesions on a) *Oryza sativa* cv. IR31917-45-3-2, b) *O. minuta* Acc. 101141, c) and d) F_1 hybrids.

Although the majority of F_1 hybrid clones showed the hypersensitive resistant response to B1, the lesions were usually larger than those in *O. minuta* (Fig. 1c). Moreover, intermediate reactions (type 3) were occasionally observed (Fig. 1d). Thus, the F_1 hybrids showed a lower level of B1 resistance than did the *O. minuta* parent. Apparently, the genes conferring B1 resistance in *O. minuta* were either incompletely expressed in the F_1 hybrid, or dosage differences between the *O. minuta* parent and the F_1 hybrid affected expression of the resistance gene(s).

The reaction of five of the six BC_1 plants was similar to that of the F_1 hybrids. The exception was WHD IS 50-1 (2n=46), which was less resistant. A few typical susceptible lesions were observed on 4 of the 20 clones of WHD IS 50-1 inoculated with PO6-6.

Tests of the reactions of the BC_2 progeny to B1 were based on fewer (1–12) clones per genotype, because propagation of this generation by nodal cuttings was more difficult. Of the 16 BC_2 plants tested, 13 were as resistant as the F_1 and BC_1 plants, and 3 BC_2 plants were susceptible to PO6-6. One of the resistant BC_2 plants, WHD IS 75-1, had 24 chromosomes, suggesting that the resistance gene(s) had been introgressed from *O. minuta* into the *O. sativa* genome.

The BC_3 and BC_2F_2 progenies of WHD IS 75-1 were investigated further. Three BC_3 progeny of WHD IS 75-1 were resistant to PO6-6, and three were susceptible. The BC_2F_2 progeny segregated for their reaction to B1, both when inoculated with PO6-6 in two trials, and when exposed to natural inocula in the IRBN (Table 2). The reaction types ranged from 0 to 9. When reaction types 0, 1, and 3 are considered as resistant (R) and reaction types 5, 7, and 9 as susceptible (S), segregation among the BC_2F_2 progeny was as follows: PO6-6, Trial 1 = 11R:12S; PO6-6, Trial 2 = 35R:11S; IRBN = 35R:14S. The 35R:11S segregation with PO6-6 in Trial 2 and the 35R:14S segregation in the IRBN test fit the 3R:1S ratio (c^2 = 0 and 0.17, respectively) expected for the action of a single dominant gene. Poor seedling survival was observed in Trial 1 with PO6-6.

Table 2. Reaction of BC_2F_2 progeny of *O. sativa/O. minuta* derivative WHD IS 75-1 to *Pyricularia oryzae* isolate PO6-6 and natural inocula in the IRBN.

Inoculum	Trial	Progeny (no.) with reaction type[a]					
		0	1	3	5	7	9
PO6-6	1	2	6	3	2	4	6
	2	0	11	24	3	1	7
	1 & 2	2	17	27	5	5	13
Natural, IRBN	1	1	19	15	3	3	8

[a]Reaction type: 0 = no evidence of infection; 1 = with brown specks, no sporulation; 3 = 1- to 2-mm-long irregularly shaped lesions with brown margin, lesions may coalesce at leaf edges; 5 = fewer than five 3- to 7-mm-long typical spindle-shaped lesions per inoculated leaf; 7 = five to many typical lesions per inoculated leaf, lesions often coalesce; 9 = many coalesced lesions infecting 50% or more of inoculated leaf, leaf dies because of lesions.

Table 3. Reaction of IR31917-45-3-2, *O. minuta* Acc. 101141, and their F_1 hybrids and BC_1 progeny to representative isolates of 6 Philippine Xoo races.

Genotype	Reaction[a] to					
	Race 1 (PXO 61)	Race 2 (PXO 86)	Race 3 (PXO 79)	Race 4 (PXO 71)	Race 5 (PXO 112)	Race 6 (PXO 99)
IR31917-45-3-2	R	MS-S	MS-S	MS-S	R	S
O. minuta Acc. 101141	R	R	R	R	R	R
F_1	R	R	R	R	R	R
BC_1	R	R	R	R	R	R

[a]R = resistant (lesion length 0.1–3.0 cm), MS = moderately susceptible (6.1–9.0 cm), S = susceptible (>9.0 cm).

Evaluation for bacterial blight resistance

O. minuta Acc. 101141 showed a high level of resistance to all representative isolates of the six Philippine races of Xoo (Table 3). Lesions were very small (<1.0 cm) and necrotic by 14d after inoculation. Although IR31917-45-3-2 was resistant to Xoo races 1 and 5 at maximum tillering, presumably because of the presence of the *Xa-4* gene, it showed moderate to full susceptibility to races 2, 3, 4, and 6. All F_1 hybrids and BC_1 progeny were resistant to all six races.

Among the 10 BC_2 plants resistant to Xoo races 2, 3, and 6, the euploid plant WHD IS 78-1 was of particular interest because it appeared to represent a product of the introgression of BB resistance from *O. minuta* into the *O. sativa* genome. The F_2 progeny of WHDIS 78-1 were evaluated and shown to segregate for resistance to three Xoo races. Their reaction to races 2, 3, and 6 ranged from resistant to susceptible (Table 4). Resistance to race 4 was not detected; only moderately susceptible and susceptible reactions were observed. Thus, while genes conditioning high levels of resistance to

Table 4. Reaction of WHD IS 78-1 BC_2F_2 progeny to Philippine Xoo races 2, 3, 4, and 6.

BC_2F_2 derived from WHD IS 78-1 (no.)	Reaction[a] to			
	Race 2 (PXO 86)	Race 3 (PXO 79)	Race 4 (PXO 71)	Race 6 (PXO 99)
6	S	S	S	S
1	S	S	MS	S
2	MR	MR	MS	MR
1	MR	R	MS	R
1	MR	R	S	MR
1	MR	MR	MS	S
1	–	R	–	MR
1	R	MR	S	S
1	S	MS	S	MR
1	MS	MS	S	R
1	MS	MR	S	MS
1	S	S	MS	MR
1	MS	MS	MS	MR
1	MS	MS	S	MS
1	MS	MS	MS	MS

[a]R = resistant, MR = moderately resistant, MS = moderately susceptible, S = susceptible.

races 2, 3, and 6 were apparently transferred from *O. minuta* to WHD IS 78-1, it is not clear whether a gene conditioning a high level of resistance to race 4 is present in this plant. Reaction to races 2 and 3 cosegregated in 15 of 20 progeny, that to races 2 and 6 in 13 of 20 plants, and that to races 3 and 6 in 12 of 21 plants. Since segregation patterns could be distorted by poor seed set, it is not possible to conduct linkage analysis with the F_2 progeny. However, the recovery of recombinant types for disease reaction to the three Xoo races suggests multiple genes conditioning specific resistance to races 2, 3, and 6 in WHD IS 78-1.

Discussion

The objective of this work was to transfer resistance genes from *O. minuta* to *O. sativa.* Expecting little or no recombination between the A and BC genomes, our intention was to produce MAALs, and then to induce translocations. However, before producing MAALs, we obtained BC_2 plants having 24 chromosomes and possessing resistance to B1 and BB.

Preliminary cytogenetic analysis of the two euploid BC_2 plants showed normal chromosome pairing, suggesting that recombination or translocation, rather than chromosome substitution, may have occurred (unpubl. data). In the case of WHD IS 75-1, the plant's high fertility and morphological similarity to the *O. sativa* parent further suggest that a relatively small amount of alien DNA was transferred by recombination or translocation. A low frequency of bivalent and trivalent formation was observed at pachytene, diakinesis, and metaphase I in the *O. sativa/O. minuta*

hybrids, suggesting that intergenomic pairing and recombination between the A and the BC genomes may have occurred. Similar observations were made by Kihara and Nezu (1960), Li et al (1962), and Nezu et al (1960). The translocation of an *O. minuta* chromosome arm onto a complete or incomplete *O. sativa* chromosome through centric break-fusion may also have occurred, either during F_1 meiosis, when chromosomes of both parents remain largely unpaired, or during BC_1 meiosis, when the majority of *O. minuta* chromosomes and occasional *O. sativa* chromosomes remain unpaired (unpubl. data).

Some of the *O. sativa/O. minuta* derivatives produced in this work have been subjected to analysis using biochemical and molecular markers. Enzyme polymorphism between *O. sativa* and *O. minuta* was studied and used by Romero (1989) to monitor introgression of *O. minuta* genetic material in the BC progeny. Repetitive DNA sequences specific to *O. minuta* were cloned at the University of Missouri (H. Aswidinnoor, University of Missouri, pers. comm.) and at IRRI and used to examine some of the BC progeny (IRRI 1990).

The BB-resistant plant WHD IS 78-1 showed morphological characters apparently inherited from *O. minuta.* In addition, this plant showed seven *O. minuta*-specific isozyme bands: SDH-M2, EST-M1, EST-M2, PGD-M1, PGI-M2, PGI-M3, and PGI-M5 (Romero 1989). *Pgd-M1, Sdh-M1* and *Sdh-M2, Est-M1,* and *Est-M2* may be homoeoalleles of the *Pgd-2, Sdh-1, Est-2,* and *Est-5* loci in *O. sativa* (unpubl. data). In *O. sativa,* gene *Sdh-1* is located on chromosome 6 (Ranjhan et al 1988), *Est-2* on chromosome 3 (Nakagahra and Hayashi 1976, Sano and Barbier 1985, Sano and Morishima 1984), and *Est-5* on chromosome 1 (Wu et al 1988); *Pgd-2* remains unlocated. The chromosomal locations of the homoeologous *O. sativa* loci suggest that a considerable amount of introgression, perhaps involving more than one *O. minuta* chromosome, occurred in WHD IS 78-1. DNA extracted from WHD IS 78-1 also showed strong hybridization to two *O. minuta*-specific repetitive DNA probes (IRRI 1990; unpubl. data). The Bl-resistant plant WHD IS 75-1, however, closely resembled the *O. sativa* parent and showed no isozyme or repetitive DNA markers from *O. minuta.*

Evidence for introgression has been detected in other *O. minuta* derivatives. Of the 14 BC_3 progeny with 24 chromosomes derived from the BB-resistant BC_2 plant WHD IS 55-1 (2n=35), 5 showed hybridization with a repetitive DNA probe specific to *O. minuta,* 1 showed 2 isozyme markers inherited from the wild species, and 8 showed no evidence of introgression based on these analyses (IRRI 1990). These results further indicate that there may be significant amounts of recombination between the homoeologous chromosomes in this wide cross.

Future work with these disease-resistant lines will focus on characterizing the resistance genes and reducing the alien DNA transferred. For the Bl-resistant line WHD IS 75-1, segregation of resistance among the selfed progeny suggests that the resistance may be conditioned by a major gene. However, further tests will be done to confirm the genetics of its resistance to Bl. The resistance spectrum of the line will be tested with a range of isolates and in various Bl nurseries. The histopathology of the advanced lines will also be examined to determine the mechanism of resistance, *O.*

minuta Acc. 101141 has been shown to be resistant to Bl in Almora, Uttar Pradesh, India (J. C. Bhatt, Indian Council for Agricultural Research, pers. comm.) and in Taichung, Taiwan, China (H. S. Tsay, Taiwan Agricultural Research Institute, pers. comm.).

For the BB-resistant line WHD IS 78-1, genes conditioning specific resistance to races 2, 3, or 6 have apparently been transferred. Derivatives from this line will be crossed with IR24 to eliminate the resistance, presumably conferred by the *Xa-4* gene inherited from IR31917-45-3-2. The reaction of the derivatives to races 1 and 5 of the BB pathogen will then be assessed.

For both resistant families, allelism tests will be conducted with known resistance genes. Restriction fragment length polymorphism analysis (McCouch et al 1988) is currently being used to characterize the alien genetic material present in WHD IS 75-1 and its segregating progeny. This information should allow us to make inferences about the extent of recombination occurring in this cross, to select lines with a minimum amount of *O. minuta* DNA, and to find markers closely linked to the gene(s) controlling resistance. The establishment of linked markers should permit efficient selection of progeny carrying the resistance gene(s), and would also be the first step toward molecular cloning of the gene(s).

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Inheritance of resistance to rice tungro spherical virus in rice (*Oryza sativa* L.)

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Three rice varieties resistant to rice tungro spherical virus (RTSV) and one susceptible variety were used to determine the mode of inheritance of RTSV resistance and the allelic relationships of the genes involved. Varieties Utri Merah, Kataribhog, and Pankhari 203 were found to be completely resistant to RTSV. To assess resistant and susceptible seedlings, enzyme-linked immunosorbent assay was employed. F_1 populations invariably showed susceptibility to RTSV. The F_2 data suggest a single recessive gene for resistance in Utri Merah, whereas resistance in Kataribhog and Pankhari 203 is controlled by three complementary recessive genes. Resistance in Kataribhog and Pankhari 203 appears to be allelic, whereas the single recessive gene of Utri Merah is nonallelic to the genes in Kataribhog and Pankhari 203.

Tungro, known as *penyakit merah* (red disease) in Malaysia, is one of the most widespread and destructive rice viral diseases in Southeast Asia. The disease is attributed to the rice tungro bacilliform virus (RTBV) and the rice tungro spherical virus (RTSV); RTBV causes the disease and RTSV intensifies it (Hibino et al 1978). The dearth of information on the inheritance of resistance to the disease is probably the main reason for the absence of a modern high-yielding cultivar resistant to it. Identification of resistance sources, detection of genes, and information about the inheritance of resistance are prerequisites to a successful breeding program. The present study was undertaken to collect information on the inheritance of RTSV resistance in rice.

Materials and methods

Three tungro-resistant varieties—Utri Merah, Kataribhog, and Pankhari 203 (Ling 1979)—were crossed with TN1, a highly susceptible variety, during the 1985 main season at the Malaysian Agricultural Research and Development Institute, Bumbong Lima, Seberang Perai. In addition, crosses were made among the resistant varieties to study their allelic relationships. F_2 and F_3 seeds were produced in subsequent seasons

and used in this study. Green leafhopper (GLH) *Nephotettix virescens* that had been reared on TN1 for more than 100 generations was used as the vector for the virus. RTSV was inoculated on 10-d-old seedlings of F_1 and F_2 populations and on the parental materials by caging 2 viruliferous vectors (after an acquisition access period of 24 h) with individual seedlings in glass chimneys. The same method was followed to inoculate RTSV into F_3 families, but instead of 2 viruliferous vectors, 40 were placed with individual families of 20 seedlings in mylar cages. In combinations where Pankhari 203, a GLH-resistant variety having the *Glh-1* gene, was involved, the Pankhari 203 vector biotype was used to eliminate the vector resistance factor. Three weeks after inoculation, the seedlings were assessed individually for RTSV infection by enzyme-linked immunosorbent assay (ELISA). However, the seedlings of each F_3 family were pooled to facilitate the detection of homozygous resistant families. For convenience of presentation, the actual values of RTSV concentration from the ELISA reader were divided by the value of controlled samples of each microplate, then multiplied by 100. Antiserum for RTSV was supplied by T. Omura, National Agriculture Research Center, Tsukuba, Japan.

Results

Kataribhog, Utri Merah, and Pankhari 203 invariably showed complete resistance to RTSV. All seedlings of these varieties showed zero concentration of RTSV compared with 95.3 in TN1 (Table 1). The F_1 progenies of all crosses had RTSV infection (Table 2), and the RTSV concentration was nearly as high as that in the susceptible parent (Table 3), suggesting that RTSV resistance in Utri Merah, Kataribhog, and Pankhari

Table 1. Concentration of RTBV and RTSV in parental varieties.

Variety	Virus concentration[a]	
	RTBV	RTSV
Kataribhog	64.5	0
Utri Merah	10.8	0
Pankhari 203	38.0	0
TN1	88.9	95.3

[a]Converted value from ELISA reader.

Table 2. Reaction of F_1 populations to RTSV.

Cross	Seedlings tested (no.)	RTSV reaction
Utri Merah/TN1	30	Susceptible
Kataribhog/TN1	26	Susceptible
Pankhari 203/TN1	31	Susceptible

203 is recessive. The F_2 populations of the three crosses did not segregate in the same mode. In Utri Merah/TN1, the ratio of resistant to susceptible seedlings fit 1:3 (Table 4), indicating that a single recessive gene is responsible for RTSV resistance in Utri Merah.

Data from F_3 families of this combination confirmed the F_2 findings (Table 5). It was not possible to classify the infected families into segregating and homozygous susceptibles, because seedlings of each family were assessed together, not individually by ELISA, and only homozygous resistant families were detected. The F_2 and F_3 data

Table 3. RTSV concentration in F_1 and F_2 populations.

Parents and progenies	RTSV concentration			
	Mean deviation	Standard	Minimum	Maximum
		Utri Merah/TN1		
Utri Merah	0.0	0.0	0.0	0.0
TN1	99.07	7.21	73.31	108.92
F_1	75.92	18.26	31.93	101.81
F_2	40.03	42.48	0.0	120.12
		Kataribhog/TN1		
Kataribhog	0.09	0.11	0.01	0.43
TN1	101.92	19.71	64.73	148.0
F_1	70.75	21.98	45.39	110.32
F_2	61.46	38.94	0.0	137.87
		Pankhari 203/TN1		
Pankhari 203	0.35	0.24	0.05	1.0
TN1	74.52	17.10	35.8	96.45
F_1	48.85	30.22	6.45	104.75
F_2	24.78	33.69	0.0	105.10

Table 4. Reaction of F_2 populations to RTSV.

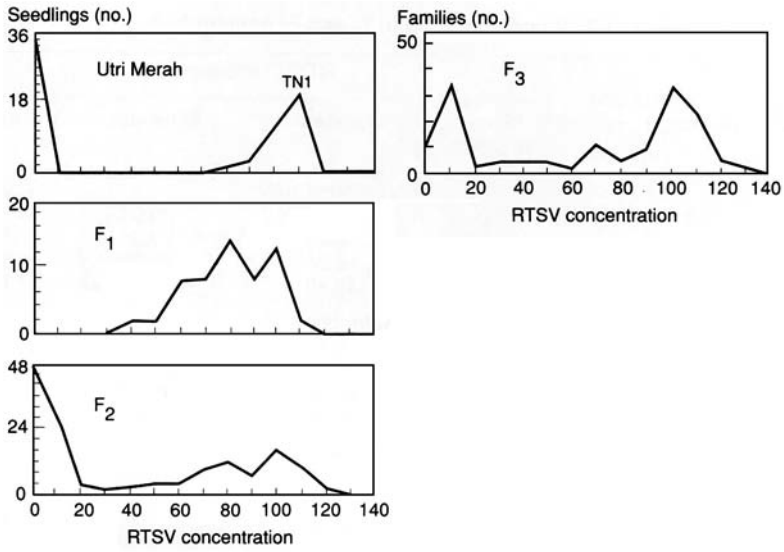
Cross	Resistant (no.)	Susceptible (no.)	Total (no.)	χ^2		p value
				1:3	1:63	
Utri Merah/TN1	92	246	338	0.772	–	0.50–0.30
Kataribhog/TN1	11	326	337	–	5.332	0.05–0.02
Pankhari 203/TN1	15	213	228	–	34.142	<0.01

Table 5. Reaction of F_3 families to RTSV.

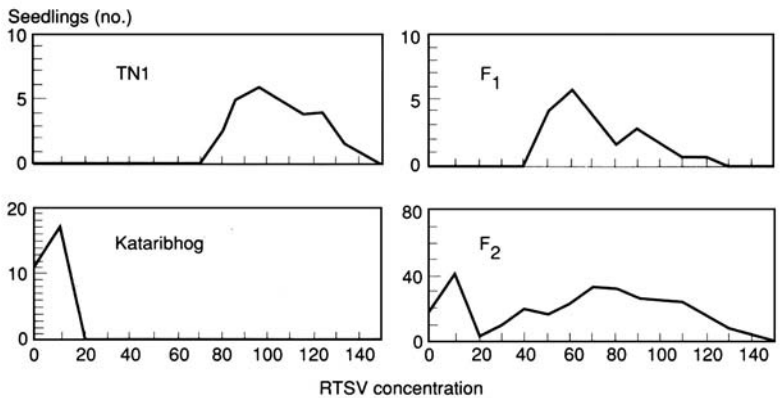
Cross	Resistant (no.)	Susceptible (no.)	Total (no.)	χ^2		p value
				1:3	1:63	
Utri Merah/TN1	43	116	159	0.253	–	0.70–0.50
Kataribhog/TN1	2	157	159	–	0.093	0.80–0.70
Pankhari 203/TN1	2	158	160	–	0.101	0.80–0.70

fit the 1:3 ratio ($p > 0.30$ and $p > 0.50$, respectively, Table 4, 5). The frequency distribution of F_2 and F_3 seedlings with various levels of RTSV concentration was bimodal, as found in typical Mendelian inheritance (Fig. 1).

The F_2 and F_3 results of Kataribhog/TN1 showed a satisfactory fit to the 1:63 ratio ($p > 0.02$ and $p > 0.70$, respectively; Table 4, 5), indicating that three complementary recessive genes control RTSV resistance in Kataribhog. The RTSV concentration in the F_2 population also showed a bimodal distribution (Fig. 2). Likewise, the F_2 and F_3



1. Frequency distribution of seedlings with various levels of RTSV concentration from Utri Merah/TN1.

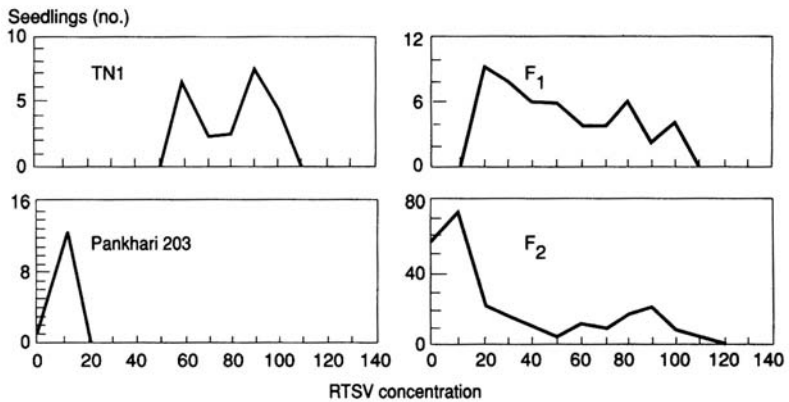


2. Frequency distribution of seedlings with various levels of RTSV concentration from Kataribhog/TN1.

results of Pankhari 203/TN1 showed a 1:63 ratio ($p < 0.01$ and $p > 0.70$, respectively; Table 4, 5), implying that three complementary recessive genes also control RTSV resistance in Pankhari 203. A mode of RTSV concentration similar to that found in the two other crosses was observed in the F_2 population (Fig. 3).

The allelic relationships among resistant varieties are illustrated in Table 6 and Figures 4 and 5. The data on F_1 and F_2 populations of Pankhari 203/Kataribhog reveal that the three complementary recessive genes responsible for RTSV resistance in each of these varieties are allelic. All 30 F_1 seedlings tested from this combination showed complete resistance to RTSV; in the F_2 population, no segregation was observed, and all 280 seedlings tested showed RTSV resistance (Table 6).

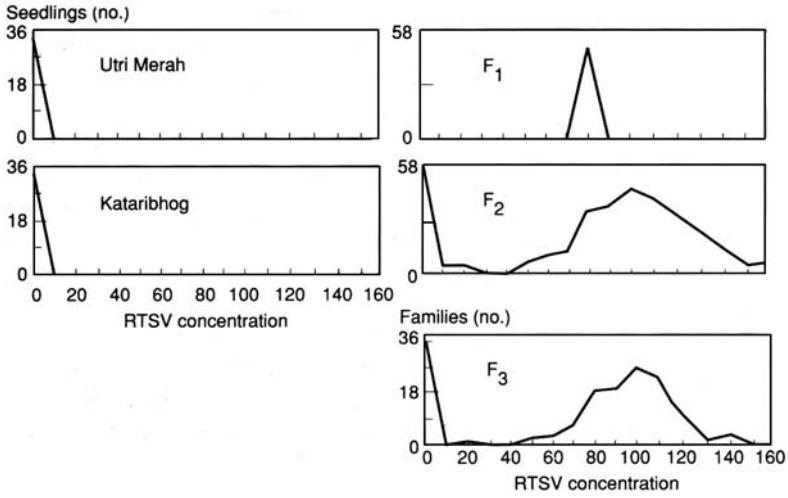
In the two other combinations—Utri Merah/Kataribhog and Utri Merah/Pankhari 203—the F_1 populations were totally infected with RTSV; in the F_2 populations, the data show a close fit to a 1:3 ratio ($p > 0.95$; Table 6). The segregations observed in the F_2 populations reveal no allelic relationship between Utri Merah and Kataribhog (Fig. 4), or between Utri Merah and Pankhari 203 (Fig. 5).



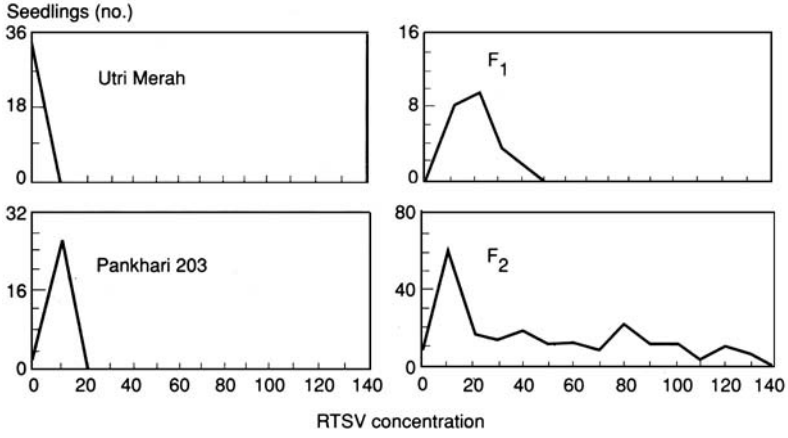
3. Frequency distribution of seedlings with various levels of RTSV concentration from Pankhari 203/TN1.

Table 6. Reaction to RTSV of F_1 and F_2 populations from crosses between resistant varieties.

Cross	F_1			F_2			c^2 1:3	p value
	Resistant	Susceptible	Total	Resistant	Susceptible	Total		
Pankhari 203/ Kataribhog	30	0	30	280	0	280	–	
Utri Merah/ Pankhari 203	0	30	30	91	259	350	0.137	> 0.95
Utri Merah/ Kataribhog	0	30	30	88	260	348	0.008	> 0.95



4. Frequency distribution of seedlings with various levels of RTSV concentration from Utri Merah/ Kataribhog.



5. Frequency distribution of seedlings with various levels of RTSV concentration from Utri Merah/ Pankhari 203.

Discussion

Studies on the genetics of tungro resistance in rice, based on visual assessment of inoculated seedlings (IRRI 1966, Lande 1975, Seetharaman et al 1976, Shastry et al 1972), have shown that resistance is dominant, and that F_2 populations inherit in a ratio of 9 resistant:7 susceptible, which suggests 2 pairs of genes having cumulative effects. Most of the resistance sources used by the authors mentioned above were also resistant to the vector.

RTSV can spread as an independent virus (Aguiero et al 1986). In some cases it is not possible to distinguish visually seedlings infected with both viruses from those infected with only one. Therefore, use of a convenient and efficient serological method for proper assessment of inoculated seedlings is necessary. Although a strong correlation was found between visual assessment and ELISA (Shahjahan 1989), this relationship may not be critical enough for a genetic study.

Recent studies by Shahjahan et al (1990) revealed that tolerance for RTBV is a polygenic trait. However, no information is available about the inheritance of resistance to RTSV. In the present study, only Utri Merah, Kataribhog, and Pankhari 203 were identified as completely resistant to RTSV. A single recessive gene was found to be responsible for RTSV resistance in Utri Merah, a traditional rice variety from Indonesia. We identified Utri Merah as an ideal source of RTSV resistance (Shahjahan 1989) and showed that the variety is also able to restrict RTBV multiplication. Kataribhog and Pankhari 203 are also suitable as RTSV resistance sources. It would be advantageous to use Pankhari 203 as a RTSV resistance source because it is also resistant to the vector. In addition, varieties resistant to RTSV may be able to inhibit the transmission of RTBV because the vector is unable to transmit RTBV without acquisition of RTSV.

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Genetics of rice resistance to brown planthopper *Nilaparvata lugens* (Stål) and relative contribution of genes to resistance mechanisms

M. Bharathi and S. Chelliah

Single dominant genes were found to govern resistance to brown planthopper (BPH) in rice accessions ARC6650, ASD11, Manoharsali, PTB33, T7, Vazhaipoo Samba (V.P. Samba), and Velathil Cheera (V. Cheera), while a single recessive gene conditions resistance in ARC10550. There was no cytoplasmic influence on BPH resistance in these varieties. The dominant gene in ASD11, Manoharsali, T7, V.P. Samba, and V. Cheera segregated independently of *Bph-3* in Rathu Heenati. The dominant gene in ARC6650 and PTB33 was found to be allelic to *Bph-3.* The recessive gene in ARC10550 was nonallelic to *bph-4* in Babawee. The dominant gene in Manoharsali, T7, V.P. Samba, and V. Cheera segregated independently of the single dominant gene in ARC6650, but was allelic to the dominant gene for resistance in ASD11. The decreased preference of BPH for orientation, feeding, and oviposition, and the increased effect of antibiosis of ASD11, PTB33, and V.P. Samba indicated the presence of minor genes or modifiers besides the single dominant genes in these accessions. The allelic relationship of the new dominant gene in ASD11, Manoharsali, T7, V.P. Samba, and V. Cheera with *Bph-6* in Swarnalata is not known, but it is apparently a new resistance gene for the South Asian BPH population.

The brown planthopper (BPH) *Nilaparvata lugens* (Stål) is one of the most devastating pests of rice in tropical and temperate Asia. Because of the development of biotypes that could damage rice cultivars possessing a narrow genetic base, it has become imperative for a crop to have a broad genetic background to retard or prevent such a catastrophe. Identification of new resistance genes is one of the most reliable strategies: by rotating varieties having different major genes conferring BPH resistance, we can slow down biotype selection. Furthermore, by pyramiding nonallelic genes, the cultivars will have a longer useful life in the field because of their resistance to BPH damage.

In view of the differential populations of BPH in Southeast and South Asia, identification of new rice genes controlling resistance to South Asian populations has become necessary. Hence, we studied the inheritance pattern of new genes, their allelic relationships with known genes, and their relative contribution to resistance.

Materials and methods

Eight BPH-resistant rice accessions were crossed with TN1, a highly susceptible variety. To determine the mode of inheritance, the F_1s and F_2s of all crosses, and the F_3s of seven crosses were evaluated for BPH resistance using the seedling bulk test method (Athwal et al 1971).

To verify any cytoplasmic influence on inheritance of resistance, reciprocal crosses between TN1 and the resistant accessions were made, and the F_1 reactions were studied.

To determine the allelic relationships with *Bph-3* (present in Rathu Heenati) and *bph-4* (present in Babawee), which impart resistance to the Indian BPH population (biotype 4), Rathu Heenati was crossed with resistant accessions ARC6650, ASD11, Manoharsali, PTB33, T7, Vazhaipoo Samba (V.P. Samba), and Velathil Cheera (V. Cheera), which possess dominant genes for resistance; and Babawee was crossed with ARC10550, which has a recessive gene.

Further crosses were made between ARC6650 and Kanoharsali, T7, V.P. Samba, and V. Cheera; and between ASD11 and Manoharsali, T7, V.P. Samba, and V. Cheera. The F_1s and F_2s of all crosses and the F_3s of three crosses were evaluated for BPH resistance. The F_1s and F_3s were evaluated on a line basis, and the F_2s on an individual seedling basis.

To unravel the relative contribution of resistance genes to the nonpreference and antibiosis components of resistance, parameters were studied on 10-, 45-, and 60-d-old potted plants of the test accessions. For orientational response and ovipositional preference of BPH, 45-d-old potted plants were used, while the feeding preference of nymphs was studied using 10-d-old plants. Twelve antibiosis parameters were studied on 60-d-old plants.

Results and discussion

Of the eight resistant accessions tested, four possessed dominant genes that have not hitherto been named; ARC10550 alone possessed a single recessive gene.

Genetics of resistance

The results on the genetics of resistance to BPH in the test accessions are presented in Table 1. The F_1s of all crosses except TN1/ARC10550 were resistant, indicating that resistance in the seven other accessions is governed by dominant gene(s). The F_1 of TN1/ARC10550 was susceptible, suggesting that resistance in ARC10550 is governed by recessive gene(s). The F_2s of all crosses except TN1/ARC10550 segregated in a ratio of 3 resistant:l susceptible, indicating that a single dominant gene governs resistance in them. The segregation of the F_3s (except TNl/ARC10550) into 1 resistant:2 segregating:1 susceptible confirmed that their BPH resistance is controlled by a single dominant gene.

The F_2 of TN1/ARC10550 segregated into 1 resistant:3 susceptible, showing that resistance in ARC10550 is governed by a single recessive gene.

Table 1. Reaction to BPH of F_1s and F_2s in crosses of TN1 with resistant accessions.[a]

Cross	F_1 reaction	F_2 seedlings (no.)		c^2 (3:1)	F_3 lines (no.)			c^2 (1:2:1)
		R	S		R	Segregating	S	
TN1/ARC6650	R	254	106	3.79	44	59	23	7.06
TN1/ARC10550	S	50	98	6.09[b]		Not tested		
TN1/ASD11	R	126	52	1.69	44	87	49	0.48
TN1/Manoharsali	R	189	58	0.30	55	137	52	3.76
TN1/PTB33	R	109	47	2.19	33	56	19	3.78
TN1/T7	R	184	68	0.53	55	99	39	2.78
TN1/V.P. Samba	R	168	46	1.40	36	58	41	3.04
TN1/V. Cheera	R	134	38	0.78	17	50	29	3.17

[a] R = resistant, S = susceptible. [b] c^2 for 1:3 ratio.

Table 2. Reaction to BPH of F_1s from crosses of TN1 with resistant rice accessions.

Cross	F_1 reaction	Cross	F_1 reaction
ARC6650/TN1	Resistant	PTB33/TN1	Resistant
ARC10550/TN1	Susceptible	T7/TN1	Resistant
ASD11/TN1	Resistant	V.P. Samba/TN1	Resistant
Manoharsali/TN1	Resistant	V. Cheera/TN1	Resistant

To study cytoplasmic influence, if any, on BPH resistance in these rice accessions, reciprocal crosses were made using TN1 as the male parent and the resistant accessions as the female parents. The F_1 reactions were similar to the F_1 reactions of the crosses where TN1 was used as the female parent (Table 2). This suggests that there is no cytoplasmic influence on BPH resistance in the accessions studied.

Allele tests. To study the allelic relationships between the dominant genes in the seven accessions and gene *Bph-3* in Rathu Heenati, the seven and Rathu Heenati were crossed. The F_1, F_2, and F_3 reactions are presented in Table 3.

The F_1s of all crosses were resistant. The F_2s of Rathu Heenati/ARC6650 and Rathu Heenati/PTB33 were almost totally resistant, with no marked segregation. This indicated that the resistance gene conditioning resistance in ARC6650 and PTB33 is allelic to *Bph-3.* The lack of segregation in the F_3 of Rathu Heenati/PTB33 confirmed that the dominant resistance gene in PTB33 is allelic to *Bph-3.* Studies on the genetics of resistance in Hyderabad, India, indicated that resistance in PTB33, ARC6650, ARC14636 B, and ARC7080 is governed by dominant genes; recessive genes control resistance in six other sources (Kalode and Krishna 1979).

The F_2s of the crosses of Rathu Heenati with ASD11, Manoharsali, T7, V.P. Samba, and V. Cheera segregated in a 15 resistant:1 susceptible ratio, indicating that resistance in these 5 accessions is controlled by a dominant gene that is nonallelic to and independent of *Bph-3.* This conclusion was confirmed in the F_3s, which segregated in the ratio of 7 resistant:8 segregating:1 susceptible (Table 3).

Table 3. Reaction to BPH of F_1s, F_2s, and F_3s in crosses of Rathu Heenati with other resistant accessions.[a]

Cross	F_1 reaction	F_2 seedlings (no.)		c^2 (15:1)	F_3 lines (no.)			c^2 (7:8:1)
		R	S		R	Segregating	S	
Rathu Heenati/ARC6650	R	96	1			Not tested		
Rathu Heenati/ASD11	R	188	6	1.57	58	74	14	3.16
Rathu Heenati/Manoharsali	R	315	27	1.58	86	111	19	3.09
Rathu Heenati/PTB33	R	114	5	–	69	0	0	–
Rathu Heenati/T7	R	95	11	3.08	38	55	11	4.53
Rathu Heenati/V.P. Samba	R	105	12	3.21	42	57	10	2.28
Rathu Heenati/V. Cheera	R	167	17	2.81	52	80	14	5.48

[a]R = resistant, S = susceptible.

Table 4. Reaction to BPH of F_1s, F_2s, and F_3s in crosses between resistant accessions.[a]

Cross	F_1 reaction	F_2 seedlings (no.)		c^2 F_3 (15:1)	F_3 lines (no.)			c^2 (7:8:1)
		R	S		R	Segregating	S	
ARC6650/T7	R	198	9	1.28	80	91	15	1.05
ARC6650/Manoharsali	R	226	20	1.48		Not tested		
ARC6650/V.P. Samba	R	260	20	0.38		Not tested		
ARC6650/V. Cheera	R	210	19	1.64		Not tested		
ASD11/Manoharsali	R	210	4	–		Not tested		
ASD11/T7	R	239	5	–	142	3	0	–
ASD11/V.P. Samba	R	219	0	–	129	2	0	–
ASD11/V. Cheera	R	163	4	–		Not tested		

[a]R = resistant, S = susceptible.

The cross Babawee/ARC10550 was made to study the allelic relationship between *bph-4* in Babawee and the single recessive gene in ARC10550. While the F_1 was susceptible, the F_2 segregated in the ratio of 3 resistant: 13 susceptible (chi-square = 5.53), suggesting that the recessive gene governing resistance in ARC10550 is nonallelic to and independent of *bph-4*. The F_3 was not tested. Khush et al (1985) reported that ARC10550 resistance to the Bangladesh (South Asian) BPH biotype is governed by a single recessive gene, designated *bph-5*.

Crosses within resistant accessions. Crosses were made between ARC6650 and T7, Manoharsali, V.P. Samba, and V. Cheera, and between ASD11 and Manoharsali, T7, V.P. Samba, and V. Cheera (Table 4). All the F_1s were resistant. The F_2 populations from the crosses between ARC6650 and T7, Manoharsali, V.P. Samba, and V. Cheera segregated into 15 resistant: 1 susceptible, indicating that the dominant resistance gene in T7, Manoharsali, V.P. Samba, and V. Cheera is nonallelic to and independent of the dominant resistance gene in ARC6650. The F_3s of ARC6650/T7 segregated into 7

resistant:8 segregating:1 susceptible, confirming that the resistance gene in T7 is nonallelic to the gene that controls resistance in ARC6650.

The F_1s of crosses between ASD11 and Manoharsali, T7, V.P. Samba, and V. Cheera were resistant. The F_2 populations were essentially resistant, and there was no segregation. This indicated that the dominant resistance gene in ASD11 is allelic to the dominant resistance gene in these four cultivars. The F_3s from ASD11/T7 and ASD11/V.P. Samba did not segregate, confirming that the dominant resistance genes in T7 and V.P. Samba are allelic to the dominant gene in ASD11.

Kabir and Khush (1987) reported that a dominant gene, *Bph-6,* in Swarnalata and a recessive gene, *bph-7,* in T12 govern resistance to the BPH population in Bangladesh. Whether the dominant gene governing resistance in ASD11, Manoharsali, T7, V.P. Samba, and V. Cheera is allelic to *Bph-6* must be verified. However, the present work indicates a dominant resistance gene hitherto not identified in the five resistant accessions studied. After studying the allelic relationship of this new gene with *Bph-6,* there is good reason to name it as a new resistance gene for the South Asian population of BPH.

Relative contribution of genes to resistance mechanisms

Data on the influence of resistance genes on orientational response of adult insects, feeding preference of nymphs, and ovipositional preference of BPH are presented in Table 5. *Bph-3* reduced adult orientation and preference for oviposition in PTB33, but exerted a smaller influence in ARC6650. This observation indicates that PTB33 might possess minor genes or modifiers that act cumulatively, leading to decreased preference of BPH for it. Similarly, the lower preference of BPH for ASD11 and V.P. Samba than for Manoharsali, T7, or V. Cheera suggests the presence of minor genes or modifiers in ASD11 and V.P. Samba.

Table 5. Relative contribution of resistance genes of rice accessions to nonpreference in BPH.

Accession	Gene	Preference by BPH for		
		Orientation[a] (%)	Feeding[b] (%)	Oviposition[c] (no. of eggs laid)
ARC6650	*Bph-3*	8.1	8.4	32.8
PTB33	*Bph-3*	6.5	4.7	20.8
ARC10550	*bph-5*	5.4	2.8	21.3
ASD11	New dominant gene	7.1	7.5	37.5
Manoharsali	New dominant gene	7.7	9.4	33.5
T7	New dominant gene	8.2	8.5	46.8
V.P. Samba	New dominant gene	6.3	6.7	35.3
V. Cheera	New dominant gene	8.8	8.8	28.0

[a]Macropterous adults alighting on 45-d-old plants at 3 d after release. [b]Alighting response of BPH nymphs on 10-d-old plants at 2 d after release. [c]Eggs laid by macropterous adults on 45-d-old plants at 3 d after release.

Table 6. Relative contribution of resistance genes of rice accessions to antibiosis against BPH.

Accession	Gene conferring resistance	Level of antibiosis in each biological parameter [a]											
		Nymphal survival (%)	Total nymphal duration (d)	Eggs laid (no.)	Egg hatch-ability (%)	Brachyp-terous adults developed (%)	Female insects developed (%)	Longevity (d)		Volume of honeydew excreted (µg)	Population buildup (no.)	Growth index	Resistance index
								Female	Male				
ARC6650	*Bph-3*	32.5 b	19.5 f	126.5 b	52.0 a	45.0 ab	42.5 a	7.0 a	6.4 ab	6.0 bc	48.3 cd	1.66 b	2580
PTB33	*Bph-3*	20.0 ab	23.8 a	84.0 a	55.0 ab	37.5 a	37.5 a	5.9 a	4.9 a	2.3 a	26.0 abc	0.84 ab	31339
ARC10550	*bph-5*	15.0 a	23.2 b	86.0 a	51.0 a	40.0 a	40.0 a	5.6 a	5.7 ab	2.5a	23.0a	0.65 a	58767
ASD11	New dominant gene	32.5 b	21.8 cd	117.0 b	66.0 bc	42.5 ab	45.0 a	6.8 a	4.5 a	4.3 ab	39.0 abc	1.50 ab	3729
Manoharsali	New dominant gene	30.0 b	20.2 ef	124.0 b	79.5 d	47.5 ab	52.5 a	7.1 a	6.7 ab	6.0 bc	82.8 e	1.49 ab	1483
T7	New dominant gene	30.0 b	19.3 f	134.0 b	55.0 ab	52.5 ab	47.5 a	6.8 a	6.3 ab	5.0 ab	66.3 de	1.54 ab	1915
V.P. Samba	New dominant gene	22.5 ab	22.4 bc	121.0 b	68.0 bcd	37.5 a	45.0 a	6.4 a	6.3 ab	4.8 ab	28.5 ab	0.99 ab	11487
V. Cheera	New dominant gene	32.5 b	20.9 de	124.0 b	67.0 bcd	47.5 ab	47.5 a	6.5 a	6.1 ab	5.3 ab	40.8 bcd	1.55 ab	3223

[a]Exhibited by 60-d-old plants of the reslstant accessions. In a column, means followed by the same letter are not significantly different at the 5% level by Duncan's Multiple Range Test.

Data on the relative contribution of resistance genes to the antibiosis components against BPH are presented in Table 6. *Bph-3* in ARC6650 exerted lower influence than *Bph-3* in PTB33 on nymphal survival, fecundity, brachypterous and female insects, longevity of adult insects, quantity of the honeydew, population buildup, and growth index, which led to a decreased resistance index. Such an effect suggests that the increased influence might have been due to a minor gene in PTB33. Despite possession of a single dominant gene for BPH resistance, ASD11 and V.P. Samba showed increased influence on many of the antibiosis factors, which also suggests that minor resistance genes or modifiers are present in these two accessions.

These results indicate that, although an identified resistance gene is present in a number of rice cultivars, the complementary effects of minor genes or modifiers cause the resistance potential of individual cultivars to vary. This information places increased importance on choice of specific resistant cultivars for resistance breeding.

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Genetic analysis of resistance to whitebacked planthopper *Sogatella furcifera* Horvath in rice

Min Shaokai, Li Ximing, Xiong Zhenmin, and Hu Guowen

Six varieties resistant to whitebacked planthopper were obtained through 3 replicated screenings from 850 Chinese rice germplasm accessions. Genetic analysis showed that a single dominant gene controls resistance in these varieties. The resistance genes for improved variety HA79317-7 and Dianri 336-3 are allelic to *Wbph-1* and *Wbph-2,* respectively, and the resistance genes for four Yunnan local varieties—Guiyigu, Biangu, Daqigu, and Dahuagu—are nonallelic to *Wbph-1, Wbph-2, Wbph-3,* and *Wbph-5* but allelic to each other. The new resistance gene is proposed as *Wbph-6(t).*

The whitebacked planthopper (WBPH) *Sogatella furcifera* Horvath is one of the important insect pests of rice. In recent years, it has occurred seriously in most rice-growing areas in South China. Varietal resistance has been considered to be the most promising strategy for control of this pest. So far, five genes (four dominant and one recessive) for resistance have been identified. This paper reports an additional gene for resistance.

Materials and methods

The six resistant varieties—Guiyigu, Biangu, Daqigu, Dahuagu, HA79317-7, and Dianri 336-3—used in this study were obtained from replicated screening (three times) during 1983–84. They were crossed with the highly susceptible check variety Taichung Native 1 (TN1), and the F_1, F_2, and BC_1 were investigated for resistance to WBPH to determine the mode of inheritance.

The test varieties were also crossed with resistant checks N22, which is homozygous for resistance gene *Wbph-1* (Sidhu et al 1979); IR30659-2-165, with *Wbph-2* (Saini et al 1982); ADR52, with *Wbph-3* (Hernandez and Khush 1981); and N'Diang Marie, with *Wbph-5* (Wu and Khush 1985). The F_1, F_2, and testcross (F_1/TN1) were studied to determine the allelic relationships of the resistance genes of the test varieties during 1985–86 (using N22 and IR30659-2-165 as resistant checks), and during 1987–

88 (using ADR52 and N'Diang Marie as resistant checks). During 1987-88, the test varieties that had the new resistance gene were crossed with each other to determine allelic relationships.

The materials were tested for resistance to WBPH using the bulk seedling test. The seedlings were uniformly infested at the 2-leaf stage (about 7 d after sowing) with 7-9 first- to second-instar WBPH nymphs per seedling. The reaction was recorded when more than 95% of the susceptible checks were killed, generally 7 d after infestation. The seedlings were scored as resistant (the reactions similar to those of the resistant checks) or susceptible (completely killed or severely wilted). The reactions of the P and F_1 generations were scored on the row basis, and those of the F_2 and BC_1 or testcross populations on an individual seedling basis.

Results

The reactions of the F_1, F_2, BC_1, and testcross progenies to WBPH investigated in 32 crosses are shown in Tables 1-5.

Table 1. Reaction to WBPH in F_1, F_2, and BC_1 populations of crosses between 6 resistant varieties and TN1.

Cross	F_1 reaction[a]	F_2 seedlings (no.)		c^2[b] (3:1)	$BC_1(F_1/TN1)$		c^2[b] (1:1)
		Resistant	Susceptible		Resistant	Susceptible	
TN1/Guiyigu	Resistant	284	99	0.12	59	36	5.09
TN1/Biangu	Resistant	272	70	3.51	64	44	3.34
TN1/Daqigu	Resistant	269	71	2.86	54	37	2.81
TN1/Dahuagu	Resistant	282	97	0.04	56	50	0.24
TN1/HA79317-7	Resistant	259	94	0.42	52	34	3.36
TN1/Dianri 336-3	Resistant	300	80	2.95	50	33	3.08

[a] 25-30 seedlings for each cross. [b] $c^2 = 3.84$, $P = 0.05$; $c^2 = 6.63$, $P = 0.01$.

Table 2. Reaction to WBPH in F_1, F_2, and testcross populations of crosses between 6 resistant varieties and N22.

Cross	F_1 reaction[a]	F_2 seedlings (no.)		c^2[b] (15:1)	Testcross (F_1/TN1)		c^2[b] (3:1)
		Resistant	Susceptible		Resistant	Susceptible	
N22/Guiyigu	Resistant	307	14	1.65	101	22	2.95
N22/Biangu	Resistant	379	21	0.52	88	18	3.22
N22/Daqigu	Resistant	328	13	3.05	92	19	3.27
N22/Dahuagu	Resistant	282	10	3.51	81	39	3.21
N22/HA79317-7	Resistant	327	0	—	118	0	—
N22/Dianri 336-3	Resistant	350	15	2.50	83	19	1.88

[a] 30 seedlings for each cross. [b] $c^2 = 3.84$, $P = 0.05$.

Inheritance of resistance

The F_1 seedlings in all crosses with TN1 were resistant, indicating that resistance was dominant in all six varieties. All of the F_2 and BC_1 populations segregated in the ratio of 3 resistant:1 susceptible and 1 resistant:1 susceptible, respectively. These results indicate that resistance to WBPH in each of the six varieties is governed by one dominant gene.

Allelic relationships

The allelic relationships of the resistant genes in the six tested varieties to *Wbph-1, Wbph-2, Wbph-3,* and *Wbph-5* were identified.

Crosses with N22. The F_1s from six crosses with N22 were resistant to WBPH (Table 2). The F_2 and testcross populations from the cross with HA79317-7 did not segregate for susceptibility, indicating that the single dominant gene in this variety is allelic to *Wbph-1.* The F_2 and testcross populations from other crosses all segregated in the ratio of 15 resistant:1 susceptible and 3 resistant:1 susceptible, respectively, indicating that the single dominant gene for resistance in Guiyigu, Biangu, Daqigu, Dahuagu, and Dianri 336-3 is nonallelic to and independent of *Wbph-1.*

Crosses with IR30659-2-165. The F_1s from five crosses with IR30659-2-165 were resistant (Table 3); the F_2 and testcross populations from the cross with Dianri 336-3 did not segregate for susceptibility, showing that the single dominant gene in this variety is allelic to *Wbph-2;* the F_2 and testcross populations from other crosses all segregated in the ratio of 15 resistant:l susceptible and 3 resistant:l susceptible, respectively, showing that the single dominant gene for resistance in Guiyigu, Biangu, Daqigu, and Dahuagu is nonallelic to and independent of *Wbph-2.*

Table 3. Reaction to WBPH in F_1, F_2, and testcross populations of crosses between 6 resistant varieties and IR30659-2-165.

Cross	F_1 reaction[a]	F_2 seedlings (no.)		c^2[b] (15:1)	Testcross (F_1/TN1)		c^2[b] (3:1)
		Resistant	Susceptible		Resistant	Susceptible	
IR30659-2-165/ Guiyigu	Resistant	331	29	1.71	96	24	1.34
IR30659-2-165/ Biangu	Resistant	342	33	3.74	83	17	3.00
IR30659-2-165/ Daqigu	Resistant	368	18	1.29	75	14	3.60
IR30659-2-165/ Dahuagu	Resistant	304	12	2.84	73	37	3.33
IR30659-2-165/ HA79317-7	Resistant	275	10	3.20	59	10	3.52
IR30659-2-165/ Dianri 336-3	Resistant	325	0	—	102	0	—

[a] 24–30 seedlings for each cross. [b] $c^2 = 3.84$, $P = 0.05$.

Crosses with ADR52 and N'Diang Marie. The F_1 from four crosses with ADR52 and N'Diang Marie were resistant (Table 4). The F_2 and testcross populations from all of the crosses segregated in the ratio of 15 resistant:1 susceptible and 3 resistant:l susceptible, respectively, indicating that a single dominant gene for resistance in Guiyigu, Biangu, Daqigu, and Dahuagu is nonallelic to and independent of *Wbph-3* and *Wbph-5.*

Crosses among Guiyigu, Biangu, Daqigu, and Dahuagu. To determine the allelic relationship of the resistance gene in Guiyigu, Biangu, Daqigu, and Dahuagu, the performances of the F_1, F_2, and testcross populations in six crosses were investigated (Table 5).

Table 4. Reaction to WBPH in F_1, F_2, and testcross populations of crosses between 4 resistant varieties and ADR52 and N'Diang Marie.

Cross	F_1 reaction[a]	F_2 seedlings (no.)		c^{2b} (15:1)	Testcross (F_1/TN1)		c^{2b} (3:1)
		Resistant	Susceptible		Resistant	Susceptible	
ADR52/Guiyigu	Resistant	266	11	2.08	98	23	2.01
ADR52/Biangu	Resistant	290	17	0.16	74	16	2.13
ADR52/Daqigu	Resistant	352	24	0.00	85	18	2.72
ADR52/Dahuagu	Resistant	294	26	1.61	74	17	1.62
N'Diang Marie/ Guiyigu	Resistant	280	19	0.00	96	19	3.97
N'Diang Marie/ Biangu	Resistant	436	28	0.01	76	16	2.45
N'Diang Marie/ Daqigu	Resistant	293	21	0.04	54	14	0.49
N'Diang Marie/ Dahuagu	Resistant	41 0	28	0.00	112	30	0.94

[a] 24–30 seedlings for each cross. [b] c^2= 3.84, P = 0.05; c^2 = 6.63, P = 0.01

Table 5. Reaction to WBPH in F_1, F_2, and testcross populations of crosses among 4 resistant varieties.

Cross	F_1 reaction[a]	F_2 seedlings (no.)		Testcross (F_1/TN1)	
		Resistant	Susceptible	Resistant	Susceptible
Guiyigu/Biangu	Resistant	405	2	78	0
Guiyigu/Daqigu	Resistant	311	1	79	0
Guiyigu/Dahuagu	Resistant	412	3	107	0
Biangu/Daqigu	Resistant	290	0	101	0
Biangu/Dahuagu	Resistant	320	1	69	0
Daqigu/Dahuagu	Resistant	289	1	84	0

[a] 24–30 seedlings for each cross.

The F_1s from all the crosses were resistant. The F_2 and testcross populations from the six crosses did not segregate for susceptibility, indicating that the single dominant genes in these varieties are allelic to each other.

Discussion

The genetic analysis of six Chinese resistant rice varieties revealed that two improved ones derived from IR lines—HA79317-7 and Dianri 336-3—have *Wbph-1* and *Wbph-2,* respectively. Each of the four Yunnan local tall indica varieties Guiyigu, Biangu, Diqigu, and Dahuagu has the same new dominant resistance gene nonallelic to *Wbph-1, Wbph-2, Wbph-3,* and *Wbph-5.* It is suggested that this new single dominant resistance gene be identified as *Wbph-6(t),* and Guiyigu should be the standard variety.

Yunnan, believed to be one of the areas in the world where rice originated, is rich in rice germplasm resources. The search there for beneficial genes for breeding should be further strengthened.

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Discussion

Session 5: Genetics of disease and insect resistance

C—*Khush:* Dr. Oka questioned why *O. minuta* was used in wide crosses when *O. officinalis* is diploid and a better wild species to use. In our wide hybridization program we are systematically exploring the possibility of transferring genes from all the wild species. We have studied the crosses of *O. sativa* with *O. minuta, O. latifolia,* and *O. brachyantha,* and we shall continue to explore crosses of *O. sativa* with other wild species.

C—*Chang:* I believe that particular accession of *O. minuta* was used because of its outstanding resistance. The other reason was to broaden the base of wide hybridization by including a tetraploid species. We now have more than 2,300 accessions of wild rices, the bulk of which were donated by Dr. Oka and his co-workers. Recently there has been great demand for wild species, and we have tried our best to meet the requests. But most wild species are low seed producers. Thus seed supply is a constraint in evaluating the wild relatives of rice.

Q—*Oka:* 1:63 and 1:3 F_2 ratios were found. Perhaps there might be four recessive genes for tungro resistance. What is your gene model?

A—*Shahjahan:* In Utri Merah a single recessive gene conveys rice tungro spherical virus (RTSV) resistance, whereas three complementary recessive genes are responsible for RTSV resistance in Kataribhog and Pankhari 203. These three genes can express only when they are all together. If one is alone or two are together, no expression is observed. We have found only 1 resistant plant in every 64 plants in the F_2.

Q—*Nguyen Van Huynh:* Please clarify the role of minor or modifier genes in the decreased preference of brown planthopper (BPH) for resistant varieties.

A—*Chelliah:* In some cases, although the same major gene controls resistance to BPH in more than one rice variety, the level of resistance imparted varies significantly. While we can use standard techniques like the chi-square test to study the number of major genes involved, it is difficult to identify the minor genes. In view of this, in the present study the behavioral and biological factors concerning BPH on resistant varieties with the same gene were considered to reflect the role of minor genes.

C—*Sano:* Recent genetic surveys have suggested great diversity among the wild species, and that different wild species might carry different types of resistance genes. So the extraction of alien genes from different taxa seems to be very important.

SESSION 6

Tissue and Cell Culture

Application of somaclonal variation and in vitro selection to rice improvement

J. Bouharmont, A. Dekeyser, V. Van Sint Jan, and Y.S. Dogbe

Somaclonal variation was observed in the greenhouse and in the field on plants regenerated from calli and on their progeny. Many plantlets were recovered from cells cultivated on medium with 1.5% NaCl. Several selected plants showed even higher salt tolerance. A few cell lines survived at a sublethal NaCl concentration (1.75%). However, the progeny of one plant from such selection did not express improved salt tolerance. Adding $Al_2(SO_4)_3$ to the culture medium reduced cell proliferation. Calli were also affected by other medium modifications required for Al solubilization. Some plants regenerated from calli selected on a modified medium, with or without Al, expressed a degree of Al tolerance. Selection for cold tolerance was attempted by long-term culturing of calli at sublethal temperatures (11-13 °C). Some plants regenerated from cell selection and tested under hydroponic conditions showed improved cold tolerance. Progeny of plants regenerated for the different stresses are being field-tested in Africa. Although incomplete, these experiments confirm the potential of somaclonal variation in rice improvement and the applicability of in vitro selection for stress tolerance.

Somaclonal variation, induced by in vitro culture, is well documented in many plant species. Several papers have reported numerous variants regenerated from cell cultures of rice. Although the origin and nature of the variations observed are diverse and generally obscure, induction of point mutations has been proved.

The implications of somaclonal variation for plant breeding are also disputed. Although some resultant modifications in agronomic traits have proved beneficial, only a few have been effectively applied for varietal improvement. Thus, somaclonal variation represents a new source of genetic diversity available for breeding.

Somaclonal variation can be used in two ways. The first approach consists of selecting from the population of regenerants from cell culture. Several examples have been described, mainly for disease resistance in sugarcane and potato. The second way depends on the possibility of selecting cell lines resistant to some physical or chemical stresses. and then recovering whole plants with the same resistance in the field. Several applications of the second approach have been described, and research at institutions in several countries has been initiated, mainly for improving salt tolerance in rice.

Induced mutations allow increased genetic variability when some characters are not available; in cultivated varieties, mutagenesis at the plant or cellular level can modify single agonomic traits without loss of existing gene combinations.

Materials and methods

A large number of rice varieties from several countries of Africa and Asia as well as from the International Rice Research Institute and the West Africa Rice Development Association were compared for their behavior in vitro. The genotypes actually used for experiments combine good agronomic characters for some African areas, high levels of callus induction, and efficient plantlet regeneration after in vitro culture for several months. All experiments were based on calli induced from mature embryos excised from hydrated caryopses.

LS medium, proposed by Linsmaier and Skoog (1965), was used. For callus induction in the dark, LS medium was supplemented with 2-4-dichlorophenoxyacetic acid (2,4-D:0.5 mg/liter), naphthaleneacetic acid (NAA:1 mg/liter), benzylamin-opurine (BAP:l mg/liter), sucrose (3%), and agar (0.7%) or gelrite (0.3%). For plant regeneration, LS mineral solution was used at half concentration and supplemented with 2,4-D (0.01 mg/liter), NAA (1 mg/liter), BAP (1 mg/liter), and sucrose (2%); in both cases, pH was 5.7. For selection for A1 tolerance, mineral composition and pH were modified.

Most somatic embryos differentiating in the calli were morphologically different from the sexual ones; small plantlets had a single primary root and were easily separated from the callus. They were transferred to soil in the greenhouse in Europe; some were sent to Africa, where they were cultivated in a nursery.

Results and discussion

The main objective was in vitro selection of mutations appearing in cell cultures. First, observations were made to estimate the importance of somaclonal variation occurring in cultivated cells and regenerated plants.

Somaclonal variation

Variation appeared very early after callus induction, resulting in differences in color, compactness, growth rate, and embryogenesis. Many calli gave rise to chlorophyll-deficient plantlets in addition to green ones. At least some chlorophyll mutants must have resulted from chloroplast modifications already present in the seed embryo or induced during the first cell divisions in the explant, because some somatic embryos were chimeric. On the other hand, white plantlets or sectors were observed in the seed progeny of some regenerated plants, probably indicating segregation of mutated plastids during embryo sac differentiation or early embryo development.

A large number of plants of varieties I Kong Pao and Tatsumi Mochi were regenerated from calli cultivated for 5–7 mo and were grown in the greenhouse.

Unfavorable conditions induced some modifications and increased variation in plant height, duration, and fertility. Differences were very clear among families derived from cell lines of the same variety. Characters involved were flowering date, shoot number, panicle number, plant size, panicle exsertion, panicle length and shape, flag leaf orientation, seed shattering, and sterility.

Seed was harvested from regenerated plants in the greenhouse and sent to Togo. R_2 plants were observed in 88 lines derived from different regenerated plants. The germination rate and flowering date were recorded, as well as some morphological characteristics and the incidence of blast (Bl) caused by *Pyricularia oryzae.* Some progenies were significantly different from the original genotypes in flowering date, plant height, and panicle morphology, indicating that somaclonal modifications had been transmitted through the seed. Some R_2 lines were very homogeneous, while others still segregated for one or several characters. Interesting phenotypes with modifications in tillering, seed number, caryopsis length and morphology, and Bl resistance were observed. Their stability must be verified through several cycles. Crosses will be made to investigate the inheritance of the variations observed.

Salt tolerance

High concentrations of NaCl and other salts are the main limitation to rice cultivation on very large areas, According to Paul and Ghosh (1986), 4 million ha of ricefields in India alone are affected by salinity. Salinity is also a major problem in West African lowlands. Many wild species are known to be highly salt tolerant, and a number of physiological mechanisms have been described. Classical breeding methods have succeeded in enhancing the salt tolerance of rice and some other crops. Salt-tolerant plants have been regenerated in tomato and a few other crops after selection of cell lines. In flax, McHughen (1987) showed that the selected character was not directly related to a modification of the cell physiology, but was a consequence of enhanced plant vigor. Nevertheless, the applicability of in vitro selection was demonstrated, and other experiments have shown that salt tolerance at the plant level is sometimes related to cellular characteristics. Such a correlation has been confirmed in rice. For critical NaCl concentrations in culture medium, callus proliferation was lower in susceptible varieties (Srimalaysia or I Kong Pao) than in the tolerant Nona Bokra.

In nontolerant genotypes, cell proliferation is impeded when calli are maintained on a medium containing 15 g NaCl or more/liter. Calli of I Kong Pao were cultivated for 4 mo on a medium containing 15 g NaCl/liter with monthly transfers to fresh medium. Under these conditions, most calli started to regrow after about 3 mo. Such recovery was probably due to a physiological adaptation of the cells rather than to a genetic modification. Eleven percent of the plantlets regenerated from these calli and cultivated on a medium with 15 g NaCl/liter survived more than 3 wk, but all unselected plants died. Progenies of selected lines were sent to Casamance, Senegal, for testing on saline soils.

Lethal NaCl concentrations varied slightly according to the genotype. Cell proliferation always stopped at 17.5 g NaCl/liter. Calli generally died after 3–4 mo, but

secondary proliferations appeared on some necrotic cultures. Four cell lines (3 of Nona Bokra and 1 of I Kong Pao) lived for 6 mo under selective conditions and produced a few plantlets after transfer to a nonsaline medium. Five plants derived from the salt-tolerant callus of I Kong Pao were cultivated in the greenhouse for seed increase, and their progenies were compared with unselected lines at the plantlet stage under hydroponic conditions. No differences in dry weight between these five progenies and the original I Kong Pao were observed after culture of the plants at different salinity levels. On the other hand, calli derived from the 5 lines did not differ from unselected calli when cultivated on a medium with 15 g NaCl/liter. These results indicate that the plants regenerated from one of the selected calli of I Kong Pao were not salt tolerant, because the cells of the secondary callus, or some of the cells, were not modified, or because their adaptation was transitory and consequently not transmitted through seed. The progenies of three selected calli from variety Nona Bokra were sent to Senegal for field testing.

Similar selection experiments were performed with other varieties; 20 calli were selected, and a number of available plantlets are to be tested for salt tolerance. Investigations on the selected calli have also been initiated to control their own tolerance and to discriminate ion toxicity from osmotic effects by replacing NaCl with mannitol.

Aluminum tolerance

Aluminum toxicity is a widespread problem in tropical Africa and South America. Aluminum acts directly on the cell and on the whole plant. Its presence also leads to deficiencies in P, Ca, Mg, and Fe. In addition, solubilization of Al ions is possible only at low pH. Therefore, plant tolerance for Al may result from several modifications at the cellular level: tolerance for Al ions themselves or for low pH, or capability to maintain the ion balance in the cell. The mineral composition of culture media was modified for selecting Al-tolerant lines: elimination of KNO_3, lower K_2HPO_4 and $CaCl_2$ concentrations, and substitution of $FeSO_4$ for chelated Fe. The pH was adjusted to 4 and a higher gelrite concentration was used when $Al_2(SO_4)_3$ $18H_2O$ concentration increased. Aluminum concentrations used in the first selection experiments ranged from 0.25 to 2 mM (83 to 670 mg/liter).

Calli from 2 varieties susceptible to Al were grown for 20 wk on several media to test the influence of factors such as pH, mineral nutrients, and Al concentration. Preliminary observations indicated that a lower pH did not affect callus growth much, while changes in ion balance had a strong negative influence. Adding $Al_2(SO_4)_3$ slowed down callus growth and increased the frequency of necrotic calli. These results suggest that a medium containing at least 1 mM Al is efficient for in vitro selection.

After transfer to a regeneration medium with no Al, calli produced secondary cell proliferations and plantlets. Therefore, it may be possible to regenerate plants after a 20-wk stress; regeneration did not depend on the composition of the medium.

Regenerants from calli selected on various media were cultivated in hydroponic solution containing Al. Generally, deficiency symptoms increased with Al concentra-

tion. Root and leaf growth was progressively inhibited. However, a few plants tolerated the stress and flowered. One plant each of varieties I Kong Pao and Aiwu formed panicles when placed in 1 mM Al solution. One plant of I Kong Pao obtained from selection on medium with 1.25 mM Al flowered in a solution with the same concentration. Two other Aiwu plants, although selected on modified Al-free medium, were tolerant of Al; they flowered when placed in 0.5 or 0.75 mM solution. More plants were regenerated and must be analyzed; their progenies must be tested.

These first data indicate that plants tolerant of Al can be selected in vitro, not only by culturing cells in the presence of Al ions, but also on a modified mineral medium. This suggests that the trait selected could be due to the capability to support an ion imbalance linked to Al-rich environments, rather than the tolerance for toxic ions themselves.

Cold tolerance

In the mountains of Central Africa, low temperatures affect rice in two ways. First, a mean temperature decrease with altitude is responsible for late flowering: second, short and often local night temperature drops cause spikelet sterility and floral malformations in sensitive genotypes.

Cold tolerance proceeds from many factors: some are related to cellular characteristics and selectable in vitro. Improved clones of ornamental plants of which floral induction mechanisms appear modified have been isolated in vitro. In addition, culture at low temperatures could promote proliferation of mutated cells that are tolerant of stress.

The implication of cellular traits for rice cold tolerance was confirmed by analyzing the response of calli derived from different genotypes cultivated at low temperatures. Callus development stopped almost completely at 13 °C for tolerant varieties Tatsumi Mochi and Yunnan III and at 15 °C for Srimalaysia and KH998. Necrotic calli were more frequent and more apparent in susceptible genotypes subjected to low temperature.

In the first experiment, calli from the tolerant varieties were cultivated for 6 mo at 13 or 11 °C. At 13 °C, several secondary calli of both varieties developed after several months and produced plantlets, but at 11 °C only 1 callus survived. Regenerated plants obtained from the selected calli were grown in the greenhouse, and their progeny were tested in Burundi at high altitude.

In the second experiment, calli from cold-tolerant variety Nanking 11 and the Egyptian genotype 170/15 were subjected to the same selection conditions (13 °C) for 6 mo. A few plantlets were recovered from two calli selected from Nanking 11 and two from 170/15. More progenies were obtained from both genotypes after callus culture at 15 °C. In spite of the small number of plantlets available, tests were performed to assess the expression of the character selected. Ten plants derived from calli of both varieties selected at 13 and 15 °C were transferred to soil and placed in the phytotron at 10 °C for 2 wk. The leaves of all plants regenerated from 13 °C-tolerant calli remained green, but the leaves of the progenies of 15 °C-tolerant calli and the

unselected plants were etiolated or turned brown. Plants of 170/15 from calli selected at 13 °C and cultivated for 10 d at 10 °C also differed from unselected plants in producing adventitious roots. These observations suggest that cold-tolerant plants can be regenerated from cell cultures selected at a sublethal temperature. Larger samples of regenerated plants and their progeny should be tested to check their cold tolerance stability and eventually to identify other modifications possibly linked to this trait.

Twelve progeny of Tatsumi Mochi evaluated in Togo for somaclonal variation were produced from calli selected at 13 °C. Some families showed rather high variability in flowering date, although differences among the means were not significant. The incidence of morphological variants seemed higher in Tatsumi Mochi than in I Kong Pao. Higher variation could be due to the stress or could reflect varietal differences.

Conclusions

The broad variation induced by in vitro culture in rice was confirmed by the differences found within both calli and regenerated plants. Several morphological traits observed in the progenies of these plants indicated the transmission of mutations through seed. At least some chlorophyll deficiencies were attributed to plastid alterations and were not observed until the second generation from regenerants. Similar cytoplasmic and nuclear mutations were reported previously (Sun et al 1983); several could be used in rice breeding programs.

Sterility and semisterility were frequent in plants derived from cell culture, even when no stress was applied. Generally, seed sterility decreased rapidly in subsequent generations. Sterility probably resulted from alterations in chromosome number or structure.

Calli were submitted to various types of stress to develop cell lines more cold resistant and salt or Al tolerant. Secondary calli survived on the selective media and developed into plantlets, which were tested for stress tolerance. The response to stress was variable. Stress tolerance increased for some regenerants, while for others no difference appeared between selected and unselected individuals.

The absence of tolerance in plants derived from cell lines selected in vitro is frequently mentioned in the literature and is tentatively explained by transient physiological changes or gene amplification. Another explanation is that regenerants are formed from more than one cell and result in chimeric plants, from which mutated cells forming tolerant tissues are eliminated. To prevent the complete failure of selection, selection pressure should be lower to produce a larger number of regenerants. This selection, less efficient at the cellular level, could be completed by screening of the regenerated plants.

For some rice varieties, plant regeneration is possible from microcalli cultured in liquid medium. Microcalli seem to be a valuable material for more efficient selection because of the larger number of “units” selectable and the better contact between cells and chemicals. On the other hand, plant regeneration on selective media might reduce the occurrence of tissues that have not undergone mutation. Finally, anther culture and

in vitro selection at the haploid level would allow isolation of mutations that are not expressed in diploid cells.

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Diallel analysis of callus growth and plant regeneration in rice

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The genetic characteristics of callus growth and plant regeneration in japonica rice varieties were studied in a 6 × 6 diallel cross. Estimates of genetic parameters following Hayman's method showed a high value of additive gene effects for callus growth, which were recognized as incomplete dominance and negative action. On the other hand, dominance effects (recognized as overdominance) were larger than additive effects for plant regeneration. Epistatic effects were observed in this character. Graphical analysis using Wr and Vr for callus growth showed that Norin 1, Somewake, and Daikoku 1 possess dominant genes that cause slow callus growth, while Kuju, Sasanishiki, and Murasaki-ine, whose callus growth was more vigorous, have recessive genes. The distribution of callus growth in the F_2 between Kuju and Somewake showed 3:1 segregation. Analysis of variance for combining ability showed a higher value for additive genetic variation, which represents high general combining ability for callus growth, and a relatively higher value for dominance variation, which represents high specific combining ability for plant regeneration. The reciprocal F_1s between Norin 1 and Somewake showed excellent capacities for plant regeneration (average: 62%). These results show that the capacity for plant regeneration can be improved through incorporation of the genes.

The application of tissue culture technology to rice improvement depends on culturability, i.e., callus formation, maintenance, and plant regeneration. Studies of physiological and environmental factors affecting culturability were first conducted by Saka and Maeda (1969) and Henke et al (1978). In our earlier studies (Abe and Futsuhara 1984, 1986), we pointed out significant variations among genotypes in the abilities to form callus and to regenerate plants, and we identified rice genotypes amenable to tissue culture. Furthermore, we suggested that the capacities for callus growth and organ differentiation are under genetic control. In cereal tissue culture, a few workers have suggested a genetic basis for in vitro culture response (Chaligari et al 1987, Charmet and Berrnard 1984, Komatsuda et al 1989, Miah et al 1985). However, precise

information on the inheritance of capacities for callus growth and plant regeneration in rice has not been reported.

The aim of the present study was to provide precise information about the genetic control of callus formation and plant regeneration and their inheritance in rice seed callus cultures.

Materials and methods

Six japonica rice cultivars—Kuju, Sasanishiki, Murasaki-ine, Norin 1, Somewake, and Daikoku 1—were selected from the results of previous experiments in which they showed different callus formation and plant regeneration abilities (Abe and Futsuhara 1986). A full set of diallel crosses was performed among the six cultivars. Twelve seeds of the 36 combinations, including the seeds from 6 selfed parents, were tested for callus formation and plant regeneration with 2 replications. Murashige and Skoog's (MS) basal medium was used. In vitro culture conditions and medium composition were the same as described by Abe and Futsuhara (1986), except that test tubes (1.6 × 18 cm) containing 10 ml agar medium were used for callus formation. The fresh weight of calli formed from seeds was measured 35 d after inoculation. Plant regeneration was scored at about 40 d after the transfer of calli to the MS regeneration medium, each liter of which was supplemented with 0.02 mg 2,4-dichlorophenoxyacetic acid, 10 mg kinetin, and 2 g casein hydrolysate.

The experiment followed a randomized complete block design with two replications. Each genotype was represented by 12 seeds for callus growth; 40–50 calli, which were subcultured once for proliferation, were tested for plant regeneration. Data on callus growth and plant regeneration were statistically analyzed according to the methods described by Hayman (1954a,b) and Jinks (1954). Variance (Vr) and covariance (Wr) analysis was conducted. Calculation in the diallel analysis was performed using Kumagai's (1967) computer program. For plant regeneration analysis, the data were transformed by the arcsin $(P)^{1/2}$. Narrow and broad sense heritabilities were calculated by the method of Mather and Jinks (1971). From the estimates of Vr, Wr, VOLO, and Error, graphs for Vr and Wr were drawn. The limiting parabolas were calculated from the equation

$$Wr^2 = (VOLO - E) \times Vr$$

where VOLO is the variance of the parents.

Furthermore, the frequency distribution of callus growth in the F_2 population of cross Kuju/Somewake was analyzed. The culture medium and culture conditions were the same as in the F_1 analysis for callus growth.

Results

The capacities for callus growth and plant regeneration of the parents and their F_1s were first tabulated. Then the variance, covariance, genetic component of variation, and analysis of variance of the diallel table were estimated.

Callus growth

The mean fresh weights of callus formed from the scutellum of rice seeds of the six parents and their reciprocal crosses are given in Table 1. Calli of Kuju, Sasanishiki, and Murasaki-ine appeared to be fresh and pale yellow, and showed better growth. Norin 1, Somewake, and Daikoku 1 formed smaller calli, and the callus tissues were partly brownish. Kuju showed the best callus growth, followed by Sasanishiki, Murasaki-ine, Norin 1, and Somewake, and Daikoku 1 showed the least callus growth, confirming our earlier observations (Abe and Futsuhara 1986) with the exception of Sasanishiki, which showed better callus growth than Murasaki-ine in the present study. Almost all the F_1s showed values between the parental ones. In F_1s having Daikoku 1, Somewake, or Norin 1 as a parent, callus growth was suppressed: those with Kuju as a parent showed enhancement.

Plant regeneration

The capacity for plant regeneration varied widely among the 6 parents and 30 reciprocal crosses (Table 2). Somewake, Sasanishiki, and Daikoku 1 had relatively high capacities for plant regeneration (32.5, 22, and 22.5%, respectively); Kuju and Norin 1 had low capacities (2% each); Murasaki-ine was intermediate (10%). The reciprocal F_1s of Somewake and Norin 1 showed very high capacities (64 and 60%,

Table 1. Mean values of callus growth in 6 parents and their hybrid F_1s.[a]

Female parent	Mean fresh callus wt (mg) for hybrid with indicated male parent					
	Kuju	Sasanishiki	Murasaki-ine	Norin 1	Somewake	Daikoku 1
Kuju	406	371	281	251	175	194
Sasanishiki	306	361	263	243	139	156
Murasaki-ine	353	243	250	166	105	103
Norin 1	184	193	146	171	84	87
Somewake	182	101	86	94	56	48
Daikoku 1	201	179	64	117	25	19

[a]Figures are means for 12 individuals, each cultured for 35 d.

Table 2. Frequencies of calli differentiating shoots in 6 parents and their hybrids.[a]

Female parent	Differentiating shoots (%) in hybrid with indicated male parent					
	Kuju	Sasanishiki	Murasaki-ine	Norin 1	Somewake	Daikoku 1
Kuju	2	12	6	16	12	30
Sasanishiki	10	22	16	12	38	28
Murasaki-ine	8	14	10	18	42	28
Norin 1	6	14	30	2	60	28
Somewake	24	40	37.5	64	32.5	2
Daikoku 1	22.5	45	27.5	35	20	22.5

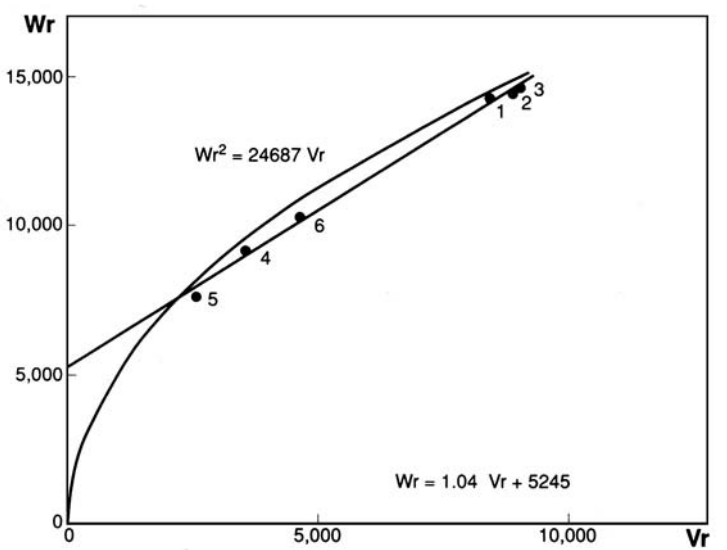
[a]25 calli were tested in each cross.

respectively). Average frequencies of plant regeneration from the calli of reciprocal F_1s of Murasaki-ine/Somewake, Sasanishiki/Daikoku 1, and Sasanishiki/Somewake exceeded those in the parents and were all more than 34%.

Variance and covariance

The adequacy of the additive-dominance model of gene action was tested by the analysis of variance of Wr-Vr. The variation of Wr-Vr for callus growth was not significant, which satisfies the hypotheses of the model. The relationship between variance (Vr) and parent-offspring covariance (Wr) for callus growth is shown in Figure 1, which assesses the genetic relationship among the parents. The regression coefficient (b = 1.04) is significantly different from zero but not from unity, indicating the absence of epistasis. Furthermore, the regression line passes far above the origin, indicating the influence of partial dominance in the inheritance of callus growth. Somewake, Norin 1, and Daikoku 1, located near the origin, possess dominant alleles suppressing callus growth, while Kuju, Sasanishiki, and Murasaki-ine, located away from the origin, possess recessive alleles, which cause relatively high callus growth.

The relationship between Vr and Wr for plant regeneration is shown in Figure 2. Clearly, the lines are far from a slope of b = 1, indicating that gene interaction (epistasis) plays a part in controlling this character. The regression coefficient (b = 0.38 ± 0.47) is not significantly different from zero or from unity, indicating the existence of epistasis and nonallelic interaction. Analysis of the variance of Wr-Vr showed inadequacy of the additive-dominance model of gene action. Somewake, located far

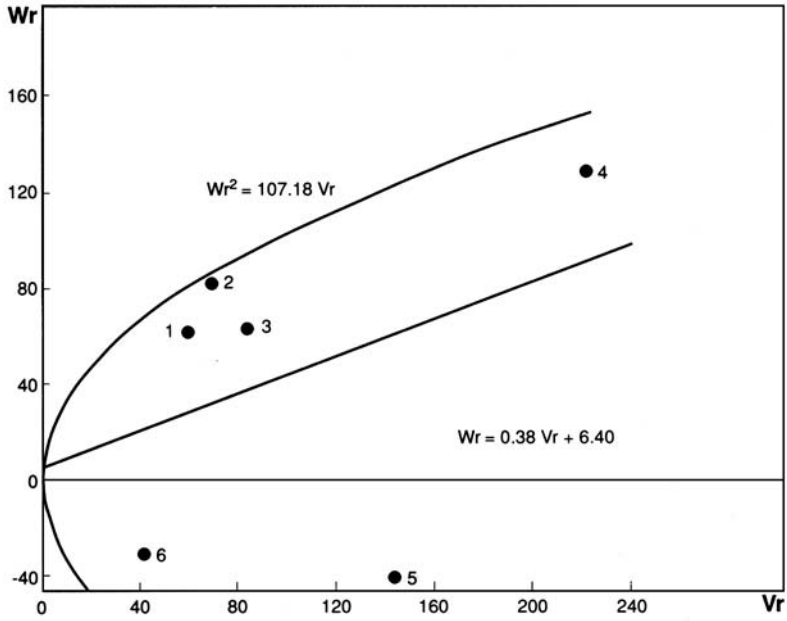


1. (Vr, Wr) graph for callus growth in 6 × 6 diallel cross in rice. 1 = Kuju, 2 = Sasanishiki, 3 = Murasaki-ine, 4 = Norin 1, 5 = Somewake, 6 = Daikoku 1.

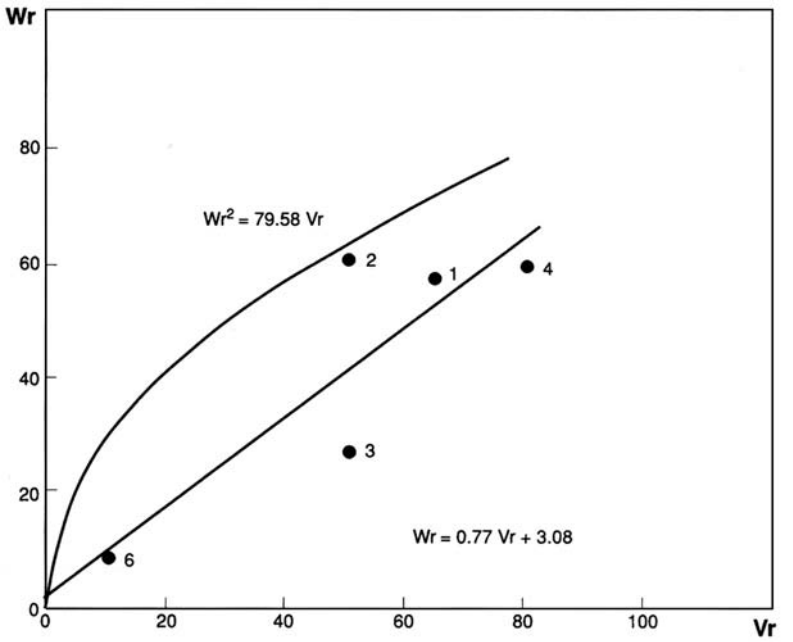
below the regression line, had an extreme Wr-Vr value. Thus, the array of Somewake was excluded from the diallel table, and a noninteracting 5 × 5 subdiallel was reconstituted. Analysis of the 5 × 5 subdiallel showed a regression coefficient (b = 0.77 ± 0.28) significantly different (5% level) from zero but not from unity, indicating the absence of epistasis (Fig. 3). Daikoku 1, which had a relatively high capacity for plant regeneration, was closer to the origin. This fact indicates that the dominant alleles were concentrated in Daikoku 1, while Norin 1 and Kuju, which had lower capacities for plant regeneration and were located away from the origin, had higher proportions of recessive alleles.

Genetic component of variation

In callus growth, the additive genetic variance (D) was larger than the dominance variances (H_1 and H_2) (Table 3). The existence of partial dominance detected in the graphical analysis was confirmed by the fact that $(H_1/D)^{1/2}$ was greater than zero and lower than unity. The ratio of the number of dominant alleles to that of recessive ones, estimated by $[(4DH_1)^{1/2}+F]/[(4DH_1)^{1/2}-F]$, was greater than unity, indicating that the parents used in this study have more dominant alleles than recessive alleles for callus growth. The ratio of 1.925 for h^2/H_2 suggests the involvement of two groups of genes having dominance. The estimated values of broad and narrow sense heritability were



2. (Vr, Wr) graph for plant regeneration from callus tissues in 6 × 6 diallel cross in rice. 1 = Kuju, 2 = Sasanishiki, 3 = Murasaki-ine, 4 = Norin 1, 5 = Somewake, 6 = Daikoku 1.



3. (Vr, Wr) graph for plant regeneration from callus tissues in 5 × 5 subdiallel without Somewake.

Table 3. Estimation of genetic components of variation for callus growth.

Parameter	Estimated value ± SE	Parameter	Estimated value ± SE
D	24686.91 ± 214.11	$(H_1/D)^{1/2}$	0.305
F	2953.03 ± 523.06	(h^2/H_2)	1.925
H_1	2291.22 ± 543.53	$H_2/4H_1$	0.230
H_2	2103.26 ± 485.55	$[(4DH_1)^{1/2} + F]/[(4DH_1)^{1/2}–F]$	1.489
h^2	4048.86 ± 326.81	h^2(ns)	0.933
E	258.66 ± 80.92	h^2(bs)	0.978

high (0.978 and 0.933, respectively), probably because of the major role of additive gene effects in rice callus growth.

Estimates of the genetic components of variation for plant regeneration are presented in Table 4. The dominance variances (H_1 and H_2) were equal to or larger than the additive genetic variance (D), indicating that dominance effects as well as additive effects are important for this character. The mean degree of dominance $(H_1/D)^{1/2}$ was greater than unity, especially in the 6 × 6 diallel, indicating predominance of overdominance effects on plant regeneration. The *F* value was near zero, and the ratio of dominance to recessive alleles, $[(4DH_1)^{1/2}+F]/[(4DH_1)^{1/2}–F)$, was almost unity, indicating that the proportion of dominant and recessive alleles is equal. The ratio of 1.69 for h^2/H_2 suggests the involvement of two groups of genes for plant regeneration.

Table 4. Estimation of genetic components of variation for plant regeneration.

Parameter	Estimated value[a] ± SE	
	6 × 6 diallel	5 × 5 subdiallel
D	107.19 ± 57.98	79.58 ± 10.97
F	41.47 ± 141.65	–0.63 ± 27.41
H_1	312.83 ± 147.19	88.84 ± 29.63
H_2	286.65 ± 131.49	74.43 ± 26.87
h^2	147.67 ± 88.50	125.80 ± 18.14
E	14.83 ± 21.91	15.87 ± 7.48
$(H_1/D)^{1/2}$	1.708	1.057
(h^2/H_2)	0.515	1.690
$H_2/4H_1$	0.229	0.209
$[(4DH_1)^{1/2} + F]/[(4DH_1)^{1/2}-F]$	1.255	0.993
h^2(ns)	0.340	0.578
h^2(bs)	0.888	0.806

[a]Estimated after transformation of arcsin $(P)^{1/2}$.

Table 5. Analysis of variance of 6 x 6 diallel table for callus growth.

Source of variation	Degrees of freedom	Mean square	F[a]
a	5	132048.513	255.265 **
b	15	2620.572	5.066 **
b_1	1	15093.223	29.177 **
b_2	5	1081.216	2.090
b_3	9	2089.919	4.040 **
c	5	2590.767	5.008 **
d	10	1678.792	3.245 **
Error	35	517.319	

[a]** = significant at the 1% level.

Dominance effects (H_1 and H_2) and average dominance $(H_1/D)^{1/2}$ in the 6 × 6 diallel were overestimated, probably because of epistasis caused by Somewake. The value of broad sense heritability was higher than that of narrow sense heritability for this trait, probably because of the involvement of dominance effects.

Analysis of variance of the diallel table

Analyses of variance for callus growth and plant regeneration are given in Tables 5 and 6. For callus growth, highly significant additive genetic variation (a) was observed. Dominance variation (b) was significant, although the mean square and *F* value of b were not high compared with those of a. The b_1 and b_3 terms were significant, while the b_2 term was not. Reciprocal differences (c and d) were also significant. For plant regeneration, additive genetic variation and dominance variation were equally significant, while reciprocal differences (c and d) were not significant in the 6 × 6 diallel table. However, in dominance variation in the 5 × 5 subdiallel, b_1 was significant, while b_2

Table 6. Analysis of variance of 6 × 6 diallel table for plant regeneration.

Source of variation	Degrees of freedom	Mean square	F[a]
a	5	581.018	19.594 **
b	15	316.306	10.667 **
b_1	1	561.251	18.928 **
b_2	5	108.186	3.649 **
b_3	9	404.712	13.649 **
c	5	52.190	1.760
d	10	57.334	1.934
Error	35	29.653	

[a]** = significant at the 1% level.

Table 7. Analysis of variance of 5 × 5 subdiallel table without Somewake for plant regeneration.

Source of variation	Degrees of freedom	Mean square	F[a]
a	4	505.805	15.863 **
b	10	106.235	3.332 **
b_1	1	425.736	13.352 **
b_2	4	80.081	2.512
b_3	5	63.257	1.984
c	4	38.155	1.197
d	6	44.078	1.382
Error	24	31.886	

[a]** = significant at the 1% level.

and b_3 were not (Table 7). Comparison between callus growth and plant regeneration showed that additive genetic variation was high in callus growth, while in plant regeneration additive genetic variation and dominance variation were equally high.

In the segregation test of the F_2 population between parents Kuju and Somewake. a bimodal distribution for callus growth was observed. The segregation ratio of Somewake and Kuju types was 77:20, which fitted the expected 3:1 ratio ($\chi^2 = 0.993$). Most of the F_2 had low callus growth similar to that of Somewake, while fewer F_2s had high callus growth like Kuju.

Discussion

We aimed to elucidate the inheritance of callus growth and plant regeneration in rice seed callus using diallel analysis. Callus growth, which is influenced by two blocks of genes, was characterized by predominantly additive genetic effects that led to a low level of mean degree of dominance, by a high value of heritability, and by a negative action of dominant genes. These characteristics were identical to those reported by Miah et al (1985) in anther culture of rice and by Komatsuda et al (1989) in culture of immature barley embryos. From the analysis of F_2 distribution of callus growth between KuJu and Somewake, it appears that callus growth is controlled by a single

dominant gene or by genes at closely linked loci that suppress callus growth. To obtain high callus growth, the dominant gene that suppresses callus growth must be excluded.

Plant regeneration was characterized by dominance effects as well as additive gene effects. It was influenced by two blocks of genes. The dominance gene action was positive. However, complex gene action involving nonallelic interaction was found in the original 6 × 6 diallel. Genotypes that have more dominant alleles could be used in the improvement of plant regeneration from callus culture.

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Notes

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Genetic analysis for salt tolerance of in vitro regenerant progenies of rice

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The inheritance of salt tolerance was studied in the first seed progeny (R_1) of in vitro-selected regenerants in salt-susceptible rice varieties IR20 and IR50. Regenerants (233) obtained from control and stressed calli had varying degrees of seed sterility; 12 were almost completely sterile. On screening the 221 R_1 sexual progenies, 1 line from IR20 and 1 from IR50 showed complete salt tolerance in terms of seedling survival in the seedbox screening trial. Of the remaining progenies, 51 segregated for tolerance and susceptibility, the proportion of tolerant variants being generally 25% or less. This suggests a recessive bias for the mutation conferring salt tolerance in variants from these lines. This study indicated that salt-tolerant rice variants can be generated by in vitro screening, and systematic progeny analysis can help identify true genetic mutants that may have some worth in salt tolerance breeding programs.

The world rice crop is greatly affected by soil salinity. About 60 million ha of riceland in South and Southeast Asia are rendered nonarable by soil salinity (Akbar and Ponnamperuma 1980). The most economic and sustained means of overcoming this problem is the development of salt-tolerant varieties.

The natural variability in salt tolerance that rice germplasm offers has been exploited by conventional plant breeders in developing a number of salt-tolerant cultivars over the last two decades. In recent years, improvements in rice tissue culture techniques have opened up new possibilities for in vitro mutant selection. Tissue culture is now widely employed to select for stress-tolerant variants in crop plants (Chaleff 1983, Maliga 1985, Stavarek and Rains 1984, Tal 1983). However, tissue culture selection remains largely empirical, and the variants generated do not always breed true. For developing salt-tolerant varieties, it is important to identify true genetic mutants; this can be done by studying the stability of the induced salt tolerance through a few sexual generations (Pua and Thorpe 1986, Scowcroft 1985). With this in view, the inheritance of salt tolerance in progenies regenerated from stressed and nonstressed rice callus tissues was analyzed.

Materials and methods

Callus was induced in induction medium (Murashige and Skoog [MS] medium + 2 mg 2,4-dichlorophenoxyacetic acid [2,4-D]/liter + 0.5 mg kinetin/liter) from mature embryos of salt-susceptible IR20 and IR50. Callus pieces, about 100 mg, were then transferred to salinized medium (MS + 2 mg 2,4-D/liter + 0.5 mg kinetin/liter + 10 g NaCl/liter) in test tubes 30 dafter induction for screening salt-tolerant variant cells. For each variety, 250 test tubes containing salinized medium were inoculated to start the screening process. The calli were taken through 6 passages in salinized medium, each passage lasting 3-4 wk. At the end of each passage, some of the surviving calli were transferred to regeneration medium (MS + 2 mg kinetin/liter + 0.5 mg naphthalene acetic acid/liter), while the calli in the remaining tubes were subcultured in the same medium. Approximately one-sixth of the calli surviving after the first passage, one-fifth after the second passage, one-fourth after the third, one-third after the fourth, one-half after the fifth, and all calli after the sixth passage were put in regeneration medium to regenerate plantlets. A control was run on medium devoid of NaCl. The regenerated plantlets (R_0) were washed free of medium in running tapwater and kept in test tubes inside a glass box under strong light for 2 d, with their roots immersed in water, for hardening. Then they were transferred to earthen pots (25 cm) in the nethouse. The regenerated plants were grown to maturity, and the seeds of the next generation (R_1) were harvested from individual plants.

The R_1 seeds were sown at random in rows in wooden seedboxes (45 × 30 × 10 cm) with 5 cm between rows. Each row had about 100 seeds, In each seedbox, one row of IR20 and one of Pokkali were also sown as susceptible and resistant checks, respectively. The seedboxes were set in galvanized iron trays containing water. The water level was kept just below the level of seed placement, so as not to hamper germination.

When the seedlings were 15 d old, the water in the galvanized iron tray was removed and replaced with 0.5% NaCl solution. The level was adjusted to about 1 cm above the soil level in the seedbox and maintained every day with normal irrigation water. To ensure even distribution of salt water, the soil between the rows of seedlings was poked with a thin stick at regular intervals. When all the IR20 seedlings were dead (after about 5 d), the resistant (surviving) and susceptible (dead) seedlings in each line were counted. The percentage of resistant plants in each progeny line was estimated.

Results

A total of 233 plants (Table 1) were regenerated after growing IR20 and IR50 callus in salinized or control medium for varying lengths of time. Generally, seed sterility of the regenerants tended to increase with duration of culturing and salt stress (Table 2). When the majority of the regenerants from the control calli of IR20 and IR50 had 0–30% seed sterility, the majority of the regenerants from stressed calli showed seed sterility of 30–100%. Twelve of the regenerants were almost completely sterile, and seeds to raise the R_1 generation could be collected from only 221 regenerated plants.

Table 1. Regenerants from IR20 and IR50 callus after screening for varying times in stressed[a] and control media.

Passages (no.) in medium	Regenerants (no.) transferred				Total
	IR20		IR50		
	Control	Stressed	Control	Stressed	
1	21	18	17	14	70
2	18	12	12	10	52
3	10	8	6	8	32
4	9	8	9	6	32
5	8	6	7	6	27
6	6	5	5	4	20
Total	72	57	56	48	233

[a]Stressed with 10 g NaCl/liter of medium.

Table 2. Frequency distribution of R_1 progeny plants from IR20 and IR50 for seed sterility percentage.

Seed sterility (%)	IR20 (no.)		IR50 (no.)	
	Control	Stressed[a]	Control	Stressed[a]
0.0– 10.0	3	0	5	0
10.1– 20.0	14	4	12	1
20.1– 30.0	12	5	13	4
30.1– 40.0	6	6	14	0
40.1– 50.0	6	9	3	8
50.1– 60.0	4	13	2	10
60.1– 70.0	4	6	2	14
70.1– 80.0	6	7	1	6
80.1– 90.0	14	5	0	2
90.1–100.0	3	2	4	3
Total	72	57	56	48

[a]Stressed with 10 g NaCl/liter of medium.

Of the 221 lines, only 1 of IR20 (derived from callus stressed for 3 passages) and 1 of IR50 (derived from callus stressed for 4 passages) were completely salt tolerant at the seedling stage. In addition, 51 lines segregated for salt tolerance and susceptibility (Table 3): 20 lines from stressed calli and 9 from the control in IR20, and 12 lines from stressed calli and 10 from the control in IR50. All the other R_1 lines evaluated were completely susceptible, and none of their seedlings survived the screening test.

The pattern of segregation in each of the segregating R_1 lines of IR20 and IR50 (Table 4) showed that generally only 25% or fewer of the seedlings were resistant, suggesting a recessive bias for the mutation conferring salt tolerance. The segregation pattern did not fit into any of the Mendelian ratios in any of the lines. However, two lines each from IR20 and IR50 broadly showed a 15:1 segregation, 3 lines from IR50

Table 3. Segregating and nonsegregating R_1 progenies in the salt-tolerance screening trial.[a]

Passages (no.) in medium	IR20 (no.)				IR50 (no.)			
	Control		Stressed[b]		Control		Stressed[b]	
	S	NS	S	NS	S	NS	S	NS
1	1	20	3	14	1	16	1	13
2	1	16	5	6	2	9	2	7
3	1	8	3	5	1	5	1	6
4	3	6	3	4	2	5	3	3
5	2	5	3	3	2	4	2	3
6	1	5	3	3	2	3	3	1
Total	9	60	20	35	10	42	12	33

[a] S = segregating, NS = nonsegregating. [b] Stressed with 10 g NaCl/liter of medium.

Table 4. Frequency distribution of R_1 progenies showing proportions of tolerant variants.

Tolerant variants (%)	R_1 segregating progenies (no.)
0.00- 5.00	16
5.01-10.00	8[a]
10.01-15.00	15
15.01-20.00	4[b]
20.01-25.00	3
25.01-30.00	1
30.01-35.00	1
35.01-40.00	0
40.01-45.00	0
45.01-50.00	3[c]
Total	51

[a] 4 segregated at 15:1. [b] 3 segregated at 13:3. [c] 3 segregated at 1:1.

showed a 13:3 segregation, and 3 lines from IR50 showed a 1:1 segregation for tolerance and susceptibility at the seedling stage, based on survival.

Discussion

Among the 221 R_1 progeny lines derived from fertile IR20 and IR50 regenerants (R_0), all seedlings in 2 lines, 1 each from IR20 and IR50, survived seedbox screening. These progenies were derived from stressed calli. The complete tolerance in these two lines may be explained by dominant mutations. The appearance of dominant mutation in cell cultures was suggested by Ling et al (1985) for disease resistance in rice and by Nabors et al (1980) for salt tolerance in tobacco.

Among the 51 segregating progeny lines, the number showing tolerant variants was greater from stressed callus than from the control (Table 5). This suggests that

Table 5. Fertile R_1 progeny lines derived from calli screened for different periods, having tolerant variants.

Passages (no.) in medium	IR20				IR50			
	Control		Stressed[a]		Control		Stressed[a]	
	no.	%	no.	%	no.	%	no.	%
1	1	4.8	3	17.6	1	5.9	1	7.1
2	1	5.9	5	45.5	2	18.2	2	22.2
3	1	5.3	4	50.0	1	16.7	1	14.3
4	3	33.3	3	42.9	2	28.6	4	66.7
5	2	28.6	3	50.0	2	33.3	2	40.0
6	1	16.7	3	50.0	2	40.0	3	75.0
Total	9		21		10		13	
Percentage of total fertile regenerants		13.0		38.2		19.2		28.9

[a]Stressed with 10 g NaCl/liter of medium.

incorporating salt in the screening medium helps in the better realization of salt-tolerant variants. There was an increase, although not regular, in the number of progenies with tolerant variants with increase in the number of passages through the stressed or control medium. An increase in the number of regenerated salt-tolerant variants with increased duration in the stressed medium was previously observed (TCCP 1988).

This study strongly indicates that the acquired salt tolerance in otherwise salt-susceptible varieties IR20 and IR50 has a genetic basis, because it was expressed in the first seed progeny (R_1). Only truly mutant traits, which could be the result of point (Mendelian) mutation, or polygenic trait alteration, or of a maternally inherited mutation, will be retained permanently through several generations (Gould 1986). Further analysis in the R_2 and R_3 of the progenies isolated in this study may be necessary to assess their real worth for developing salt-tolerant varieties. The progenies are being evaluated at Tamil Nadu Agricultural University, Coimbatore. Preliminary indications are that variants derived from stressed calli are more stable for salt tolerance in the R_2.

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Somaclonal male sterile mutants and their expression in indica rice

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During 1984–88, 48 male sterile (MS) mutants were found in 9 varieties of indica rice, 20 of which belonged to the R_1 generation and 28 to the R_2. Both pollen-free (nuclear gene-controlled) and pollen abortive (cytoplasmic gene-controlled) MS mutants were obtained. Two pollen-free MS mutants of IR54, which were induced by somatic cell culture in different years and different generations, were completely uniform. In one MS mutant of the pollen abortive type, 54257, sterility could be maintained and restored: Zhen shan 97, Er Jiu-ai, and 162-5 (a stable somaclone from IR52) could maintain this mutant's male sterility; and IR24, IR36, IR54, and Shuang Er-zhan, among others, could restore its fertility. After six to seven backcrosses (BCs), the capacity of Zhen shan 97, Er Jiu-ai, and 162-5 for maintaining the male sterility of 54257 was stable, showing that such male sterility is controlled by a cytoplasmic gene. This is the first time a cytoplasmic male sterile (CMS) mutant of rice was derived from somaclones. When Zhen shan 97 B as the maintainer was backcrossed to 54257, the BC hybrid 54257/Zhen shan 97 A was expressed as a CMS line with wild abortive cytoplasm. But when 162-5 as the maintainer was backcrossed to 54257, the BC hybrid 54257/162-5 A was expressed quite differently.

Hybrid rice occupied than 11 million ha in China and contributed 50% of the total rice yield in 1988. Hybrid rice yield averaged 6.6 t/ha, higher by 25% than that of conventional varieties (Yuan 1988). The great success of hybrid rice in China has created huge economic and social benefits (Yuan 1986). However, more than 90% of the maternal parents of the hybrids are derived from the same male sterile (MS) cytoplasmic origin, viz., wild abortive (WA) type. The extensive use of a single source of cytoplasm for hybrid maize in the United States led to epidemics of spot disease caused by *Helminthosporium maize,* which severely lowered maize production there. Although a direct relationship between the WA-type cytoplasm in rice and susceptibility to a major disease or insect has not yet been established, the extensive use of a single source of MS cytoplasm may make hybrid rice vulnerable to an epidemic (Virmani et al 1986) and may also limit the breeding of better hybrids.

Some shortcomings are present in WA-type cytoplasm such as low disease and insect resistance and poor quality. Therefore cytoplasmic male sterile (CMS) lines other than WAcytopIasm have been studied for some time. Besides using conventional methods of establishing CMS lines, including backcross breeding (intraspecific and interspecific crossing) and mutagenic induction using physical and chemical agents, new male steriles were successfully induced or transferred recently in some important crops by anther culture, somatic cell culture, and protoplast fusion: Kaul (1986) obtained MS rice from doubled haploid rice by anther culture; MS cytoplasm in *Raphanus sativus* L. was transferred to *Brassica napus* by protoplast fusion and somatic cell hybridization (Akamatsu et al 1988, Sakai and Imamura 1988), and a new CMS rape was obtained; Akagi (1989) fused the nuclei of CMS rice, in which the nuclear material was first destroyed by X-rays, and the one in normal rice by protoplast fusion and transferred the cytoplasm of the CMS rice into the normal one to get a new MS rice; and we reported a MS mutant derived from somatic cell culture in indica rice (Ling et al 1987). In this paper, we report on the genetic expression of this MS mutant.

Materials and methods

Fourteen indica rice varieties (Qing Er-ai, Gui-Chou 2, Nan-Zao-zhan, Xin Jin-zhan, Aichimao, Shao li-zhan, Xin-hei, K-59, 162-5, IR24, IR36, IR50, IR52, and IR54) were used. The explants for inoculation were young embryos, mature seeds, young panicles (stage between initiation of the 2nd rachis and spikelets, 0.5–2 cm long), and anthers (late uninuclear stage).

Tissue culture

Anther culture employed callus-inducing N6 medium supplemented with 3% sucrose, 2 mg 2,4-D/liter, and 10% (vol/vol) coconut milk; Murashige and Skoog's plant regeneration medium with 3% sucrose, 2 mg 6-benzylaminopurine/liter, 0.5 mg kinetin/liter, and 1 mg naphthalene acetic acid (NAA)/liter; and rooting medium that was half Murashige-Skoog's with 2% sucrose and 0.1 mg indolebutyric acid/liter.

Somatic cell culture (including mature seed and young panicles) employed callus-inducing Murashige-Skoog's medium with 3% sucrose, 2 mg 2,4-D/liter, and 1 mg kinetin/liter; and plant regeneration medium with 3% sucrose, 2 mg NAA/liter, and 2 mg kinetin/liter.

The preparation of media, surface sterilization of the explants, sterile manipulation, and culture conditions were the same as in Ling and Yoshida (1987). Callus subculture from both somatic cell culture and anther culture lasted for 1 mo.

Identifying the fertility of regenerated plants and their offspring

Plantlets with healthy roots were transferred into a seedbed to promote survival. They were planted in the field after the new leaves and roots were well-developed. The regenerated plants were called the R_1 generation, their seed offspring were the R_2, and so on. We paid special attention to the sterility of plants in the R_1 and R_2. Most of the

sterile plants in the R_1 and R_2 were emasculated and pollinated by hand to check whether the female was normal. Meiosis of some sterile plants was also observed to eliminate the influence of chromosomal variation. In the R_1, planting distance was 20-30 cm between plants and 60 cm between rows, to promote growth and facilitate observation. The R_2 was planted at 5 × 30 cm; seedlings were transplanted in individual plant progeny plots, each of which contained 60-70 plants at 1 plant/hill. Fertility segregation was recorded each season. Testcrosses of different varieties with the sterile plants were made. The original sterile plants were kept for further research.

Results and discussion

The MS mutants obtained in the R_1 and R_2 generations had different expressions.

Types of male sterile mutants and their expression

The MS plants obtained from somaclones during 1984-88 could be classified as either pollen-free or pollen abortive, and as nuclear gene-controlled or cytoplasmic gene-controlled.

Pollen-free somaclonal male sterile mutants. The following MS mutants belonged to the pollen-free type: 91-20 (R_2 of IR54 in 1985), S5541 (R_1 of IR54 in 1986), P10-1 (R_2 of Xin Jin-zhan in 1987), and 7P201 (R_2 of Qing Er-ai in 1988). In these MS mutants, the anthers were small and shrunken, and the pollen sacs were empty. No meiosis was observed in the anthers of young panicles. In some materials, e.g., 91-20, even pollen mother cells (PMCs) were rare. Probably, pollen abortion occurred before meiosis of the PMCs. Either no seed (under self-pollination), very few seeds (open pollination), or many seeds (controlled hand pollination) were set (Table 1), showing that the female was normally fertile and that all of these plants were MS. The F_1s from these mutants were fully or partially fertile; none were MS. The male sterility of these mutants might be controlled by nuclear genes only.

Although both 91-20 and S5541 were derived from somatic cell culture of IR54, the former was from segregation in the R_2 in 1985 and the latter from the R_1 in 1986. In spite of their different origins in different years and generations, their phenotypes were so similar that it was very difficult to distinguish them. The plants were short and leaves were dark-colored, with many brown spots. They flowered earlier, and no pollen was found in the anthers. When 91-20 and S5541 were individually crossed with IR36 (as the male parent), the F_1s were fertile, and the F_2s segregated in the same ratio (Table 2). Not only were the phenotypes of 91-20 and S5541 similar; their genotypes might also be the same. In other words, the same MS mutant was induced in different generations (R_1 and R_2) and different years (1985 and 1986) by somatic cell culture of IR54. This fact shows that in vitro culture can cause not only one point on a pair of genes to mutate to form a heterozygous mutant in the R_1 (so the MS plants, like 91-20, segregate in the R_2) but also two sites on a pair of genes to mutate to form a homozygous mutant, like S5541 that emerges in the R_1. The mutant S5541 is a typical example of homozygous point mutation derived from somatic cell culture of rice. In

Table 1. Pollen fertility and seed setting in MS mutants and their hybrids.

Mutant and type	Fertility (%) of MS mutant		Male parents (♂) and seed setting[c] in MS mutants (F_0) and F_1 generation								
	Pollen[a]	Seed[b]									
Pollen-free											
91-20 (IR54)	No	0	♂	IR54	IR36	IR24	ID18	ID14	EJ	ZS	SE 91
R2			F_0	32.0	15.0	11.1	18.8	6.3	11	20	2.5 13
			F_1	R	R	R	R	R	R	R	R R
S554 (IR54)	No	0	♂	IR54	IR36	IR52	BH	EJ			
R1			F_0	4.3	17.0	4.3	10.3	8.4			
			F_1	R	R	R	–	R			
H10-1	No	0	♂	IR52	IR24	EJ					
XJZ	No		F_0	10.1	17.8	18.5					
R2			F_1	R	R	R					
7P3-1	No	0	♂	IR24		IR52		EJ	QE		
QE			F_0	35.2		21.3		18.5	28.9		
R2			F_1	131 R; 3M		R		R	41R;1M		
Pollen abortive											
826s	0.8	0.2	♂	EJ							
(IR8)			F_0	6.7							
R1			F_1	R							
CH-s	0.4	0	♂	IR54	IR56		IR24				
(IR54)			F_0	12.0	17.9		10.1				
R1			F_1	R	R		R				
54257	0	0.25	♂	IR54	IR36	IR24	162-5	GC	EJ	ZS	SE
(IR54)			F_0	27.6	17.9	20.5	49.5	17	21	30	18.2
R2			F_1	R	R	R	M	M	M	M	R
$9S_1$	0.8	2.1	♂	IR24		ZS	EJ				
(TN1)			F_0	21.0		18.9	19.1				
R1			F_1	M		R	R				
M	0.5	1.2	♂	IR24		IR36	EJ				
(MH)			F_0	16.7		21.5	19.1				
R1			F_1								

[a]Frequency of pollen stained by I-KI solution. [b]Open pollination condition. [c]Controlled hand pollination; R = restorer (fertile), M = maintainer (sterile). EJ = Er Jiu-ai, ZS = Zhen Shan 97, SE = Shuang Er-zhan, QE = Qing Er-ai, GC = Gui-Chou 2, XJZ = Xin Jin-zhan, MH = Min hui 63.

classical genetics, the same mutant occurring repeatedly in different generations and years would be next to impossible, but this did happen in the somaclones from somatic cell culture of IR54 in this experiment. The homozygous mutants were repeatedly observed in somaclonal variation (Oono 1985). In the somaclones of IR36, we found homozygous mutants including many complex physiological and quantitative characters (Ling et al 1986). How these mutants happened and why the homozygous mutants occurred frequently in somaclones are worth further study.

Pollen abortive somaclonal male sterile mutants. The pollen abortive MS mutants obtained were CH-s (R_1 of IR54 in 1984), 54072 (R_1 of IR54 in 1985), 826-s (R_1 of IR8 in 1985), 9s-1 (R_1 of TNl in 1986), 54257 (R_2 of IR54 in 1986), and M63 (R_1 of Min hui 63 in 1988). They can be divided into two groups according to the fertility of the

Table 2. Segregation patterns for seed fertility in R_2 and F_2 of some male sterile mutants.

Mutant or cross		Fertility reaction			c^2	P
		Fertile (no.)	Sterile (no.)	Total		
91-20 (R_2)	Observed	33	2	35	0.0176	0.995
	Expected	32.8	2.2	35		(15:1)
91-20/IR24 (F_2)	Observed	176	18	194	3.0174	0.995
	Expected	181.9	12.1	194		(15:1)
91-20/IR36 (F_2)	Observed	222	16	238	0.0867	0.995
	Expected	223.2	14.8	238		(15:1)
S5541/IR34 (F_2)	Observed	602	49	651	1.8054	0.995
	Expected	610.3	40.7	651		(15:1)
54257 (R_2)	Observed	139	8	147	0.1569	0.995
	Expected	137.8	9.2	147		(15.1)
54257/IR36 (F_2)	Observed	1126	81	1207	0.4436	0.995
	Expected	1131.6	75.4	1207		(15:1)

F_1 of crosses with normal varieties as male parents. In one, the male sterility of the mutant could not be maintained by backcrossing, and the BC hybrid segregated for fertility (Table 1). In the other, the male sterility of the mutant could be maintained by backcrossing (Table 1,3).

In the first group, for example in 9s-1 (from TN1), both plants in the F_1 of 9s-1/IR24 were sterile, but the successive BC hybrids segregated for fertility. Only 1 plant among 22 hybrids of 9s-1/IR24 (BC_1F_1) and 2 plants of 37 in the BC_2F_1 were sterile. The MS nature of this group could not be identified because the numbers were not enough for testcrossing.

Mutant 54257 belonged to the second group. In the spring of 1986, among 57 lines of the R_2 derived from young panicle culture of IR54, one (code 54257) was found to segregate for fertility. Of 147 plants of 54257, 8 were sterile. The pollen grains of the MS plants were not stained by 1% I-KI solution. The frequency of seed setting under free pollination conditions was 0.25%. In the autumn of 1986, the MS plant was crossed with 13 varieties as the male parents, and the frequency of seed setting was from 8.8 to 49.5% (Table 1), showing that 54257 was MS. In the spring of 1987, expression of fertility in the hybrid F_1 was different: some F_1 were sterile and others were fertile. Zhen shan 97, Er Jiu-ai, and 162-5 (a stable somaclone of IR52) could maintain the male sterility of 54257 (Table 3); and IR24, IR36, and Shuang Er-zhan could restore the fertility of the mutant (Table 1). For the sterile hybrid F_1 of 54257, backcrosses with the original male parent varieties were made 6-7 times during 1986-90. The F_1 of every BC was still MS (Table 3), showing that 54257 was a MS mutant controlled by a CMS gene.

The MS lines of BT type (Shinjyo 1969) and WA type (Yuan 1977) are controlled by CMS genes. Both were bred by hybridization. Here the CMS mutant 54257 was derived from somatic cell culture. This is apparently the first example of a CMS line

Table 3. Seed and pollen fertility of F_1 hybrids of 54257 and its backcross offspring.

Cross and generation		Plants			Fertile pollen (no.) with given fertility percentage			Seed fertility[b]		
		Total (no.)	MS (no.)	%	0-10	11-50	51-100	TG	SS	SS (%)
54257/162-5	F_1	16	16	100	50	33	17	85	0.17	0.2
	BC_1F_1	23	23	100	50	50	0	77	2.5	3.3
	BC_2F_1	152	137	90.1	29.4	47	23.5	93.6	2.4	2.56
	BC_3F_1	162	156	96.3	25	36	39	88.1	7.2	8.2
	BC_4F_1	28	28	100	17.8	67.9	14.3	57.1	4.3	7.5
	BC_5F_1	825	825	100	28.4	58.9	12.6	63.8	1.9	2.9
	BC_6F_1	332	332	100	29.6	40.2	30.2			
54257/ZS97	F_1	6	6	100	100	0	0	75.1	4.7	6.3
	BC_1F_1	11	11	100	100	0	0	79.5	2	2.5
	BC_2F_1	17	17	100	100	0	0	54.2	2	3.7
	BC_3F_1	180	172	95.9	100	0	0	78	2.4	3.1
	BC_4F_1	98	97	98.9	100	0	0	82	4.6	5.6
	BC_5F_1	241	241	100	100	0	0	85.2	6.4	7.6
	BC_6F_1	30	30	100	100	0	0	75.8	1.0	1.3
	BC_7F_1	47	47	100	100	0	0			
(54257/IR24)	F_1							137	118	86.5
(54257/SEZ)	F_1							83	38	46.0

[a]MS = male sterile plants, TG = total number of grains per panicle, SS = total number of seeds setting per panicle.
[b]By free pollination.

obtained by biotechnology, not only in rice but in any crop—a fact that has significance for hybrid rice production and cytoplasmic genetics.

Expression of male sterile mutant from somatic cell culture

The inheritance of both nuclear and CMS-controlled MS mutants and the relationship of restoring and maintaining 54257 were investigated.

Inheritance of male sterility in mutants. The F_1s of the cross between the MS mutants (91-20 and 54257) and IR24 or IR36 were fertile, but the F_2 segregated for fertility. The segregation of fertile and sterile plants in the R_2 and F_2 of MS mutants 91-20 and 54257 and the chi-squares, are listed in Table 2. The ratio of 15 fertile to 1 sterile exactly fits the expected Mendelian value of 9:3:3:1, demonstrating that the sterility of MS mutants 91-20 and 54257 might be controlled by two pairs of independent recessive genes. Only individuals with homozygous recessive genes were phenotypically MS.

Restoring or maintaining relationship between 54257 and certain varieties. The varieties that could maintain the male sterility of 54257 were Zhen shan 97 B, Er Jiu-ai B (maintainers of MS lines with WA cytoplasm), and 162-5 (a stable somaclone of IR52). Backcrossings between 54257 and these maintainers (as the male parent) were undertaken for 6-7 generations. Although the BC hybrids in every generation were sterile (Table 3), their expression differed depending on the maintainer (BC parent).

When Zhen shan 97 B and Er Jiu-ai B as the BC parents were backcrossed to 54257 (correspondingly, the MS line called 54257/ZS97 A or 54257/EJ A), the pollen of the BC hybrid (MS line) was completely sterile, not stained by I-KI solution, and without seed set (Table 3). Expression of the MS line 54257/ZS97 A was like that of the CMS line with WA cytoplasm.

When 162-5 as the BC parent (maintainer) was backcrossed to 54257 (correspondingly, the MS line called 54257/162-5 A), the hybrid (54257/162-5 A) was sterile; no seeds were set, but the pollen grains were partially stained by I-KI solution. In each BC generation of 54257/162-5 A, the pollen of 25–50% of the individuals was not completely stained by I-KI, 33–36% individuals were partially stained (the frequency of staining was 11–50%), and the remainder were fully stained (Table 3). Partial staining of pollen of 54257/162-5 A was consistently observed over six generations (Table 3). In this case, the MS line 54257/162-5 A was quite different from the one with WA cytoplasm.

IR54, IR36, IR24, and Shuang Er-zhan could restore the fertility of MS mutant 54257 (Table 3). 54257/162-5 A was different from 54257/ZS97 A in the relationship of restorer. The variety Test 64 is an excellent restorer of MS lines with WA cytoplasm and is widely used for hybrid rice production in China. When Test 64 as the male parent was crossed with 54257/ZS97 A and 54257/162-5 A, the fertility of the two F_1s differed (Table 4). Seed set was more than 60% in 54257/ZS97 A //Test 64 but less than 15% in 54257/162-5 A //Test 64. It appears that Test 64 can restore 54257/ZS97 A but not 54257/162-5 A.

Thus the mutant 54257-5 A differed from both BT type and WA type in at least two respects. First, pollen abortion is a stable characteristic of a MS line in rice. The pollen grains in CMS lines of BT type are staining abortive (stained by I-KI but having no function), and in CMS lines of WA type the pollen is not stained. The opposite case has never been found (Shinjyo 1969, 1975; Virmani et al 1986; Yuan 1977). But in the mutant 54257 there are two types of pollen staining. When Zhen shan 97 B as the maintainer was crossed with 54257, the pollen of the hybrid (54257/ZS97 A) was not stained. With 162-5 B as the maintainer, the pollen of the hybrid 54257/162-5 A was

Table 4. Effect of maintainers on capacity for restoring fertility of mutant 54257.[a]

Seed setting range (%)	Seed setting frequency in F_1			
	54257/162-5 A//Test 64		54257/ZS97 A//Test 64	
	Plants (no.)	Plants (%)	Plants (no.)	Plants (%)
0 – 15	7	63.6	0	0
16 – 30	2	18.2	0	0
31 – 60	2	18.2	0	0
61 –100	0	0	10	100

[a] ZS97 A = Zhen shan 97 A.

Table 5. Frequency of somaclonal male sterile variation in different years and generations.

Year	R_1			R_2			Total MS lines (no.)	Varieties found with MS variation[a]
	Total plants (no.)	MS plants		Total lines (no.)	MS lines			
		no.	%		no.	%		
1984	84	1	1.19				1	IR54
1985	141	2	1.42	124	1	1.82	3	IR8, IR54
1986	115	2	1.73	45	1	2.2	3	IR24, IR54
1987	471	3	0.64	58	2	3.4	5	IR26, QE, GC, XJ, NZ
1988	510	12[b]	2.35	247	24	11.6	36	IR26, QE, GC
Total	1321	20	1.51	474	28	5.91	48	9 varieties 14 times

[a]QE = Qing Er-ai, GC = Gui-Chou, XJ = Xin Jin-zhan, NZ = Nan Zao-zhan. [b]Including R_1 plantlet from anther culture.

partially staining abortive. The second difference is in the response of 54257 to Test 64 as restorer or maintainer. To Test 64, there is only one response, restoring fertility, in the MS line of WA type; but in 54257 there are two responses depending upon the maintainer (ZS97 B or 162-5 B) (Table 4).

The frequency of male sterile mutants in somaclones. During 1984-88, 48 cases of MS mutants were found. The frequency of MS mutants in different years and generations varied. In the R_1, the frequency was 1-2% (but 0.64% in 1987), and in the R_2 it was 1–3% (but 11.6% in 1988; Table 5). That the frequency in the R_2 was higher than in the R_1 showed that most MS mutants were single-locus mutants, and the expression of male sterility in the phenotype should be in the second generation by segregation. Among nine varieties, MS mutants in IR54 appeared three times in both the R_1 and R_2. The MS mutant found in IR54 was a nuclear-controlled, pollen-free type and CMS type (pollen abortive).

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Regeneration and transformation of protoplasts in indica rice

Xiang-hui Li, A.P. Aryan, Cheng-bin Xiang, and T.W. Okita

To improve the frequency of protoplast regeneration from both indica and japonica rice using standard cell culture methods, calli were initiated on N6 medium from young inflorescence tissues of indica cultivar DGZ and japonica cultivar JO 2428. Embryogenic calli were subsequently used to establish suspension cultures in AA2 or modified N6 media. After subculturing for 8-10 wk, the embryonic suspension lines yielded cytoplasmically densed protoplasts at 4-10 × 10^6 /ml. Of the various media tested, the highest plating efficiency of protoplast division was observed on modified KM medium containing 0.2% agarose. The amount and frequency of regenerable calli were substantially increased (about threefold) by subculturing in modified N6 liquid media for about 3-4 wk before plating on hormone-free NGSS medium. The frequency of plant regeneration of highly compact cells was 15-28%. Studies aimed at obtaining stable transformed protoplasts via polyethylene glycol-mediated gene transfer using several marker genes are under way.

Following recent successes with rice protoplast culture (Kyozuka et al 1988, Lee et al 1989, Masuda et al 1989 and references cited therein) and gene transfer (Toriyama et al 1988, Zhang et al 1988) techniques by several laboratories, we directed our efforts toward improving the frequency of protoplast regeneration from both indica and japonica subspecies, a limiting step in the process, using standard protoplast culture methods. Based on protoplast regeneration in indica rice, cotransformation of indica cultivar DGZ was performed using polyethylene glycol (PEG). In this paper, we report plant regeneration from protoplasts of DGZ and japonica cultivar JO 2428 using different media and transformation with the *GUS* reporter gene.

Plant regeneration from rice protoplasts

Calli were initiated from young inflorescence tissues of DGZ and JO 2428 on solid N6 medium containing 2 mg 2,4-dichlorophenoxyacetic acid (2,4-D)/liter, 3% sucrose, and 0.8% agar. After subculture for 5-6 wk, 1.5 g of calli having a slight yellowish color

and exhibiting high regenerative capacity were suspended in AA2 liquid medium. The suspension cell lines were subcultured in flasks on a rotary shaker at 100 rpm under dim light, with the medium replaced every 5–7 d. The embryogenic cell suspension lines were then maintained in AA2 medium or grown in a modified N6 medium containing the amino acids of AA2; in unmodified N6; or in GM, D2, or R2 media. After subculturing for 9–10 mo, the cell lines grown in AA2 medium and modified N6 medium yielded cytoplasmically densed protoplasts at a density of 4–10 × 10^6/ml. The amino acids specified by AA medium were absolutely necessary for the compact growth of highly cytoplasmically densed cells and produced a higher yield and percentage of viable protoplasts than did the other media (GM, N6, R2) without the amino acid supplements. The addition of casein hydrolysate (0.025%) and coconut water (5%), although not absolutely necessary, resulted in faster growth of the liquid suspension cultures and, hence, was routinely performed on AA2 or modified N6 media.

The protoplasts of JO 2428 and DGZ released by an enzyme mixture solution were cultured in either KM (Kao 1977), D2, N6, or R2 medium with or without agarose using normal protoplast culture techniques. The plating density of the protoplasts was 1–6 × 10^5/ml. The osmoticum was varied somewhat by the presence of glucose in a range between 0.4 and 0.52 M.

The highest plating efficiencies (34% for JO 2428, 37% for DGZ) of protoplast growth were observed on modified KM medium solidified with 0.2% agarose (Table 1). A somewhat lower plating efficiency was observed for both genotypes on KM medium without agarose. This observation differed significantly from that of Sun et al (1990), in which the growth of DGZ protoplasts was strikingly dependent on the presence of agarose in KM. D2 and R2 media were also suitable for the growth of JO 2428, which yielded plating efficiencies similar to KM medium with agarose (Table 1).

After incubation for 3 wk, fresh KM medium containing 18 g glucose/liter and 20 g sucrose/liter was added to reduce the osmoticum, enhancing the growth rate of the cells. After 3–4 wk, the calli (about 1 mm in size) were grown for 3–4 wk in liquid N6 medium containing 1 mg 2,4-D/liter, followed by a similar period on solid N6G

Table 1. Plating efficiencies of japonica rice JO 2428 and indica rice DGZ.

Variety	Culture medium	Frequency (%) of cell division at 10–11 d
JO 2428	KM	25
JO 2428	KM + agarose	34
JO 2428	D2	32
JO 2428	R2 + agarose	32
DGZ	KM	23
DGZ	KM + agarose	37

Table 2. Differentiation and plant regeneration of JO 2428 and DGZ protoplasts.

Variety	Media	Frequency (%) of differentiation	Frequency (%) of plant regeneration
JO 2428	KM + agarose – N6SS	56.9	27.8
JO 2428	KM – N6SS	37.2	21.8
DGZ	KM + agarose – N6SS	34.8	21.7
DGZ	KM – N6SS	Not determined	15.0

medium containing 0.6 mg 6-benzylaminopurine/liter and 2 mg 2,4-D/liter. Incubation of the calli—first in liquid N6 medium, then in N6G medium—enhanced their growth while maintaining their embryogenic character. After these treatments, the amount of embryogenic calli capable of regeneration was substantially increased, because no plants were obtained from calli pre-incubated under these culture conditions. Protocalli, which were yellowish and highly compact, were then plated onto hormone-free N6SS medium. Within 3-4 wk, green spots appeared on many of the calli. In JO 2428, the frequency of differentiation was 37.2–56.9% (amount of green spots with shoots/number of calli × 100), while for DGZ it was 34.8% (Table 2). In several instances, many of the small green shoots did not develop into whole plants. The frequency of plant regeneration at 1 mo after transferring onto N6SS was 21.8–27.8% for JO 2428 and 15.0-21.7% for DGZ (Table 2). Calli derived from protoplasts incubated on modified KM media with agarose showed substantially higher frequencies of differentiation and regeneration than cells incubated in the absence of agarose. Approximately 30 protoplast-derived JO 2428 plants were transferred to the greenhouse. All had normal morphology, flowered, and set seed. Likewise, more than 15 protoplast-derived DGZ plants were grown to maturity. The method described here is direct and relatively simple, as it does not require the use of feeder cells forprotoplast growth and viability.

Gene transfer to rice cells mediated by protoplast cotransformation

Protoplasts from the DGZ suspension cultures were cotransformed with p35S-GUS (a plasmid containing the b-glucuronidase reporter gene under the control of the CaMV 35S promoter and 3' Nos sequences) together with a second plasmid containing the *CAT* reporter gene under the control of either CaMV 35S or wheat gliadin promoter. We added the mixed DNAs (20 µg of GUS DNA, 20 µg of CAT DNA, and 40 µg of carrier calf thymus DNA) in a total volume of 100 µl to each milliliter of protoplasts (density = 1×10^6).

Transformation was by direct gene uptake mediated by PEG according to Negrutiu et al (1987). After treatment, protoplasts were cultured in KM media as above.

Table 3. Detection of ß-glucuronidase activity in rice cells.[a]

Date	DNA treatment	MU concentration with spectrofluorescence (OD volume)			Fluorescence per 50 h (OD volume)
		2 min	60 min	50 h	
16 Nov	1. 35S-Gus-3' Nos 35S-CAT-3' Nos	187	1114	10094	9907
	2. 35S-Gus-3' Nos Gliadin-CAT-3' Nos	95	715	4165	4070
	3. Control (without PEG, DNA)	70	367	497	427
17 Nov	1. 35S-Gus-3' Nos 35S-CAT-3' Nos	458	2350	15840	15382
	2. 35S-Gus-3' Nos Gliadin-CAT-3' Nos	480	1600	6576	6096
	3. 35S-Gus-3' Nos	275	1580	2840	2565
	4. Control	200	520	1120	920

[a]Determined by spectrofluorimetric assay with excitation at 365 nm and emission at 455 nm according to Jefferson (1987).

Transient expression of *GUS* reporter gene in protoplasts

A relatively high activity of ß-glucuronidase (GUS) was obtained by cotransformation with DNA controlled by the CaMV 35S reporter (Table 3). In cotransformation experiments with a plasmid construct containing the gliadin promoter, GUS activity was half that of the first experiment, but it was still 10-fold higher than that of control cells not treated with DNA or PEG.

Callus formation and selection of transformed calli

Protoplasts subjected to the PEG-mediated DNA uptake treatment were grown as described above. To determine the proportion of protocalli exhibiting GUS enzyme activity, the nondestructive assay developed by Gould and Smith (1989) was employed. Calli were transferred to fresh media on plates for 2 d, then removed. Then 20 µl of sterile 4-methyl umbelliferyl-ß-D-glucuronide (MUG) was pipetted onto the media where the calli were cultured. After 24 h, stop buffer was added to the media and the calli were examined with a hand-held ultraviolet light source. About 26.4% of the calli of group 1 transfected with the 35S-Gus-3' Nos and 35S-Cat-3' Nos reporter genes exhibited blue fluorescence (Table 4), suggesting the possible presence of GUS enzyme activity. Group 2 calli obtained by PEG-mediated uptake of 35S-Gus-3' Nos and gliadin-Cat-3' Nos plasmid DNAs yielded a lower frequency of 19.8%.

To confirm that the fluorescence obtained by the nondestructive assay was indicative of GUS activity, we directly assayed this enzyme activity by measuring the product, 4-methyl umbelliferone, using a fluorometer. Table 5 shows a direct comparison of GUS activities of seven putative transformed calli and two control calli as estimated by the nondestructive and direct fluorometric assays. The level of GUS

Table 4. GUS assay by spent media of transformed callus.

Date	DNA treatment	Calli obtained (no.)	Calli assayed (no.)	Spent calli media (no.) exhibiting blue fluorescence	Positive calli (%)
16 Nov	1. 35S-GUS-3' Nos 35S-CAT-3' Nos, DNA	1579	1076	284	26.4
	2. Control (without DNA)	415	155	–	–
17 Nov	1. 35S-GUS-3' Nos Gliadin-CAT-3' Nos, DNA	1283	976	193	19.8
	2. Control (without DNA)	324	205	–	–

Table 5. GUS expression of transformed calli as determined by indirect nondestructive assay and by direct fluorogenic assay.

Simple calli (no.)	GUS staining[a]	ß-glucuronidase activity (nmol/µg protein per 50 h)
4	++++	4.080
9	+++++	5.442
21	+++++	7.032
22 (check)	+	1.942
47	+++++	5.983
49	+++++	4.700
60	++++	8.210
61	+++	20.284
42 (check)	–	1.080

[a]Semiqualitative estimate by extent of blue fluorescence: + = weak, +++++ = strongest.

activity exhibited by the putative transformed calli was 2- to 10-fold higher than that of the control calli. GUS activity appeared relatively stable over the assay period and generally corresponded to the differences in GUS activity estimated by the nondestructive assay. The presence of GUS activity in these protocalli suggests that plasmid DNA has been stably integrated in their genomes. Studies to obtain direct molecular evidence that these protocalli have been transformed are being pursued.

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Isolated microspore culture of rice at the International Rice Research Institute

F.J. Zapata, R.R. Aldemita, E.S. Ella, and Moon Soo Cho

Studies were done on isolated microspore culture of rice japonica variety Taipei 309 and indica varieties IR43 and IR36. Culture of isolated microspores from Taipei 309 in Gamborg's B5 medium containing 1 mg 2,4-dichlorophenoxyacetic acid (2,4-D)/liter, 0.5 mg 6-benzylamino purine/liter, and 0.5 mg indole-3-acetic acid/liter as growth regulators (E10 medium) produced calli. When 1 mM proline was added, callus production efficiency was increased. Only large microspores of 50-58 µm diameter with thin, light pink-colored cell walls (p-grains) developed into embryos. After 14-d culture, microcalli derived from the microspores were transferred for regeneration into Murashige and Skoog's medium containing 1 mg kinetin/liter and 1 mg naphthalene acetic acid/liter (MSYo medium). Fifty-seven plants were obtained, of which 41 were homozygous diploids and the rest haploids. In E10 medium, callus induction was obtained in IR43, while up to 8 cell divisions were observed in IR36. The initial percentage of microspore viability and the rate of decrease in viability accounted for the differing responses in vitro. Four hundred thirty-seven calli of IR43 were transferred to MSYo medium for regeneration. Of 56 plants regenerated, 32 were green; the rest were albino. High initial percentage p-grain and microspore viability were then recognized as important prerequisites for improving efficiency in isolated microspore culture. Treatment of donor plants with Alar (a feminizing agent), exposure to various photoperiods, temperature treatment of freshly detached panicles, and sucrose starvation during preculture were tried to improve p-grain recovery and microspore viability. Studies were also conducted to increase callus induction and plant regeneration, e.g., media evaluation for preculturing, quality and quantity of nitrogen source, and effects of 2,4-D concentration.

Isolated microspore culture has long been recognized as an important experimental system for basic genetic studies and crop breeding (Nitsch 1977). Since the microspore is both a single cell and haploid, it is an effective experimental system for inducing mutation and for genetic manipulation. Direct injection of DNA (microinjection) into the microspore or microspore-assisted gene transfer is potentially applicable to crop improvement (Potrykus 1988). Transformation of microspores may even be more

advantageous than wing somatic cells, since the inserted gene could be duplicated, thus eliminating segregation in future generations.

Rice plants were obtained from isolated microspore culture as early as 1980 (Chen et al 1980). However, the low induction frequencies of callus and plants were attributable to the lack of understanding of factors influencing the initiation of androgenesis and further development of microspores in vitro.

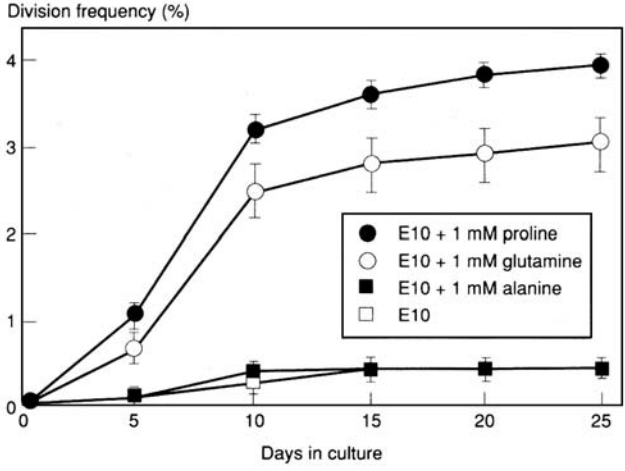
We have tried to overcome the problem of low efficiency by treatment of the donor plants and by manipulation and evaluation of media and media components.

Callus formation and plant regeneration in isolated microspore culture of three rice varieties

A number of methods have been employed to produce pollen-derived rice embryos: shedding culture, cold treatment, and preculture of anthers before isolation (Nitsch 1974). Isolation and culture in this experiment followed Nitsch (1974).

Japonica variety Taipei 309

Our initial attempt to study isolated microspore culture in rice used Taipei 309, a responsive variety. Isolated microspores from 7-d precultured anthers were cultured in E10 [modified Gamborg's (1968) B5] liquid medium, E10A (with 1 mM alanine), E10G (with 1 mM glutamine), and El0P (with 1 mM proline). Only large microspores 50–58 µm in diameter with thin, light pink-colored cell walls (p-grains) seemed to develop into pollen embryos. Eight-cell division frequency increased rapidly during the first 10 d of culture in the 4 media tested (Fig. 1). E10P yielded the highest division frequency of 3.9% and a calli plating efficiency of 3.5% (Table 1). After a 14-d culture



1. Effect of media containing various amino acids on division frequency of Taipei 309 pollen at different durations in culture (av of 4 replications ±SE).

Table 1. Plating efficiency and plant regeneration in isolated microspore cultures from precultured anthers based on 4 replications.

Callus induction medium[a]	Plating efficiency (%) 17 d after culturing[b]	Calli (no.) plated on regeneration medium	Plant regeneration efficiency (%)	
			Green	Albino
E10	0.26 a	35	5.7	5.7
E10 + 1 mM alanine	0.35 a	16	6.3	12.5
E10 + 1 mM glutamine	2.71 b	89	21.3	12.4
E10 + 1 mM proline	3.50 b	111	31.5	18.0

[a] Modified Gamborg's B5 medium with 20 g sucrose/liter, 5 g glucose/liter, 1 mg 2,4-D/liter, 0.5 mg BAP/liter, and 0.5 mg IAA/liter. [b] Treatment means followed by a common letter are not significantly different at the 5% level by Duncan's multiple range test (DMRT).

Table 2. Response of indica rices to isolated microspore culture, based on 5 replications.

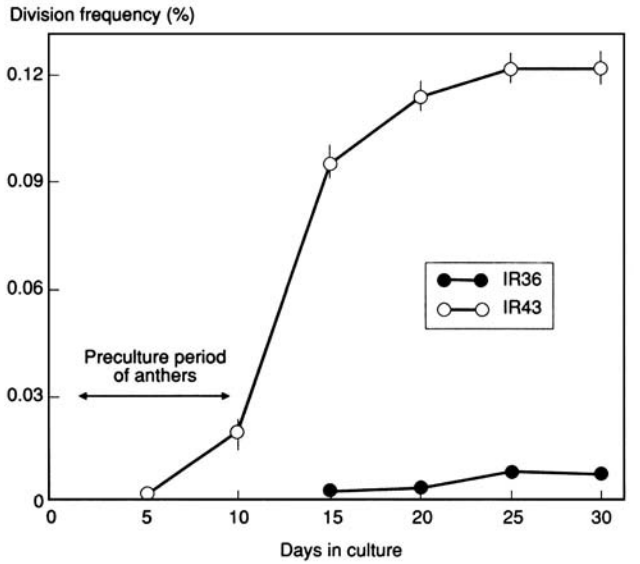
Response	Genotype	
	IR36	IR43
Division frequency (%) at day 30[a] (mean ± S.E.)	0.008 ± 0.0019	0.12 ± 0.03
Plating efficiency (%) at day 30[b] (mean ± S.E.)	0	0.05 ± 0.006
Calli (no.) plated for regeneration	0	437
Plant regeneration (no.)		
Green	—	32
Albino	—	24

[a] $\frac{\text{No. of dividing pollen}}{\text{No. of pollen plated}} \times 100$ [b] $\frac{\text{No. of pollen-formina mcroscopic calli}}{\text{Total no. of pollen plated}} \times 100$

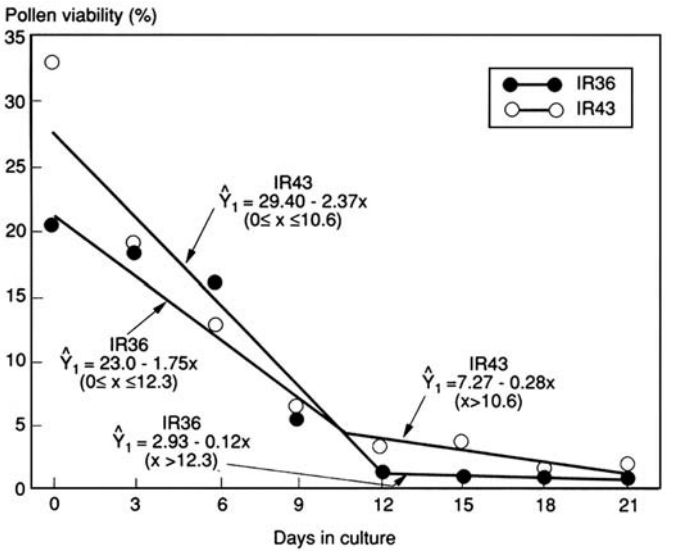
period, microcalli derived from the microspores were transferred to Murashige and Skoog's (1962) (MS) liquid medium, each liter supplemented with 1.0 mg kinetin (KIN), 1.0 mg naphthalene acetic acid (NAA), and 20 mg abscisic acid (ABA). For plant regeneration, growing calli were transferred onto ABA-free MS semisolid medium (MSYo). The proportion of albino to green plants was lower in E10P and E10G than in E10 and E10A. A total of 57 plants were regenerated from these treatments. Of these, 41 (72%) were homozygous diploids and the rest haploids.

Indica varieties IR43 and IR36

In terms of division frequency, IR43 was more responsive than IR36 (Table 2, Fig. 2). IR43 had 0.05% plating efficiency, while none of the dividing or multicellular microspores of IR36 underwent further development. IR43 also had higher initial microspore viability and a slower rate of decrease in viability during culture (Fig. 3).



2. Division frequency of pollen at various culture durations (av of 5 replications).



3. Estimated linear relationships between pollen viability and culture time in indica cultivars IR36 and IR43.

Four hundred thirty-seven calli of IR43 were transferred for regeneration to MSYo medium. Of 56 plants, 32 were green and the rest were albino. This is the first known report on callus induction and plant regeneration from isolated microspore culture in indica varieties.

Factors affecting p-grain development and microspore viability

In the preceding experiments, two significant points were observed: 1) in Taipei 309, only the p-grains with large microspores with thin, light pink cell walls divided; 2) the initial viability of the isolated microspores should be high to increase callus induction efficiency. Using these observations, we studied the development of p-grains and ways to obtain an initially high p-grain release and microspore viability.

Effect of feminizing agents

Treatment of donor plants affects the development of p-grains (Dunwell 1985). The p-grains are generally sterile as judged by their staining reaction with acetocarmine and fluorescein diacetate and in in vitro germination tests (Heberle-Bors 1982). An increase in p-grain formation can be achieved by shifting the sex balance by applying a feminizing agent. A 50-ml solution of 500-ppm Alar, a feminizing agent, applied daily for 7 consecutive days at the base of a 3- to 5-cm inflorescence of a donor plant such as IR42 gave the highest percentage of p-grains, about a 2.4-fold increase relative to the control (Table 3).

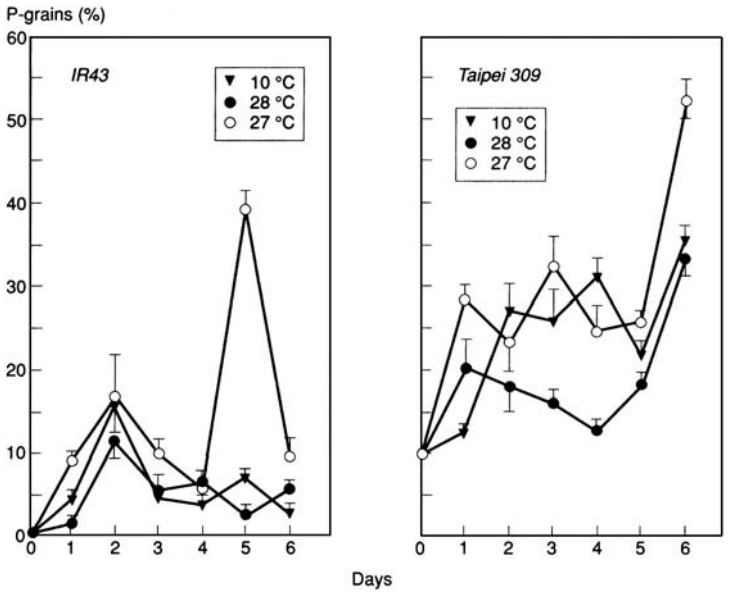
Photoperiod effects

Heberle-Bors and Reinert (1981) observed that an 8-h photoperiod, considered as a short day, induces a high percentage of p-grains in tobacco. In Taipei 309 rice, donor plants were exposed to 8-h, 12-h, and 16-h daylengths at the end of the vegetative stage. Percentage p-grain recovery and viability were best with 12-h photoperiod, but callus induction did not significantly differ among the 3 treatments. This could be due to the inherent photoperiod insensitivity of the japonica variety used.

Table 3. Effect of various Alar concentrations applied at different lengths of inflorescence on percent p-grains of IR42.

Alar (ppm)	Length of young inflorescence (cm)	% p-grains[a]
0		13.26 ± 0.33 g
50	3– 5	16.66 ± 0.39 cd
	8–10	14.17 ± 0.21 e
	13–15	13.31 ± 0.23 g
100	3– 5	24.78 ± 0.25 b
	8–10	16.37 ± 0.42 d
	13–15	13.91 ± 0.21 ef
500	3– 5	31.46 ± 0.26 a
	8–10	17.00 ± 0.19 c
	13–15	13.61 ± 0.32 fg

[a]Av of 4 replications. Means followed by a common letter are not significantly different at the 5% level by DMRT.



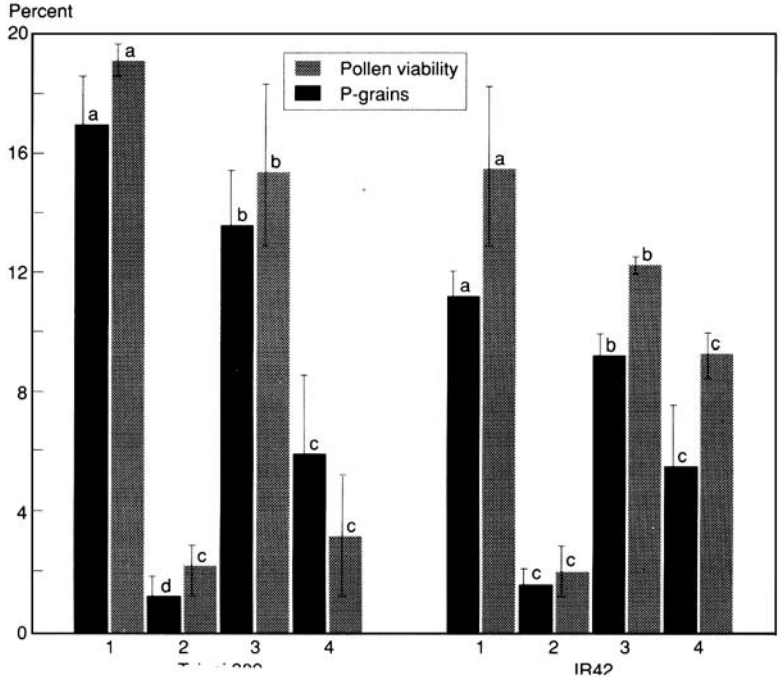
4. Effect of exposure time at different temperatures on percentage p-grains in IR43 and Taipei 309. Mean of 3 replications.

Temperature effects on detached panicles

Temperature treatments of 8–10, 18, and 25–27 °C were applied on freshly detached panicles of IR43 and Taipei 309. Initially, p-grains were low in newly detached panicles of IR43 (0%) and Taipei 309 (10%) (Fig. 4). The percentage of p-grains changed with time depending on the temperature treatment. Higher temperatures (27 °C) released a higher percentage of p-grains than did lower temperatures. Further development of the predetermined p-grain may thus be associated with temperature. The characteristic p-grain as described by Cho and Zapata (1988) is not present in freshly collected panicles; however, after exposure to 27 °C, these predetermined grains were activated to assume the distinct p-grain characteristics.

Effect of sucrose starvation during preculture

Sucrose starvation induced immature microspores to form p-grains in tobacco (Kyo and Harada 1986). This was also observed in rice, where the viability and percentage of p-grains released to the medium after 5 d of preculture were also highest in sucrose-free medium (Fig. 5). This shows that carbohydrate starvation during preculture induces anther dehiscence due to reduced osmoticum or starvation itself. This technique also facilitates microspore isolation and eliminates the debris encountered by crushing the anthers during mechanical isolation, since these microspores are readily shed onto the medium. Browning of anthers in sucrose-free medium was also reduced by the absence of 5-hydroxymethyl furfural, a by-product of sucrose after autoclaving, as observed in tobacco (Maheshwari et al 1980).



5. Percentage of p-grains and viability of pollen of Taipei 309 and IR42 from different sources. Within a response of a variety, points followed by a common letter are not significantly different at the 5% level by DMRT. 1 = So liquid, 2 = So anther, 3 = E10 liquid. 4 = E10 anther.

Three days of preculture with sucrose starvation was optimum to obtain high viability and percentage of p-grain release; beyond that, only small grains were released to the medium, thus lowering the p-grain percentage.

Nutritional requirements of isolated microspore culture

The medium in isolated pollen culture is much more complex than in anther culture. Certain factors responsible for inducing androgenesis, which might have been provided by the anthers, are missing and must be supplied through the medium. Oftentimes, separate media for preculture and pollen culture correspond to the two steps involved in callus induction.

Evaluation of basal medium suitable for preculture

Preculture time coincides with the period required for the formation of p-grains from late uninucleate to early binucleate microspores. Using IR42 and Taipei 309, different media with sucrose—Gamborg's (1968) B5 medium with 1 mg 2,4-dichlorophenoxyacetic acid (2,4-D)/liter, 0.5 mg 6-benzylamino purine (BAP)/liter, and 0.5 mg indole-3-acetic acid (IAA)/liter (E10 medium); White's (1943) medium with 0.5 mg 2,4-D/

Table 4. Effect of quality and quantity of N source on isolated microspore culture of IR43.

Medium[a]	Components (mM)		Callus induction[b] (%)
	$(NH_4)_2SO_4$	KNO_3	
N6	3.5	28	0.24 ± .05
N6-N1	0	28	0.07 ± .05
N6-N2	0	35	0.026 ± .02
N6-N3	0	40	0.014 ± .02

[a]Basal medium N6 with 2 mg 2,4-D/liter. [b]Av of 5 plates.

liter and 0.1 mg BAP/liter (WS medium); MS medium with 2 mg 2,4-D/liter, 0.5 mg NAA/liter, and 0.5 mg KIN/liter (MSN4S medium); and the corresponding media without sucrose (So, W, and MSN4)—were evaluated to determine the best preculture medium. Medium So released the highest percentage of p-grains, while W, which has the lowest osmoticum, was not as suitable for preculture. Thus it is not only the osmoticum, but also the medium and the genotype-medium interaction, that affect p-grain release during preculture (significant variety × media interaction at the 1% level).

Quality and quantity of nitrogen source

Using NO_3^- as the only N source and increasing the concentration from 28 to 40 mM in N6 medium with 2 mg 2,4-D/liter inhibited callus induction (Table 4). The control containing 3.5 mM $(NH_4)_2SO_4$ and 28 mM KNO_3 gave the highest callus production of 0.24%. This shows that the ammonium-N content in the medium cannot be replaced by increasing the nitrate content. The total N content in the medium should come from an ammonium and a nitrate source. This agrees with previous findings (Raina and Zapata, unpubl. data) that NO_3^- alone as the N source is not beneficial to rice anther culture.

Effect of 2,4-D concentration

Concentrations of 0.5, 1.0, 2.0, and 5.0 mg 2,4-D/liter in N6 medium were used in isolated microspore culture of IR43 and Taipei 309. The highest callus induction efficiency of 0.075% was obtained in Taipei 309 isolated microspore culture at 2 mg 2,4-D/liter, which is 46.7 times less than in the first experiment on Taipei 309 (3.5%) in E10P medium. In IR43, however, callus induction efficiency improved from 0.12% in E10 medium to 0.2% in N6 with 0.5 mg 2,4-D/liter. Taipei 309 is more responsive to E10P medium, while IR43 is to N6 at 0.5 mg 2,4-D/liter. This shows that different genotypes respond differently to media (Chen et al 1980).

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Cryopreservation: a method for maintaining plant regeneration capability of rice cell suspension cultures

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Efficient and reproducible plant regeneration systems from rice (*Oryza sativa* L.) protoplasts depend upon embryogenic cell suspension cultures. However, over time, suspension cultures lose their ability to release protoplasts capable of plant regeneration. Cryopreservation was investigated as a means of providing stocks of cells for re-initiation of embryogenic cell suspension cultures, thus minimizing cytological changes that may adversely affect regeneration and reducing the risk of loss from microbial contamination.

Cell cultures of the sinica (japonica) rice variety Taipei 309 were frozen 4–5 d after subculture using the following cryoprotectants/freezing regimes:

- dimethyl sulfoxide (DMSO)/freeze from 0 °C at a cooling rate of –1 °C/min to –30 °C before transferring to liquid nitrogen
- polyethylene glycol (6000), glucose, and DMSO/freeze from 0 °C at a cooling rate of –1 °C/min to –30 °C before transferring to liquid nitrogen
- glycerol, DMSO, and sucrose/freeze from 0 °C at a cooling rate of –1 °C/min to –35 °C, holding for 30 min, then transferring to liquid nitrogen

Cell survival was determined by the reduction of triphenyl tetrazolium chloride. After a rapid loss of cell viability immediately after freezing, the cells gradually started to divide. After several weeks, embryogenic callus was used to reinitiate cell suspension cultures. The adverse effects of bleaching of thawed cells were reduced by modifying the recovery medium. The types of sugar (sucrose, fructose, and glucose) in the recovery medium have a significant effect on the degree of bleaching, and hence on cell recovery.

Current successes in regenerating plants from protoplasts of the Gramineae depend upon the use of embryogenic cell suspension cultures as the source of protoplasts (Vasil 1988). Using this approach, a protoplast-to-plant regeneration system has been established for *Oryza sativa* L. (Finch et al 1991). However, with time, the capacity for plant regeneration from isolated protoplasts is lost. This necessitates regular culture initiation to ensure a constant supply of competent cells. Cryopreserved embryogenic rice cells offer an alternative source of cultures with high regeneration capability.

Liquid nitrogen storage has already been used to maintain a supply of embryogenic maize cell suspension cultures (Shillito et al 1989). Similarly, cryopreservation offers a useful adjunct to rice biotechnology.

Cryopreservation of rice cell suspensions has been reported by Sala et al (1979) and Finkle and Ulrich (1982). The first objective of the present study was to compare the effectiveness of these rice-specific cryoprotection and freezing regimes with the more widely applied method of Withers and King (1980). These preliminary investigations were conducted on a cell line of Taipei 309 (T309).

Successful cryopreservation is dependent on both the cryogenic technique and pre- and post-freezing tissue culture. Thus, a further objective of this investigation was to establish optimum culture conditions during pregrowth and recovery. This is most important, since the duration of the post-freezing recovery stages must be minimal, allowing the re-establishment of cell suspensions before the deleterious effects of prolonged culture ensue. A recent study by Kuriyama et al (1989) showed that NH_4^+ ions in the post-thaw recovery medium are deleterious to freeze-damaged rice cells. The effects of two N sources (inorganic and amino acid) on the recovery of T309 were therefore investigated. Cella et al (1982) showed that rice cells are metabolically depleted after freezing because of the loss of respiratory intermediates. The effects of different sugar combinations (sucrose, glucose and fructose) on post-freeze survival and respiration were also investigated.

Materials and methods

All experiments were conducted on a single T309 cell suspension line (1 yr old) that was initiated from mature seed scutellum embryogenic callus and cultured as described by Finch et al (1991). Unless otherwise stated, cells were subcultured in AA2 medium, and 4–5 d after subculture were separated from the liquid by filtration through a 20- or 30-µm sieve.

Pregrowth

Cells were pregrown for 5 d in either AA2 (N source provided as L-glutamine, L-arginine, glycine, or L-aspartic acid; Abdullah et al 1986) or LS2.5 (N source provided as NH_4NO_3 and glycine; Thompson et al 1986).

Cryoprotection and freezing

Cells (0.2 g) were placed into 2.0-cm^3 polypropylene cryovials (Starstedt Ltd., Boston Road, Leicester, UK), and chilled (on iced water) cryoprotectant mixtures (Table 1) were added to a volume of approximately 0.75 cm^3. All cryoprotectants were AnalaR grade with the exception of dimethyl sulfoxide (DMSO), which was spectroscopically pure. Cryoprotectant cocktails were made up in the appropriate pregrowth medium (AA2 or LS2.5), and the pH was adjusted to that of the pregrowth medium (pH 5.8). Cells were cryoprotected for 1 h on iced water (approximately 0 °C). In the case of protocol 1, cryoprotectant cocktails were added gradually in 2 stages of increasing concentration, and the stepwise addition was completed within 1 h.

Table 1. Cryoprotectant and freezing protocols.

Protocol	Cryoprotectant treatment	Freezing protocol	Reference
1	Polyethylene glycol (PEG) MW6000 2.5% wt/vol, glucose 2.0% wt/vol, dimethyl sulfoxide (DMSO) 2.5% vol/vol, followed by PEG (MW 6000) 10% wt/vol, glucose 8% wt/vol, DMSO 10% vol/vol	From 0 °C to –30 °C at –1 °C/min, then plunge samples into liquid N_2.	Finkle and Ulrich (1982)
2	DMSO 5% vol/vol (Sala et al 1979)	From 0 °C to –30 °C at –1 °C/min, then plunge samples into liquid N_2.	Finkle and Ulrich (1982)
3	DMSO 0.5 M (as volume of liquid), glycerol 0.5 M (as volume of liquid), sucrose 1.0 M	From 0 °C to –35 °C at –1 °C/min, hold at –35 °C for 30 min, then plunge samples into liquid N_2.	Withers and King (1980)

Vials were transferred to canes, and the cells were frozen in a programmable freezer (Planer Cryo 10 Series, Planer Biomed, Sunbury-on-Thames, Middlesex, UK; or Cryo Med model 1010, Cryo Med, New Baltimore, USA). Freezing procedures are shown in Table 1. Protocol 2 comprises a cryoprotection treatment described by Sala et al (1979) followed by the freezing method of Finkle and Ulrich (1982). This allows a direct comparison of two cryoprotection regimes followed by a common freezing procedure. Ice nucleation was allowed to initiate spontaneously. External ice formation occurred at –20 °C in protocol l, –10 °C in protocol 2, and –19 °C in protocol 3.

Thawing and recovery

After storage in liquid nitrogen (–196 °C) for 1–8 wk, the cells were thawed by plunging the vials in sterile water (+45 °C). Cells were transferred to filter paper bridges, washed with the appropriate culture medium (AA2 or LS2.5), and placed onto LS2.5 solidified with agarose (SigmaType 1) containing either 3% wt/vol sucrose (control); 3% wt/vol fructose; 3% wt/vol glucose; 1.5% wt/vol sucrose and 1.5% wt/vol fructose; 1.5% wt/vol sucrose and 1.5% wt/vol glucose; 1.5% wt/vol glucose and 1.5 wt/vol fructose; or 0.75% wt/vol sucrose, 0.75% wt/vol fructose, and 0.75% wt/vol glucose. The cells were subcultured at 14- or 28-d intervals. All manipulations during the recovery phase were performed under dark conditions, thereby reducing photo-oxidation (Benson and Noronha-Dutra 1988). Once cell growth was re-established, embryogenic tissue was used to re-initiate suspension cultures in AA2, which were maintained as described by Finch et al (1991).

Post-freezing cell respiration, and thus cell viability was assessed by

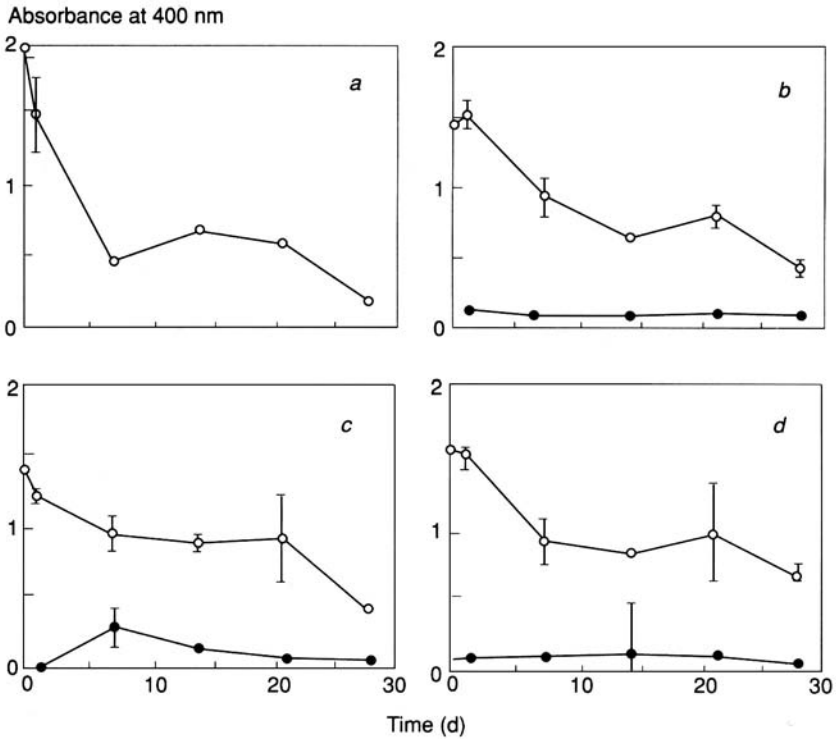
- the reduction of triphenyl tetrazolium chloride (TTC) using the method of Steponkus and Lanphear (1967). Replicates comprised cells from three cryovials that were plated onto three separate recovery dishes. Three samples of cells (50 mg) were assayed from each dish. The means and variance of each replicate were calculated.
- the morphology and color of recovering callus.

Data were analyzed using analysis of variance (Genstat 4, Lawes Agricultural Trust, Rothamsted Experimental Station, UK, statistical computer package).

Results and discussion

Cryoprotection and freezing

When establishing a cryopreservation protocol it is important to determine if cryoprotectants impair cell growth and development. Figure 1 compares the effects of cryoprotectants and freezing on post-thaw TTC reduction with a noncryoprotected control. The initial rapid decrease in cell respiration in unfrozen cryoprotected



1. Post-freeze respiration of frozen —●— and unfrozen —○— cells after no cryopreservation (a) and with cryopreservation treatments by (b) Sala et al (1979) and Finkle and Ulrich (1982), (c)Finkle and Ulrich (1982), and (d) Withers and King (1980). Error bars indicate mean variance.

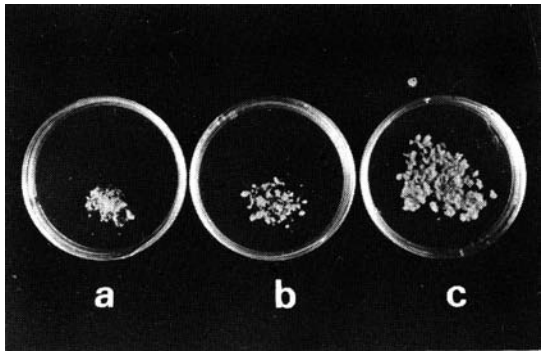
treatments (Fig. 1b, c, d) was similar to that of the noncryoprotected control (Fig. 1a). This indicates that the transfer of suspension culture cells to solidified medium, and the cryoprotection treatments both have an effect on metabolism. However, since respiration and cell growth were subsequently maintained, the cryoprotectants apparently had no long-term toxic effects. The significant ($p < 0.001$) reduction in cell respiration observed in both culture controls and unfrozen cryoprotected treatments was most probably due to nutrient depletion. This was substantiated by the finding that increased respiration occurred when cells were transferred to fresh medium. The timing of subculture intervals on semisolid recovery medium is therefore an important factor in post-thaw survival. To avoid the adverse effects of nutrient depletion, the first subculture period should be 14 d, rather than 28 d.

The respiration of thawed cells was significantly ($p < 0.001$) lower than that of unfrozen cells due to the cumulative effects of transferring cells from liquid to semisolid medium, cryoprotection, freezing, and thawing. A significant ($p < 0.001$) change in cell respiration over the 28-d post-thaw period (Fig. 1b, c, d) was probably due to the combined effects of early post-freeze injury followed by the previously observed nutrient depletion.

Table 2 shows morphological and color descriptors for post-freeze recovery in T309 exposed to the three cryopreservation protocols. Adverse symptoms of cryoinjury included callus bleaching and browning (indicators of oxidation stress) and callus wetness due to deplasmolysis injury and membrane damage. Clearly, the method of Finkle and Ulrich (1982) and the combined cryoprotectant and freezing protocol of Sala et al (1979) and Finkle and Ulrich (1982) prohibit cell growth after freezing. Although cells exposed to these regimes are viable, as shown by TTC reduction (Fig. 1b, c), they are not able to withstand deplasmolysis and oxidation injury during the later

Table 2. Morphology and regrowth of rice callus in the post-freeze period after different cryopreservation treatments.

Protocol	Callus morphology					
	Coloration		Wetness		Cell regrowth (d after thawing)	Re-initiation of cell suspension cultures (wk after thawing)
	7 d after thawing	28 d after thawing	7 d after thawing	28 d after thawing		
1	Pale, bleached	Slightly browned	Wet	Slightly wet	Not observed	–
2	Pale, very bleached	Mix of bleached and browned cell groups	Very wet	Wet	Not observed	–
3	Pale, slightly bleached	Normal callus coloration	Slightly wet	Dry	21–28	6–8



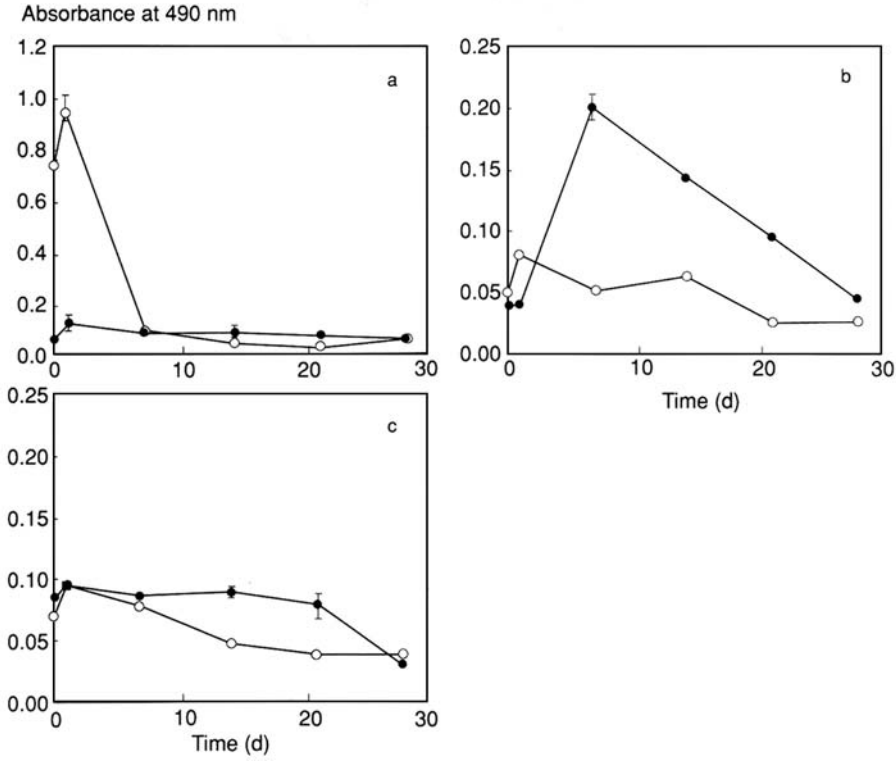
2. Morphology of rice callus (a) 0d, (b) 14d, and (c) 36d after freezing using the cryopreservation protocol of Withers and King (1980).

stages of recovery. It was not possible to re-initiate cell suspensions via callus derived from these cryopreservation treatments. In contrast, normal morphological development was resumed within 1 wk of thawing in cells that had been cryopreserved using the method of Withers and King (1980) (Fig. 2). After 6–8 wk, callus growth was sufficient to allow the re-initiation of suspension cultures.

Pregrowth nitrogen status

Two media based on different N sources have been defined for the routine culture of T309 rice. Cell suspensions are normally maintained on AA2 medium, which contains amino acids as the sole N source (Abdullah et al 1986, Muller and Grafe 1978). In contrast, callus proliferation is optimized on LS2.5 medium, which contains inorganic N. Nitrogen status has also been shown to affect rice protoplast culture. Protoplasts derived from rice cells cultured in AA medium were unable to divide when maintained on the same medium. Cell divisions were observed only in protoplasts cultured in medium containing inorganic N (Toriyama and Hinata 1985). As protoplast isolation involves "stress-inducing" treatments, similarities may occur in the N requirements of stressed, cryopreserved cells and freshly isolated protoplasts.

Post-freeze recovery is dependent on an intermediate callus stage in which the thawed cells are allowed to recover on semisolid medium. Withers (1980) suggested that the direct transfer of freshly thawed cells to liquid culture can be damaging and can exacerbate freezing injury. Clearly, in changing from pre-freeze liquid culture to post-freeze callus proliferation it is most important to consider the N requirements of the two culture states. Nitrogen metabolism in the recovering cells may require adjustment when switching from organic to inorganic substrates. Such changes in primary metabolism may have important consequences for cells that are already suffering from metabolic impairment after a freeze/thaw cycle (Cella et al 1982). Kuriyama et al (1989) showed that the viability and proliferation of thawed rice cells are depressed in the presence of NH_4^+ ions. This effect is thought to be due to the inability of freshly



3. Post-freeze respiration of pretreated (LS2.5 -o-) or non-pretreated (AA2 -•-) cells after (a) Sala et al (1979) and Finkle and Ulrich (1982). (b) Finkle and Ulrich (1982), and (c) Withers and King (1980) cryopreservation treatments. Error bars indicate mean variance.

thawed cells to control ionic gradients across plasma membranes. However, rice cells were able to utilize NH_4^+ ions once they had started to recover from cryoinjury. Schmitz and Lörz (1990) have also shown that protein synthesis is delayed when rice cells are transferred to media with high NH_4^+ levels.

The effects of cell suspension pregrowth (in AA2 or LS2.5) on post-freeze recovery on LS2.5 are shown in Figure 3. Analysis of variance revealed that pretreatment of rice cells with inorganic N-containing LS2.5 medium did not significantly increase post-freeze viability. However, further statistical analysis indicated a complex relationship between cryopreservation protocol, N pregrowth, and stage of recovery (time). The interaction between these variables was significant at $p < 0.001$.

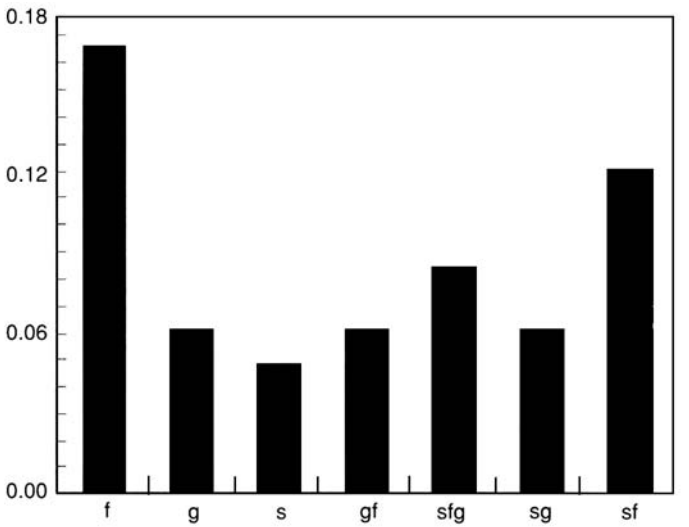
The effects of N pregrowth on cell recovery depend on time and on the cryopreservation method. Cells pregrown in LS2.5 (Fig. 3b) and cryopreserved using the combined methods of Sala et al (1979) and Finkle and Ulrich (1982) showed an initial rise in post-freeze respiration. This was possibly due to the continued maintenance of pre- and post-freeze N metabolism on a common inorganic substrate. In comparison,

cells that had been cryopreserved using the method of Finkle and Ulrich (1982) showed enhanced respiration on AA2 medium only after 7 d. Cells cryopreserved by the method of Withers and King (1980) did not show significant variation in response to pregrowth N treatment until day 14 of post-thawing recovery.

During the later stages of recovery, respiration was consistently higher for cells that had been pregrown in AA2. Nutrient depletion at 28 d becomes a limiting factor for both media types. Respiratory data are supported by the finding that cell growth was superior on AA2 than on LS2.5 medium. Suspension cultures were re-initiated only from cells that had been pregrown in organic N-based medium (AA2).

Effect of post-freeze carbon source

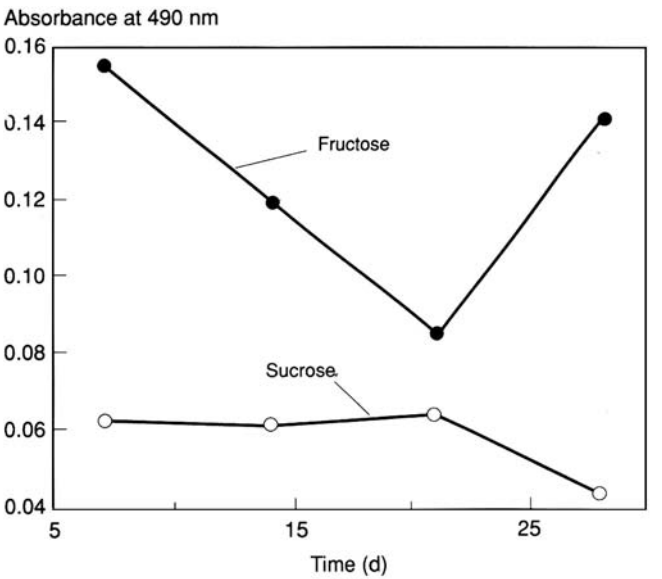
Rice is normally cultured with sucrose as the sole C source (Finch et al 1991). Sucrose is rapidly hydrolyzed by actively growing rice cells to fructose and glucose, which are utilized equally. Amino and Tazawa (1988) showed that sucrose hydrolysis is dependent on invertases, which are excreted into the culture medium. Invertase activity is also associated with rice cell walls (Schmitz and Lörz 1990), and on cell wall removal, rice protoplasts preferentially utilize glucose and fructose rather than sucrose (Amino and Tazawa 1988). As freezing and thawing can also cause cell wall and membrane disturbances, enzymes associated with sugar utilization may be impaired during early post-thaw recovery. Cella et al (1982) reported reduced glucose uptake by rice cells



4. Cell respiration 28 d after post-freeze culture on medium containing either 3% wt/vol fructose (f); 3% wt/vol glucose (g): 3% wt/vol sucrose (s); 1.5% wt/vol glucose and 1.5% wt/vol fructose (gf); 0.75% wt/vol sucrose, 0.75% wt/vol fructose, and 0.75% wt/vol glucose (sfg); 1.5% wt/vol sucrose and 1.5% wt/vol glucose (sg); or 1.5% wt/vol sucrose and 1.5% wt/vol fructose (sf).

after freezing. The membrane carrier for glucose was undamaged after cryopreservation; however, uptake may have been inhibited by a depletion in energy available for active transport, this being linked to a post-thaw decrease in glycolytic and respiratory metabolites (ATP, glucose-6-phosphate, and pyruvate). As C uptake and utilization by rice cells may be a critical factor in post-freeze recovery, the effectiveness of sucrose, fructose, and glucose in supporting recovery of cryopreserved rice was tested. To avoid "contamination" from sugars (sucrose and glucose) used in cryoprotectant mixtures, cells used were cryoprotected with 5% vol/vol DMSO (Sala et al 1979) and frozen using the method of Finkle and Ulrich (1982).

Figure 4 shows mean cell respiration for sugar treatments on day 28 of post-freeze recovery. Post-freeze respiration was better supported by the monosaccharides than by the disaccharide. Analysis of variance revealed that this effect was significant ($p < 0.001$). The presence of fructose in the recovery medium may be particularly beneficial. Figure 5 shows a time course of recovery on fructose medium compared with that on sucrose, respiration being greater on the monosaccharide substrate. This indicates that during early post-thaw recovery the C source for standard rice tissue culture may not adequately support respiration in cryopreserved cells. However, in terms of growth, the callus recovered on fructose medium became brown during the later stages of post-thaw recovery and failed to grow (Table 3). This suggests that long-term maintenance on fructose, while supporting high respiratory activity during the first stages of recovery, may be detrimental once normal metabolism resumes. A phasing of post-thaw recovery on different C sources may be more suitable: rice cells recovered initially on fructose medium (7–14d) are then transferred to standard sucrose



5. Respiration of cells on sucrose and fructose during the 28-d post-freeze period.

Table 3. Morphology and regrowth of rice callus on sucrose- or fructose-containing media during the initial post-freeze period.

Carbon source	Callus morphology at indicated time (d) after thawing			
	Coloration		Wetness	
	7d	28 d	7d	28 d
Sucrose	Pale, very bleached	Mix of bleached and brown cell groups	Very wet	Wet
Fructose	Slightly bleached	Brown	Wet	Wet

medium. This protocol is currently being investigated using the optimal cryopreservation method of Withers and King (1980).

Conclusions and prospects

Rice cells were viable after all freezing protocols, but successful recovery was achieved only after pregrowth on AA2 medium and cryopreservation by the method of Withers and King (1980). The inability to recover cells using the previously reported rice cell suspension cryopreservation protocols may have been due to varietal differences and/or to the pre-freeze culture. The significant effect of different carbohydrates on cell recovery indicates the need to develop a specialized medium containing fructose to enhance the early post-thaw survival of cryopreserved cells.

Cryopreservation studies were conducted by Sala et a1 (1979) on Roncarolo, an Italian variety; and Finkle and Ulrich (1982) used unspecified rice mutants. In both studies rice cell suspensions were maintained on media containing inorganic N (R2, Sala et a1 1979, and a modified Murashige and Skoog's formula, Finkle and Ulrich 1982, Murashige and Skoog 1962). Tissue culture regimes can greatly influence the growth characteristics of rice cells. Thus T309 cell suspension colonies contain on average 100 cells, whereas the cultures of Sala et al (1979) comprised colonies of several hundred cells. N status also influences growth, and cell colony density is greater in the presence of inorganic N than with organic N (Thompson et al 1986). Thus, cell culture characteristics will significantly influence cell viability both during and after cryopreservation.

The culture characteristics of protoplasts isolated from re-initiated cultures are currently being assessed. Cella et al (1982) isolated protoplasts from re-initiated cell suspension cultures of rice. They found that membranes were unstable in protoplasts isolated from cells in the early phases of post-freeze recovery. However, isolation was successful when protoplasts were isolated from cells that had undergone several post-thaw subculture cycles. Isolation of protoplasts from frozen and then thawed T309 is therefore not anticipated to be problematic.

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Discussion

Session 6: Tissue and cell culture

Q—*Chaudhuri:* It seems from your results that your P_5 and P_6 are least responsive to callus regeneration, and P_4 is intermediate. However, during differentiation the P_4 becomes very susceptible in different crosses. Will you please elaborate? Do you have backcross data?

A—*Abe:* P_2, P_5, and P_6 had high capacities for plant regeneration, while P_1 and P_4 had very low capacities. In the parents that had high capacities, P_6 had more dominant alleles, while P_5 evidenced overdominance, nonallelic interaction, and epistasis. The aim of this study was to provide precise information about the genetic control of callus growth and plant regeneration, so we did not have to backcross.

Q—*Guiderdoni:* Drs. Peng and Hodges recently published the results of a comparable study on indica rices. Could you (or Dr. Hodges) tell us whether the conclusions of this study are identical to those of your report, suggesting an identical type of genetic control for regeneration in japonica and indica rices?

A—*Abe:* I do not know the study by Peng and Hodges. We used japonica rice in this study because we have many japonica genotypes. We found that dominance effects as well as additive gene effects are important and that the action of dominance genes is positive. From these facts, we can improve the regenerative trait using elite genotypes that have more dominant alleles, e.g., Daikoku 1.

A—*Hodges:* Initially we observed that certain genotypes such as IR36 were recalcitrant to regeneration, but when crossed with regenerable varieties such as IR54, the hybrid was regenerable. We then did a diallel analysis involving four indica varieties. The results indicated more additive gene effects than dominance effects. I cannot explain why the indica varieties appear to be somewhat different from the japonicas regarding the nature of genetic control of regeneration. However, we have carried out an inheritance study of maize regeneration. Interestingly, the conclusions from that study are very similar to those of Dr. Abe for japonica rices, namely, that two genes or groups of genes play a majorrole incontrolling somatic embryogenesis and regeneration. Furthermore, in an unpublished study in collaboration with Drs. M. Murry and C. Armstrong of Agrigenetics Corporation we have shown that two restriction fragment length polymorphism probes account for 90-95% of the variability associated with regeneration. Together, these results clearly demonstrate the genetic control of regeneration.

Q—*Shimamoto:* How do you evaluate new lines as salt tolerant?

A—*Narayanan:* Tolerance and susceptibility are scored and compared with those of check varieties IR20 (susceptible) and Pokkali (resistant). Scoring is done

when all IR20 plants are dead. However, the comparison is rather subjective, and with some experience we can easily distinguish the tolerant from the susceptible plants.

Q—*Brar:* So far, in vitro selection for salt tolerance has not been very encouraging; salt tolerance itself is a very complex trait. Do you think we need to revise our classical selection strategy to overcome epigenetic effects, e.g., use cell suspension rather than callus cultures, include various salt ions in place of NaCl, or employ mutagenesis of cultured cells?

A—*Narayanan:* The only way to exclude physiological and epigenetic variants is by careful progeny analysis. Some reports of useful somaclonal variants generated from tissue cultures exist, and therefore we should continue with in vitro selection; but we should also concentrate on progeny analysis to isolate genetic mutants. Since tissue culture selection is still largely empirical, we cannot really say whether one method is better than the other—whether we should use suspension culture or callus culture for selection. Mutagenesis of cultured cells should enhance variability and therefore may be useful. Regarding the use of NaCl alone instead of a combination of salts, we observed in our preliminary experiments that NaCl tolerance correlated highly with tolerance for a combination of salts.

Q—*Gupta:* Various studies on salt tolerance indicate the role of cytoplasmic genes. Would it be useful to take up research work on this line?

A—*Narayanan:* We, too, have observed extranuclear effects in the expression of certain characters under salt stress. However, the organelle genome involved is unknown. It would certainly be useful to study cytoplasmic effects.

C—*Zapata:* We kept calli of Giza 173 for 3-1/2 yr on 25 g NaCl/liter. Plants regenerated from them had very poor plant type and were sensitive to 0.6 g NaCl/liter. Also, high sterility was observed.

C—*Narayanan:* I am not surprised that you got highly abnormal regenerates from 3-1/2-yr-old stressed cultures. I think it would be better to stress for a shorter period to avoid physiological adaptation of cells, and to optimize the level of stress to select only mutant cells. Our experience is that salt-tolerant variants are indeed generated by this method, and their usefulness can be determined only after analyzing a few more sexual generations. We are now in the R_3, and some lines are promising.

C—*Kinoshita:* For proof of cytoplasmic inheritance, the reciprocal difference of the inheritance modes must be examined by crossing between pollen-restored plants and normal-cytoplasm plants.

C—*Ling:* We will make these crosses.

Q—*Narayanan:* Was your tissue culture experiment specifically designed to generate male sterile mutants, or was it incidental that you got cytoplasmic male sterile mutants?

A—*Ling:* The experiment was designed to obtain male sterile mutants.

Q—*Zheng Kangle:* I wonder if male sterility is controlled by cytoplasm. How could you detect male sterility in the R_2 generation, because the R_2 generation should be the selfed progeny of R_1-plants?

A—*Ling:* Mutant 54257 is controlled by cytoplasmic nuclear genes, because it has both restorer and maintainer. In the R_2 generation, male sterile mutants can be found by segregation of the male sterile gene. This mutant must be heterozygous in the R_1 generation, which is fertile and can give seeds by self-pollination.

Q—*Rangasamy* : What was the behavior of mutant 54257 when crossed with the parent IR54?

A—*Ling:* When 54257 was crossed with its parent, IR54, the F_1 was fertile.

Q—*Gupta:* Were the protoplasts derived from calli or cell suspensions?

A—*Li Xiang-Hui:* The protoplasts were derived from suspension cells of DGZ.

Q—*Chaudhuri:* Do you have any reason to use D2 and R2 as control media when your experimental medium is KM, and do you have data using KM as the control medium?

A—*Li Xiang-Hui:* We would like to find a simple basic medium for rice protoplast culture. D2 and R2 are quite simple and have different components, so we used them as control media.

C—*Zapata:* We increased the number of green plants regenerated versus albino plants. The plants diploidized spontaneously, about 50%. The easier way to check if they come from pollen is to take them to the field and check if they segregate or not in the case of the F_1.

Q—*Fujimura:* Why is fructose suitable for recovery of the cryopreserved cells?

A—*Lynch:* Fructose is desirable in the semisolid recovery medium only in the early stages of cell recovery because of the long-term detrimental effects of maintaining callus on fructose-containing medium. The initial advantages of fructose use may relate to carbon uptake and utilization. Cella et al (1982) (Physiol. Plant. 55:279) reported inhibition of sugar by calli after freezing.

Q—*Chaudhuri:* Do you have any data on karyotype analysis of your original calli and cryopreserved calli? I find that you have used dimethyl sulfoxide (DMSO) as a cryopreservant. DMSO is known to be mutagenic or even carcinogenic. Have you studied the chromosomes?

A—*Lynch:* There are no data on the karyotypes of the original cells compared with those of cells recovered from cryopreservation. Although DMSO is a known mutagen, it has been used widely and successfully as a cryoprotectant for many plant systems. The chromosomes of cells have not been studied. The ultimate test of cryopreservation is the regeneration of plants similar to those of the original culture, and this is being studied.

Q—*Shao:* Do you have data on the efficiency of plant regeneration? How long did you store during freezing?

A—*Lynch:* The protoplast isolation characteristics, growth, and regeneration from suspension cultures initiated from thawed cells are currently being assessed.

However, successful similar studies in maize were reported by Shillito et al (1989) (Biotechnology 7:581–587). We have recovered cells after several months storage in liquid nitrogen. The duration of cell storage in liquid nitrogen is not significant to subsequent cell recovery.

Q—*Aryan:* How long can you keep the callus cryopreserved and maintain viability? Restoring of cryopreserved calli seems to take a long time, especially on recovery medium where you have to optimize various sources of nitrogen and sucrose. In contrast to producing embryogenic calli for suspension from mature rice embryos, it takes about 2-3 mo. What are the advantages of cryopreservation?

A—*Lynch:* Once the cells have been frozen and placed in liquid nitrogen (-196°C), cell metabolism stops. The material can thus be maintained indefinitely without adversely affecting cell viability. The normal time rate for development of a cell suspension culture of Taipei 309 suitable for protoplast isolation is 8–10 mo, including 1–2 mo to produce embryogenic callus from mature rice embryos. Whereas, at present there is no totally optimized cell recovery method after cryopreservation, cell regeneration is observed 21–28 d after thawing. Within 6–8 wk after thawing the callus can be used to reinitiate cell suspension cultures. These cultures are then suitable for protoplast isolation within the next 4–6 wk. The whole time scale from cell thawing to protoplast isolation is only 14 wk (4 mo). Once the rice cell recovery medium or protocol is optimized, the time scale should be substantially reduced. Since the reinitiated cell suspension culture characteristics will probably be similar to those of the original cultures, cell culture characterization time will also be reduced compared with that for a totally new cell suspension line. Cryopreservation of rice cells thus has obvious advantages over conventional cell suspension initiation.

SESSION 7

Molecular Genetics of Cytoplasmic Genomes

Structure of cytoplasmic genomes in rice

A. Hirai, M. Iwahashi, K. Sugino, A. Kanno, and T. Ishibashi

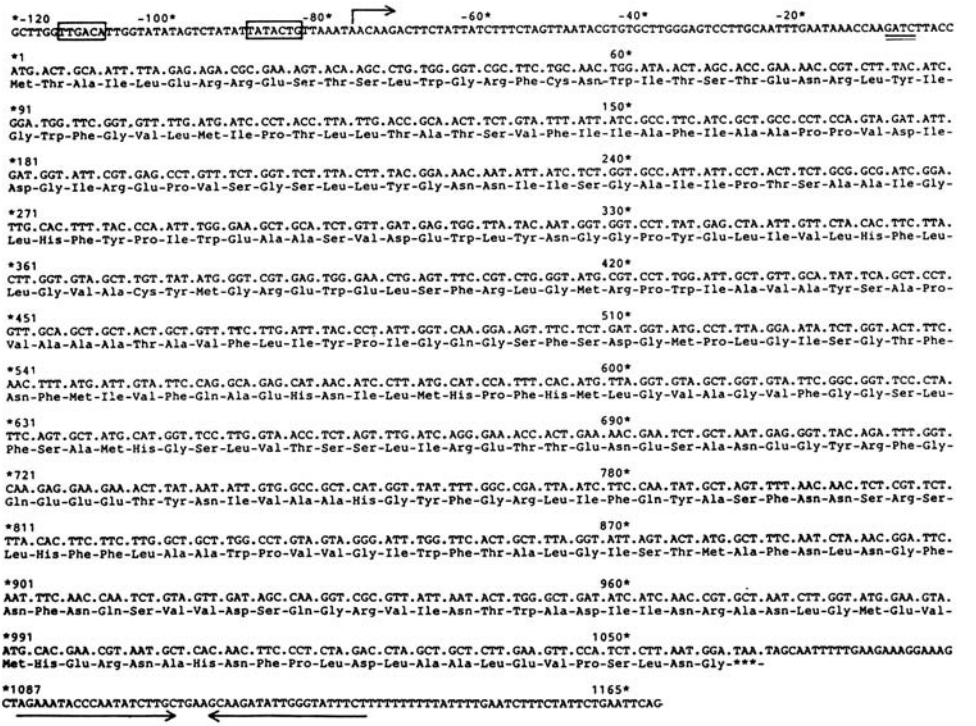
Chloroplast DNA (ctDNA) and mitochondrial DNA (mtDNA) were isolated from green leaves of rice, and fragments were cloned into plasmids or lambda DNAs. Overlapping clone banks of two DNAs were obtained. Physical and genetic maps of two cytoplasmic genomes were also constructed. For ctDNA, the complete nucleotide sequence was determined. Many sequences of ctDNA were found in mtDNA, indicating the transfer of DNA fragments from chloroplasts to mitochondria.

Cells in higher plants including rice contain genetic information in the nucleus, chloroplasts, and mitochondria. The information in the latter two organelles, i.e., cytoplasmic genes, is maternally inherited in most cases. Therefore, it is difficult to improve these genes by ordinary breeding, which is based mainly on sexual hybridization. However, chloroplast DNA (ctDNA) codes many important genes for photosynthesis, and mitochondrial DNA (mtDNA) codes genes for energy generation. Improving cytoplasmic genomes using techniques from the new molecular biology-based biosciences is thus desirable. Accordingly, we have been studying the structure of ctDNA and mtDNA from rice *Oryza sativa* L. (cultivar Nipponbare) for use in breeding programs.

Mapping and cloning the rice chloroplast genome

Chloroplast DNA has been isolated from young rice leaves. A physical map of *Sal*I, *Pst*I, and *Pvu*II fragments of the circular DNA has also been constructed (Hirai et al 1985). The location of important genes for photosynthesis such as *rbcL, atpBE, psbA, petA,* and *psuA* was determined. Most of the *Bam*HI and *Pst*I fragments were cloned into plasmids, and thus an overlapping clone bank was obtained.

Using this bank, nucleotide sequences of *rbcL* (Nishizawa and Hirai 1987), *atpBE* (Nishizawa and Hirai 1989), and *psbA* (Kanno and Hirai 1989) were determined. S_1 mapping and primer extension analyses were conducted to determine the 5' end of these messenger RNAs (mRNAs). These genes have the typical prokaryotic structure.



1. Nucleotide sequence of 32-kDa quinone-binding protein gene *psbA* from rice and deduced amino acid sequence. Numbers indicate base pairs relative to 5' end of *psbA* coding region. Possible "Pribnow box" and "-35 region" are boxed, and possible Shine-Dalgamo sequences are underlined. Sequences that can form stem and loop structure at 3' end are shown by arrows.

Analysis of *psbA* gene

The gene for the high turnover 32-kDa quinone-binding protein of the photosystem II reaction center in chloroplasts is called *psbA* and is encoded by ctDNA. The nucleotide sequence of *psbA* and the deduced amino acid sequence of the protein were determined (Kanno and Hirai 1989; Fig. 1). The protein coding regions of *psbA* from rice and tobacco have 91.7% homology in terms of nucleotide sequence, and 98.6% homology in terms of deduced amino acid sequence (Sugita and Sugiura 1984).

The 5' end of the *psbA* mRNA was determined by primer extension analysis and was mapped on the sequence 77 bp upstream from the ATG codon. Upstream of this site are sequences similar to the "Pribnow box" and the "-35 region" sequences, respectively. Transcription of *psbA* from rice starts from the same site as that of *psbA* from tobacco. The sequence ATTTTG (at positions -60 to -55) is repeated in tobacco but not in rice.

The size of the *psbA* mRNA was determined by northern hybridization. Only one band was detected by autoradiography, and the size of the mRNA was deduced to be approximately 1.2 kb.

A stem-and-loop structure can be formed in the region from 45 to 88 bases downstream from the stop codon of *psbA.* This inverted repeat has a very stable structure, having -40.2 kcal free energy/mol when it is transcribed to RNA (Tinoco et al 1973). The distance from the site of initiation of transcription to this inverted repeat coincides with the length of the mRNA (1.2 kb). In spinach, inverted repeat sequences at the 3' end of *psbA* may act as processing and stabilizing elements and are not involved in the termination of transcription (Stern and Gruissem 1987).

Complete sequence of rice chloroplast DNA

The complete nucleotide sequence of 134,525 bp DNA was determined by M. Sugiura's group using our clone bank of overlapping fragments (Hiratsuka et al 1989). The sequences determined for rice and tobacco were aligned, and rice genes were identified by homology with their tobacco counterparts (Shinozaki et al 1986). Thirty transfer RNA (tRNA) genes were identified, corresponding in ctDNA location and anticodon to those previously identified in tobacco. Ribosomal RNA *16S, 23S, 4.5S,* and *5S* genes were also conserved, as were putative protein coding genes previously identified in tobacco. A genetic and physical map is shown in Figure 2.

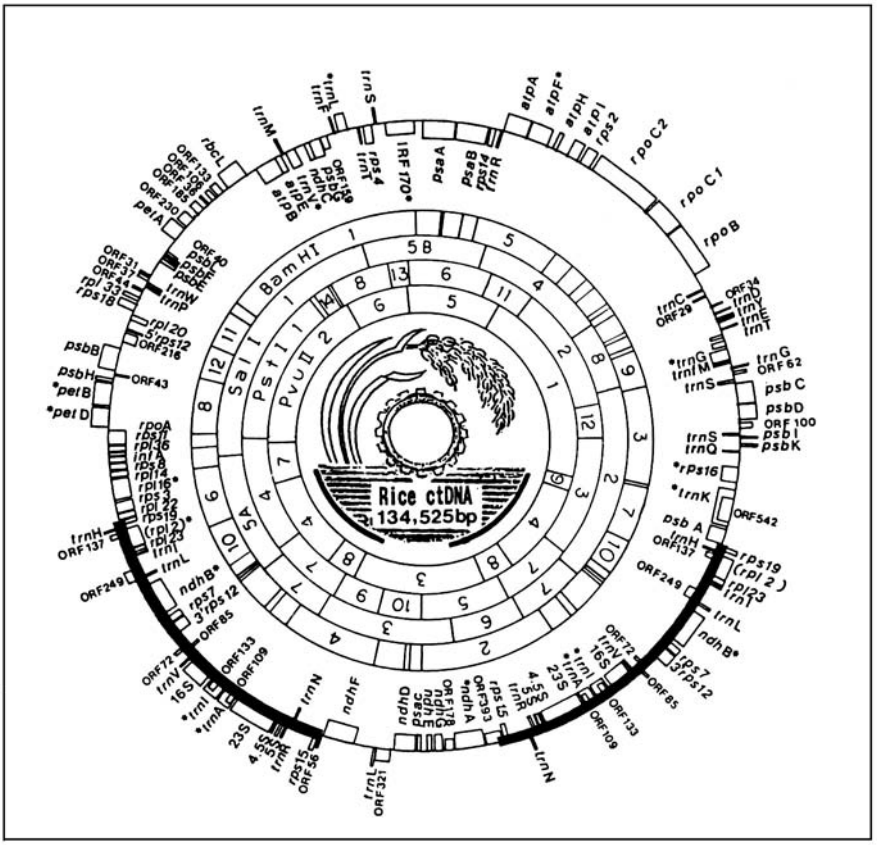
Relative to tobacco, some structural reorganization of the rice chloroplast genome is evident: the inverted repeats code additional genes; kilobase unit deletions have occurred; and major rearrangements are evident within the large single-copy region.

Structure of rice mitochondrial genome

Mitochondrial DNA was also isolated from green rice leaves; fragments of Sau3A partial digestion of the DNA were size-fractionated by sucrose density gradient centrifugation; and 14- to 18-kb fragments were cloned into lambda DNA. Thus, a lambda library of the rice mitochondrial genome was constructed.

DNA fragments containing mitochondrial genes such as *atpA, atp9,* or *cox*I from other plants were used as probes to identify rice mitochondrial clones from the library. Physical maps of the cloned DNA containing each gene were determined. Overlapping clones were identified by plaque and Southern hybridization. These were extended subsequently by "genome walking," and complete genetic and physical maps of rice mtDNA were constructed. The genetic map is shown in Figure 3.

Rice mtDNA in green leaves consists of four kinds of circular DNA. Each circular DNA shares homologous sequences with one to three other circular DNAs. If recombinations occur in homologous sequences, then new kinds of circular DNA will be observed. This explains a multicircular organization in which the various circular molecules are present in different stoichiometric amounts.



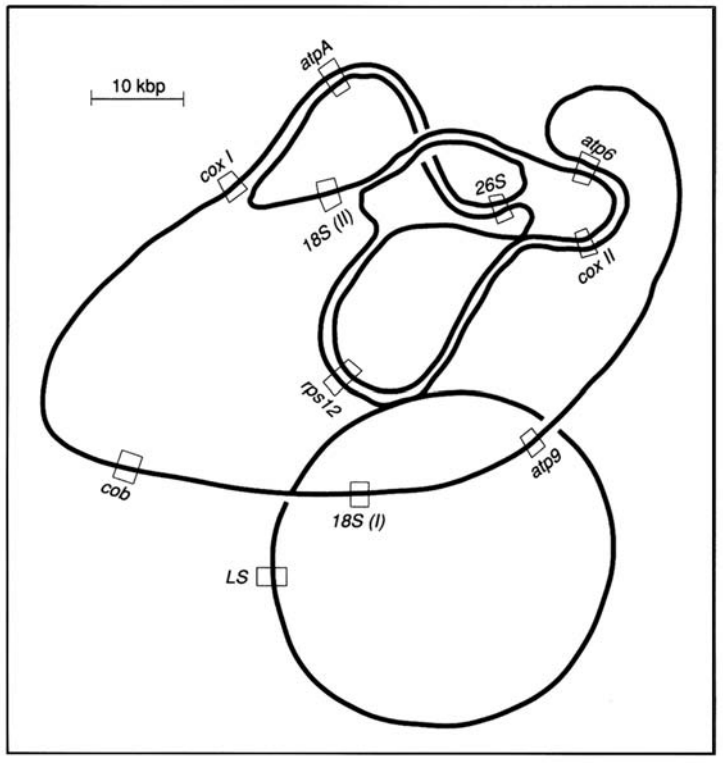
2. Genetic and physical map of ctDNA from rice, drawn from data of Hiratsuka et al (1989).

It is unknown how this complex mtDNA replicates. However, if rice mtDNA replicates by a "master circle" as in maize and in other plants, we predict from our data that the master circle of rice mtDNA has 4 repeating sequences, and its size is 528 kb.

A further characteristic of plant mtDNA is the observed areas of homology between ctDNA and mtDNA. Stern and Lonsdale (1982) described the homology of 12 kb of inverted repeat of maize ctDNA, which was identified in mtDNA.

We also detected rice ctDNA sequences in rice mtDNA. In fact, 15 out of 19 ctDNA fragments, ranging from 15 to 2.2 kb and covering the entire ctDNA, hybridized with our mitochondrial clones. The results show that the transfer of genetic information from chloroplast to mitochondrion is a widespread phenomenon. The function of these sequences is unknown.

The mitochondrial genomes of higher plants are much larger than the mitochondrial genomes of other groups of eukaryotic organisms. They have a complex organization



3. Genetic map of rice mtDNA.

generated by homologous recombination. However, we know that almost the same number of genes are coded in plant and animal mitochondrial genomes. Why are plant mitochondrial genomes so large and so complex? The answer will be obtained when the complete nucleotide sequence of the DNA has been determined.

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Rice mitochondrial genes

C. André, E.K. Kaleikau, and V. Walbot

We report the sequence of three mitochondrial genes from the IR36 line: apocytochrome b, cytochrome oxidase subunit 3, and subunit 9 of ATPase. We compare codon usage in these genes and propose sites of possible RNA editing. We also report progress in developing a "fingerprinting" technique to classify rice cytoplasm based on the *Sfi*I digestion patterns of mitochondrial DNA analyzed on pulsed field gels. Available rice genes can be localized by DNA hybridization to the large *Sfi*I fragments resolved by pulsed field electrophoresis.

Unlike the plastid genomes of angiosperms, which are relatively constant in size and coding function, mitochondrial genomes of higher plants are very divergent in size and sequence content (Newton 1988). The smallest known mitochondrial DNA (mtDNA) in an angiosperm is the 208-kb genome found in *Brassica hirta* (Palmer and Herbon 1987). While the rice genome is about twice this size (Harai et al 1990), it is very unlikely that rice mtDNA encodes more genes than does the *Brassica* genome. In fact, despite their larger sizes, the mtDNAs in higher plants are thought to contain fewer genes than the chloroplast DNA (ctDNA). This conclusion is based on the diversity of transcripts isolated from organelles and the diversity of proteins synthesized in vitro by isolated, intact organelles. The apparently limited coding capacity of mtDNA and its wide variation in size among plants (from 208 kb in *Brassica* to about 2,500 kb in some melons) suggest that much of the genome is "junk" DNA; indeed, mtDNAs from a variety of species have been shown to contain large segments of plastid DNA (Stern and Lonsdale 1982, Stern and Palmer 1984), and one segment of ctDNA has already been found in rice mtDNA (Moon et al 1988).

No higher plant mtDNA has been sequenced entirely; however, when the complete sequence is available there may be surprises such as unexpected genes. Known genes in plant mitochondria are, in general, limited to those encoding ribosomal RNAs, transfer RNAs (tRNAs), and proteins involved in ribosome assembly; cytochrome oxidase; components of electron transport; and ATP synthase. The sequences of a

number of these genes have been obtained from rice using heterologous probes to recover the appropriate rice clones from genomic libraries.

One reason for interest in the rice mtDNA genome is that cytoplasmic male sterility in many higher plants is associated with defects in the mitochondrion rather than in the plastid (Newton 1988). The cytoplasmic male sterile (CMS) trait has been exploited in crops such as maize, *Brassica,* and rice for the production of hybrid seed. To be of use to farmers, however, such hybrid seed must also inherit a nuclear-based fertility restoration system from the male parent so that the planted seed is fully fertile. This genetic relationship between a defective cytoplasm and one or more required (usually fully dominant) nuclear genes suggests that the nuclear genome of fertility-restoring lines encodes a function correcting a mitochondrial deficiency. The nature of these mitochondrial defects has been well established for the sterile T cytoplasm of maize (Dewey et al 1986) and for a CMS mutant in petunia (Young and Hanson 1987); in both cases, rearrangements in CMS mtDNA created a novel opening reading frame. In maize, expression of the novel protein (TURF13) in *Escherichia coli* confers sensitivity to methomyl, an insecticide that is also a selective toxin in sterile T maize plants (Dewey et al 1988).

Because of high intraspecific variation in mtDNA organization and in the copy number of individual genes in other plant species (Newton 1988), it is unlikely that a single map of rice mtDNA will be sufficient. Indeed, the intraspecific diversity of mtDNA is sufficiently high that restriction fragment length polymorphism (RFLP) in mtDNA can be used to classify cytoplasmic sources in other species. Catalogs of RFLP and ultimately physical maps of rice mtDNA prepared from a variety of lines may enable deduction of phylogenetic relationships among the cytoplasms and may also pinpoint regions of the genome involved in CMS. Although RFLP is high, individual restriction sites are usually conserved; novel fragments are generated by recombination—scrambling the order of genes and RFLP markers in higher plant mtDNA (Palmer 1985).

Cytoplasmic male sterility and mtDNA organization

Paralleling the initial reports on cytoplasmic male sterility in other species, however, novel restriction fragments (Kadowaki et al 1986) have been detected in three CMS cytoplasms. Interpretation of such differences is difficult, because rearrangements in mtDNA are common, creating many novel fragments in each cytoplasm. Another proposed feature of CMS lines is the presence of plasmids; these molecules have been detected in at least one source of cytoplasmic male sterility in rice (Chinsurah Boro II: Yamaguchi et al 1986). Both RFLP and plasmids are compelling evidence for diversity among mtDNA in rice cytoplasm, but the association of these features with the CMS trait must be interpreted with caution. To verify that a particular restriction fragment variant is associated with cytoplasmic male sterility would require analysis of a cytoplasmic fertility revertant and/or discovery of the impact of RFLP on a mitochondrial function such as detection of a novel protein. Similarly, because the S1 and S2

DNA episomes of cytoplasmic male sterility type S maize were initially shown to correlate well with the male sterile phenotype, it was proposed that they caused cytoplasmic male sterility. Subsequently, it was demonstrated that neither the presence of the plasmids (Escote et al 1985, Weissinger et al 1982) nor their expression (Manson et al 1986, O'Brien-Vedder et al 1989, Zabala et al 1987, Zabala and Walbot 1988) directly determines the CMS phenotype. Further data on the role of plasmids and episomes in the rice mitochondrial genome are required before concluding that the plasmids detected so far are determinants of male sterility. Furthermore, plasmids were shown to be unreliable markers for classifying maize cytoplasm when wider surveys of plasmid distribution were reported (Newton and Walbot 1985). Consequently, RFLP in the main mtDNA will probably prove to be a more reliable indicator of cytoplasmic type than the distribution of plasmids in rice mitochondria.

Sequence analysis of three rice mitochondrial genes

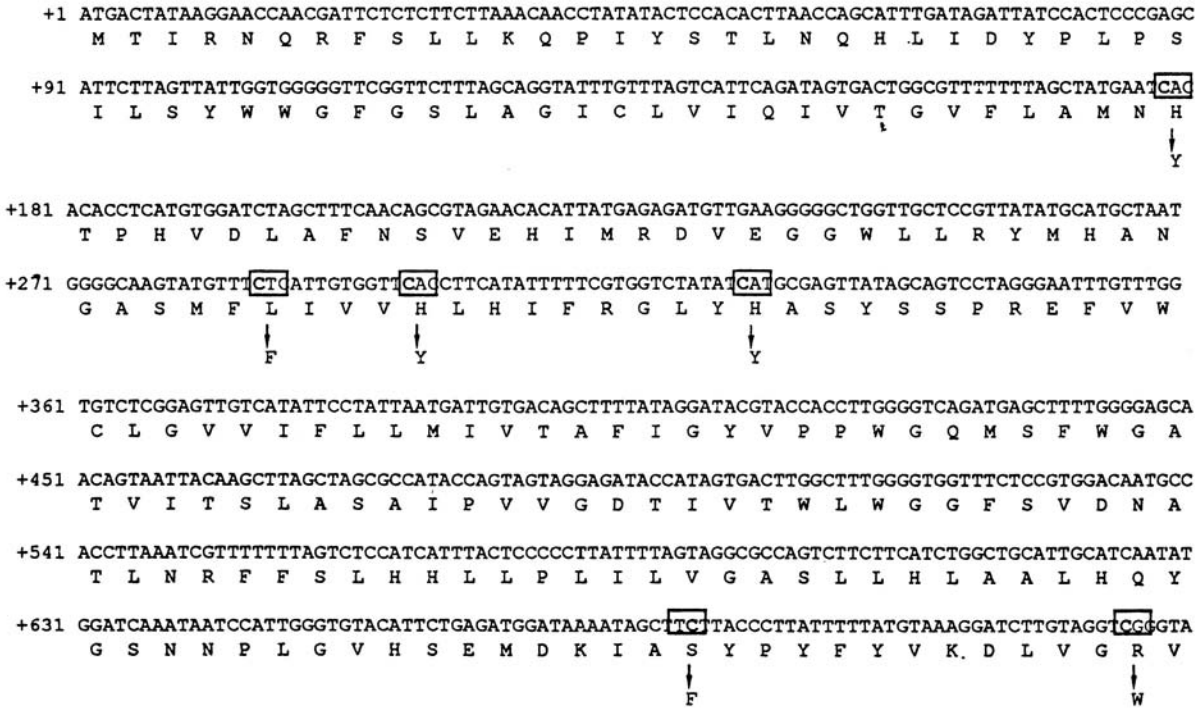
Total rice DNA, containing approximately 5% mtDNA, was partially digested with the restriction enzyme *Sau*3AI, and 15- to 20-kb pieces were cloned into the EMBL 3 lambda vector. The maize coding regions for three mitochondrial genes—apocytochrome b (*cob*), cytochrome oxidase subunit 3 (*cox*3), and subunit 9 of ATPase (*atp*9)—were used as probes to recover the rice sequences from the total DNA library. Subclones containing the coding regions for each gene were prepared and sequenced (Kaleikau et al 1990a,b,c; Fig. 1, *cob*; Fig. 2, *cox*3; Fig. 3, *atp*9); also presented is information on codon usage (Table 1) and base usage in the coding strand (Table 2).

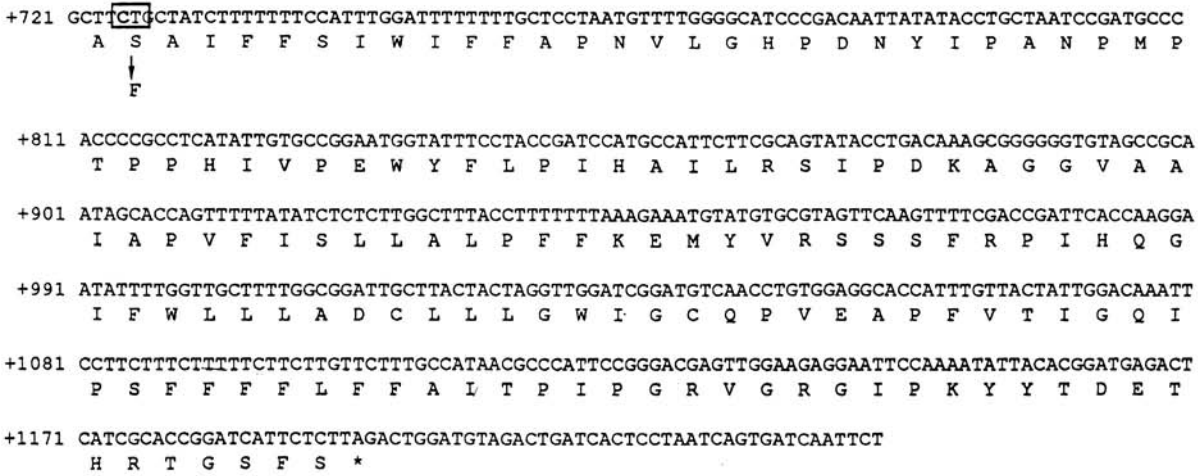
Codon usage

The three rice mitochondrial genes sequenced have a biased base composition: approximately 35% of the coding strand bases are T residues, while the 3 other bases comprise about 21–23% of the total (Table 2). In terms of codon usage (without correcting for possible RNA editing), all of the universal codons are used at least once. There is, however, a striking base bias, with a significant preference for U in the third position of codons of the most commonly used amino acids (Table 1). For example, among the alanine codons (GCN), 42.9–53.3% are GCU; similarly, the most commonly used glycine, isoleucine, leucine, phenylalanine, proline, and tyrosine codons have U in the third position. The next most commonly used codons typically end in an A residue, and for valine the most commonly used codon in the three genes is GUA.

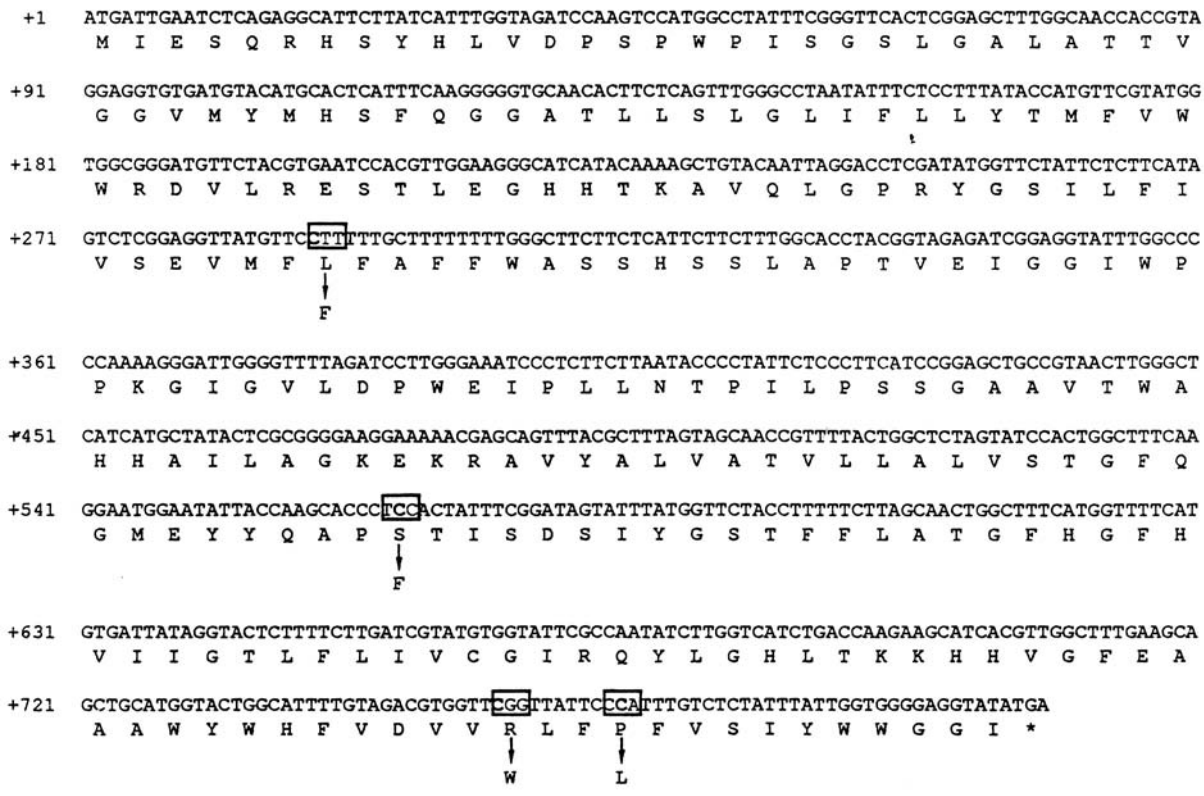
Messenger RNA processing

RNA processing by endonucleolytic cleavage occurs in the formation of mature ribosomal RNAs in plant mitochondria (Mulligan et al 1988), but messenger RNA (mRNA) processing seems to be restricted to intron removal by mRNA splicing. Splicing occurs in the *cox*2 transcripts of rice (Kao et al 1984). A recent surprise was the discovery of a novel form of mRNA processing in plant mitochondria, termed RNA editing. The existence of editing appears to be an evolutionary adaptation to allow

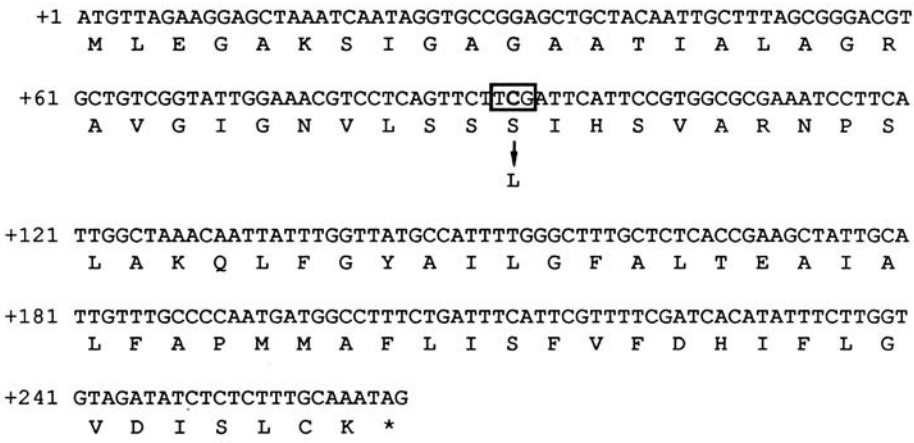

+1 ATGACTATAAGGAACCAACGATTCTCTCTTCTTAAACAACCTATATACTCCACACTTAACCAGCATTTGATAGATTATCCACTCCCGAGC
M T I R N Q R F S L L K Q P I Y S T L N Q H L I D Y P L P S
+91 ATTCTTAGTTATTGGTGGGGGTTCGGTTCTTTAGCAGGTATTTGTTTAGTCATTCAGATAGTGACTGGCGTTTTTTTAGCTATGAATCAC
I L S Y W W G F G S L A G I C L V I Q I V T G V F L A M N H
Y
+181 ACACCTCATGTGGATCTAGCTTTCAACAGCGTAGAACACATTATGAGAGATGTTGAAGGGGGCTGGTTGCTCCGTTATATGCATGCTAAT
T P H V D L A F N S V E H I M R D V E G G W L L R Y M H A N
+271 GGGGCAAGTATGTTTCTCATTGTGGTTCACCTTCATATTTTTCGTGGTCTATATCATGCGAGTTATAGCAGTCCTAGGGAATTTGTTTGG
G A S M F L I V V H L H I F R G L Y H A S Y S S P R E F V W
F Y Y
+361 TGTCTCGGAGTTGTCATATTCCTATTAATGATTGTGACAGCTTTTATAGGATACGTACCACCTTGGGGTCAGATGAGCTTTTGGGGAGCA
C L G V V I F L L M I V T A F I G Y V P P W G Q M S F W G A
+451 ACAGTAATTACAAGCTTAGCTAGCGCCATACCAGTAGTAGGAGATACCATAGTGACTTGGCTTTGGGGTGGTTTCTCCGTGGACAATGCC
T V I T S L A S A I P V V G D T I V T W L W G G F S V D N A
+541 ACCTTAAATCGTTTTTTTAGTCTCCATCATTTACTCCCCCTTATTTTAGTAGGCGCCAGTCTTCTTCATCTGGCTGCATTGCATCAATAT
T L N R F F S L H H L L P L I L V G A S L L H L A A L H Q Y
+631 GGATCAAATAATCCATTGGGTGTACATTCTGAGATGGATAAAATAGCTTCTTACCCTTATTTTTATGTAAAGGATCTTGTAGGTCGGGGTA
G S N N P L G V H S E M D K I A S Y P Y F Y V K D L V G R V
F W



1. Annotated sequence of IR36 *cob* gene. Boxes indicate codon positions where RNA editing would increase amino acid similarity with other organisms. C residues proposed to change to U in the RNA are shown in **boldface.** Proposed amino acid changes are also indicated.



2. Annotated sequence of IR36 *cox*3 gene. Boxes indicate codon positions where RNA editing would increase amino acid similarity with other organisms. C residues proposed to change to U in the RNA are shown in **boldface.** Proposed amino acid changes are also indicated.



3. Annotated sequence of IR36 *atp*9 gene. Box indicates codon position where RNA editing would increase amino acid similarity with other organisms. C residue proposed to change to U in the RNA is shown in **boldface.** Proposed amino acid change is also indicated.

higher plant mitochondria to conform to the universal codon rules. In the past it was assumed that plant mtDNA did not utilize the universal rules, because at amino acid positions with conserved tryptophan residues (TGG codon in DNA, and UGG in mRNA) in genes of fungal and animal mtDNA, some plant genes contained the codon CGG (arginine in the universal code). Furthermore, both monocot and dicot mitochondria appeared to contain only one tRNA gene for tryptophan; this tRNA gene is part of a plastid DNA segment now present in mtDNA (Leon et al 1989, Marechal et al 1987) and has the anticodon for UGG. The dilemma is resolved by mRNA editing: a few C residues are changed to U; consequently, CGG in the DNA can be edited to UGG in the mRNA, preserving universal codon usage in higher plant mitochondrial RNA (Covello and Gray 1989, Gualberto et al 1989). Editing at a few C residues changes additional codons in ways that maximize amino acid sequence conservation among higher plant mitochondrial genes.

The editing process is selective—on1y a few residues are changed—requiring an unknown mechanism to provide specificity. Much remains to be done to elucidate the regulation of this novel molecular process and to determine the extent to which it occurs in rice. In Figures 1–3 we have boxed codons that may be sites of RNA editing. These bases were chosen to maximize amino acid conservation with other organisms.

Pulsed field gel analysis of rice mtDNA

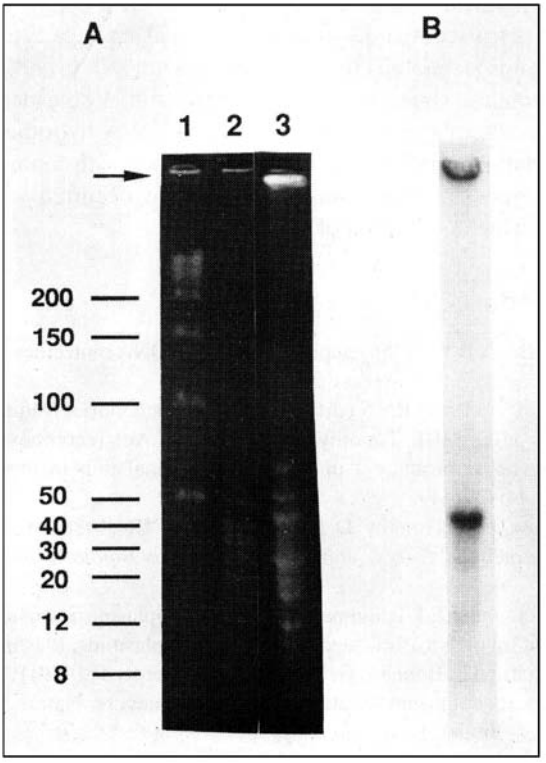
Conventional agarose gel electrophoresis cannot resolve linear DNA fragments greater than 20kb; however, fragments of up to 10Mb can be resolved by pulsed field gel electrophoresis. We have utilized contour clamped homogeneous field electrophoresis (Chu-et al 1986) to resolve S*fi*I digests of rice mtDNA (Table 1). S*fi*I is an 8-

Table 1. Codon usage for 3 rice mitochondrial genes from IR36 background.

Amino acid	Codon	*cob*		*cox3*		*atp9*	
		Count	% AA	Count	% AA	Count	% AA
Ala	GCA	7	25.0	9	42.9	1	6.7
	GCC	6	21.4	1	4.8	4	26.7
	GCG	3	10.7	1	4.8	2	13.3
	GCU	12	42.9	10	47.6	8	53.3
Arg	AGA	2	14.3	0	0.0	0	0.0
	AGG	2	14.3	1	14.3	0	0.0
	CGA	3	21.4	2	28.6	1	50.0
	CGC	2	14.3	1	14.3	0	0.0
	CGG	1	7.1	2	28.6	0	0.0
	CGU	4	28.6	1	14.3	1	50.0
Asn	AAC	3	25.0	0	0.0	1	50.0
	AAU	9	75.0	1	100.0	1	50.0
Asp	GAC	3	27.3	1	20.0	0	0.0
	GAU	8	72.7	4	80.0	2	100.0
Cys	UGC	1	25.0	0	0.0	1	100.0
	UGU	3	75.0	1	100.0	0	0.0
Gln	CAA	6	66.7	5	83.3	1	100.0
	CAG	3	33.3	1	16.7	0	0.0
Glu	GAA	5	62.5	7	77.8	2	100.0
	GAG	3	37.5	2	22.2	0	0.0
Gly	GGA	12	40.0	7	25.9	4	44.4
	GGC	3	10.0	4	14.8	1	11.1
	GGG	5	16.7	5	18.5	0	0.0
	GGU	10	33.3	11	40.7	4	44.4
His	CAC	4	22.2	2	14.3	1	50.0
	CAU	14	77.8	12	85.7	1	50.0
Ile	AUA	15	41.7	5	26.3	2	22.2
	AUC	3	8.3	3	15.8	1	11.1
	All	18	50.0	11	57.9	6	66.7
Leu	CUA	6	13.3	3	10.0	0	0.0
	CUC	7	15.6	6	20.0	2	18.2
	CUG	1	2.2	2	6.7	1	9.1
	CUU	12	26.7	7	23.3	2	18.2
	UUA	10	22.2	6	20.0	3	27.3
	UUG	9	20.0	6	20.0	3	27.3
Lys	AAA	5	83.3	3	50.0	3	100.0
	AAG	1	16.7	3	50.0	0	0.0
Met	AUG	10	100.0	6	100.0	3	100.0
Phe	UUC	11	34.4	6	33.3	2	28.6
	UUU	21	65.6	12	66.7	5	71.4
Pro	CCA	7	23.3	4	30.8	1	50.0
	CCC	4	13.3	2	5.4	0	0.0
	CCG	7	23.3	0	0.0	0	0.0
	CCU	12	40.0	7	53.8	1	50.0
Ser	AGC	6	20.7	0	0.0	0	0.0
	AGU	9	31.0	3	13.6	1	12.5
	UCA	3	10.3	3	13.6	3	37.5
	UCC	3	10.3	4	18.2	1	12.5
	UCG	0	0.0	3	13.6	1	12.5
	UCU	8	27.6	9	40.9	2	25.0
Thr	ACA	5	31.3	2	12.5	1	50.0
	ACC	4	25.0	7	43.8	1	50.0
	ACG	2	12.5	2	12.5	0	0.0
	ACU	5	31.3	5	31.3	0	0.0
Trp	UGG	12	100.0	11	100.0	0	0.0
Tyr	UAC	4	25.0	4	36.4	0	0.0
	UAU	12	75.0	7	63.6	1	100.0
Val	GUA	11	35.5	10	45.5	1	20.0
	GUC	2	6.5	2	9.1	2	40.0
	GUG	9	29.0	3	13.6	1	20.0
	GUU	9	29.0	7	31.8	1	20.0
Stop	UAA	0	0.0	0	0.0	0	0.0
	UAG	1	100.0	0	0.0	1	100.0
	UGA	0	0.0	1	100.0	0	0.0
Total		398		266		88	
Molecular weight (kDa)			44.488		29.324		8.945

Table 2. Nucleotide content of 3 IR36 rice mitochondrial genes: *Cob, Cox*3, and *atp*9.

Nucleotide	*cob*		*cox*3		*atp*9	
	Number	% of total	Number	% of total	Number	%of total
A	277	23.2	171	21.4	57	21.6
C	247	20.7	169	21.2	55	20.8
G	252	21.1	182	22.8	58	22.0
T	418	35.0	276	34.6	94	35.6
Total	1194	100.0	798	100.0	264	100.0



4. Pulsed field gel electrophoresis of rice mtDNA. The gel was run with a pulse time ramped from 2 to 10 s, for 16 h at 5 Y/cm. Panel A shows the ethidium bromide staining pattern. The top of the gel (origin) is indicated by an arrow. Sizes (in kb) are indicated to the left of the gel. Lane 1- lambda DNA (50 kb) and concatemers (100 kb, 150 kb, etc.); lane 2- other DNA molecular weight DNA markers (BRL); lane 3- IR36 mtDNA digested with *Sfi* I. Panel B shows a blot of the same gel hybridized to a *cox*1 gene probe; only the rice mtDNA hybridizes, and the hybridization is primarily to an approximately 50-kb fragment.

base recognition enzyme and hence is expected to have a site approximately every 64 kb. The rice genome, estimated to be about 500 kb, would be expected to have 5–10 *Sfi*I fragments. The genes on individual (or multiple) *Sfi*I fragments can be determined by hybridization.

In our preliminary results, we find multiple *Sfi*I fragments for both indica and japonica rice lines. The two japonica lines tested, Calmochi-101 and M-202, are similar to each other but differ radically from the indica line tested (IR36). By far the most unique pattern was demonstrated by a tissue culture line, Yamabiko, which showed fewer *Sfi*I bands and greater stoichiometric differences between bands. Yamabiko did, however, share *Sfi*I bands with the other japonica lines. Conventional gels, in which fragments from digestion with 6-bp recognition enzymes are examined, yield a far more complex pattern with 40–80 bands, and comparison of cytoplasmic types is much more tedious. We plan to compare a group of indica and japonica cytoplasms, including CMS types, using this technology to catalog RFLP with *Sfi*I. In addition, we will place the known mitochondrial genes on the *Sfi*I fragments to make the identification scheme more precise. An example of gene localization by DNA hybridization is shown in Figure 4B. Eventually this information can be combined with complete physical maps to elucidate the types of rearrangements that have occurred in the various rice cytoplasms to alter the distribution of *Sfi*I sites.

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Notes

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Organization of mitochondrial DNA in male sterile and fertile lines of rice

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Mitochondrial DNAs (mtDNAs) from fertile and cytoplasmic male sterile lines of rice were analyzed using restriction endonucleases and hybridization with cloned mitochondrial gene probes. Sterile lines and nuclear restored lines of indica rice carrying wild abortive (WA) cytoplasm were used. The rice mitochondrial genome has a complex organization that is reflected in the complexity of its restriction profile. The presence of plasmid-like molecules that can replicate independently of the main mitochondrial genome adds to the diversity of organization of rice mtDNA. In tissue-cultured cells of fertile japonica rice (Taipei 309), the organization of the mitochondrial genome can be considerably altered, probably as a result of recombination. Changes in the organization of mtDNA may also underlie the differences between fertile and WA cytoplasm.

The inability of cytoplasmic male sterile (CMS) plants to produce functional pollen is a maternally inherited trait encoded in the mitochondrion (Levings and Brown 1989). Many histological studies (Laser and Lersten 1972) have demonstrated, with few exceptions (Chu et al 1972, Shinjyo 1975), that in CMS plants, pollen abortion results from tapetal hypertrophy, which probably prevents a sufficient supply of nutrient from being delivered to the actively developing pollen (Nakashima and Hosokawa 1971).

Investigations have also been carried out to decipher the molecular events taking place in the plant in relation to the CMS phenotype. In maize in particular, analyses of mitochondria at the DNA, transcriptional, and translational level have helped to elucidate the genetic lesions responsible for different types of CMS. For example, Dewey et al (1987) demonstrated that in CMS-T maize, the mitochondrial gene *URF13-T,* which is abundantly transcribed, encodes a 13-kDa polypeptide found in the inner mitochondrial membrane. When CMS-T cytoplasm was transferred into a nuclear background possessing fertility restorer (*Rf1* and *Rf2*) genes, the abundance of this polypeptide was reduced (Forde and Leaver 1980). However, only one of the restorer genes (*Rf1*) was later shown by Dewey et al (1987) to be responsible for reducing the 13-kDa polypeptide in CMS-T maize. The presence of the *URF13-T* gene is actually linked to the susceptibility of CMS-T maize to the T-toxin of the fungal

pathogen *Helminthosporium maydis*, race T (Levings and Dewey 1988). The presence of the *URF13-T* gene coding for the 13-kDa polypeptide inhibited respiration (Matthews et al 1979) and uncoupled oxidative phosphorylation (Bednarski et al 1977) of CMS-T maize. Thus, the presence of gene *URF13-T* in relation to T-toxin sensitivity and CMS phenotype in maize has been established. The molecular basis of the different types of cytoplasmic male sterility in rice is not yet understood to the same degree.

The rice mitochondrial genome consists of heterogeneous populations of linear and circular molecules, with the majority distributed between 60 and 105 kb (Wang et al 1989). More than 95% of these molecules exist as linear forms in vitro (Wang et al 1989). However, as observed in other plants such as *Brassica* spp. (Palmer and Shields 1984), higher proportions of circular molecules may exist in vivo. Fragmentation of the larger circular molecules during mitochondrial DNA (mtDNA) isolation could account for the high proportion of linear molecules seen in vivo.

In addition to the main high-molecular-weight genome, the rice mitochondrion often contains several smaller plasmid-like molecules. This paper discusses the possible role of the main mitochondrial genome and mtDNA plasmids in WA(S_2) and other types of cytoplasmic male sterility in rice.

The presence of "recombination repeats" in the mitochondrial genome of higher plants has been well-described (Lonsdale 1984, Palmer and Shields 1984, Stern and Palmer 1984). Repeat sequences permit the reorganization of mtDNA through recombination in vivo. In *Petunia* CMS and maize CMS-T, mtDNA recombination can generate novel reading frames coding for proteins that may cause the CMS phenotype (Dewey et al 1987, Nivison and Hanson 1989). In this paper, the possibility of such recombination events mediating wild abortive (WA) cytoplasmic male sterility in rice is examined. In addition, studies on the stability of rice mtDNA in tissue culture are described.

Isolation of rice (*Oryza sativa* L.) mitochondrial DNA

Rice seedlings were grown in continuous light (2,000 lux, Thorn 36W, Pluslux 3,500) at 28+2 °C for 14–21 d. They were maintained under sterile conditions on hormone-free agar-solidified (0.8% wt/vol, Sigma) Murashige and Skoog's (1962) medium.

Cell suspensions were initiated from leaf base and mature seed scutellum according to the methods described by Abdullah et al (1986) and Finch et al (1991), respectively.

The method used for extraction of rice mtDNA was as described by Saleh et al (1989). The key steps are

1. low-speed centrifugation of rice homogenate to eliminate nuclei, chloroplasts, and other cell debris
2. high-speed centrifugation to pellet mitochondria
3. additional low-speed centrifugation to remove remaining nuclei, chloroplasts, and debris
4. DNAse I treatment to remove attached nuclear DNA contaminants from the mitochondria

5. purification of mitochondria using a sucrose gradient
6. extraction and precipitation of mtDNA from lysed mitochondria

The mtDNA extracted was digested with restriction endonucleases, electrophoresed on agarose gels, blotted onto nylon filters, and hybridized with cloned mitochondrial gene probes.

Plasmid-like molecules associated with rice mitochondria

The presence of plasmid-like mtDNA molecules has been observed in many species such as *Zea mays* (Kemble and Bedbrook 1980), sorghum (Chase and Pring 1985, Pring et al 1982), wheat (Handa et al 1984), *Brassica* (Palmer et al 1983), sugar beet (Powling 1981), *Vicia faba* (Boutry and Briquet 1982, Negruk et al 1982), and rice (Kadowaki et al 1986, Mignouna et al 1987, Saleh et al 1989, Shikanai et al 1987, Yamaguchi and Kakiuchi 1983). These plasmids exist in linear or circular forms and can apparently replicate independently of the main mitochondrial genome. Mignouna et al (1987) reported the presence of closed circular forms of plasmid-like molecules in BT cytoplasm (S_1 CMS) of rice. Such molecules are present in both CMS and fertile rice, including nuclear restored lines (F_1 hybrid of WA CMS rice) (data not shown). Because the plasmids are a common feature of rice cytoplasms, there is no simple correlation between their presence or absence and the expression of the CMS phenotype. This is particularly the case in WA cytoplasmic male sterility, where one CMS variety V41A and its maintainer V41B, which possesses a fertile cytoplasm probably unrelated to that of V41A, both contain the same set of mtDNA plasmids (Saleh et al 1989). The lack of some of these plasmids in certain fertile maintainer lines relative to the corresponding CMS lines may be due merely to the differences in their cytoplasmic backgrounds. However, further investigations regarding the function of plasmid-like molecules in rice are necessary.

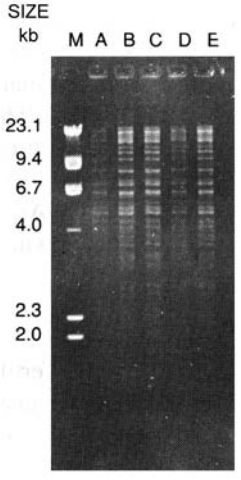
In maize CMS-S, the rearrangement of mtDNA due to excision and insertion of S1 and S2 linear molecules is associated with cytoplasmic male sterility (Small et al 1988). No such event has yet been described in rice. In fact, the plasmid-like molecules (B1, B2, B3, B4) observed in BT cytoplasm do not show any homology with the main mitochondrial genome (Shikanai and Yamada 1989; Shikanai et al 1987, 1989). The molecules also lack extensive sequence homology (Shikanai et al 1989). This eliminates the possibility of homologous recombination. However, these plasmid-like molecules have sequences in common with the 1.9- or 1.4-kb plasmid-like DNAs of maize (Shikanai et al 1989). The significance of this observation is still unknown.

Restriction patterns of rice mitochondrial DNA

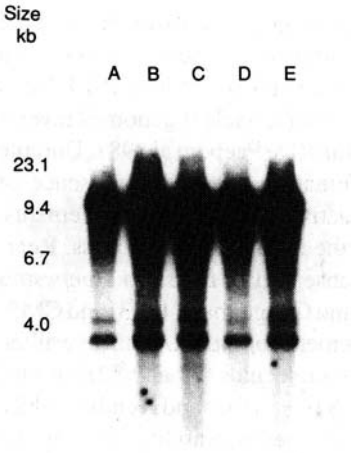
Restriction fragment analysis of mtDNA from V41A (CMS) and V41B (fertile maintainer) lines using *Bam*HI reveals minor differences in the higher molecular-weight main mitochondrial genome (Saleh et al 1989). These differences are not unexpected, since the cytoplasmic backgrounds of these lines differ. V41A carries the

cytoplasm of a naturally occurring wild rice (*Oryza sativa* f. *spontanea*), which can cause spontaneous pollen abortion. However, the fact that there were only minor differences in the restriction profiles of the two cytoplasms suggests that they may be related. The mitochondrial genome of higher plants generally exists as a dynamic system in which the presence of repeated DNA sequences permits the reorganization of the mitochondrial genome through recombination processes (Lonsdale 1984). In maize CMS-T, alterations of mtDNA are associated with reversion to fertility (Rottmann et al 1987). However, whether such events underlie the differences observed in sterile V41A and fertile V41B cytoplasm is not yet known.

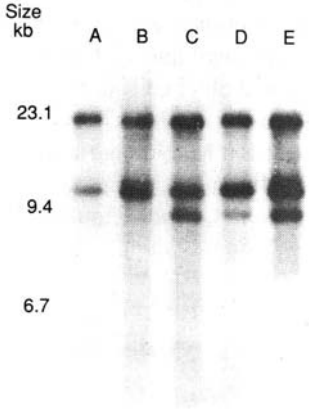
*Bam*HI restriction analysis of total mtDNA from WA CMS lines (V41A, IR54752A) and nuclear restored lines, also carrying the WA cytoplasm (IR54752A/IR54R, IR54752A/IR46R, IR54752A/IR21916-128-2-2-2-3R) showed no obvious differences (Fig. 1). Hybridization of the restricted mtDNAs with a maize *cox*I probe (Isaac et al 1985) showed identical banding patterns (Fig. 2). Differences in the relative intensity of the ≈ 4.9-kb fragment of V41A (WA CMS) and that of the nuclear restored lines (IR54752A/IR46R) are due mainly to inconsistencies in the amount of mtDNA loaded in each lane. In contrast, using the maize *cox*II probe (Fox and Leaver 1981) showed clearer differences in the hybridization pattern of WA CMS lines and nuclear restored lines (Fig. 3). Hybridization of the *cox*II gene to nuclear restored lines showed three prominent hybridization signals, whereas only two prominent signals were present in WA CMS lines (V41A and IR54752A). The smallest of the three signals (≈7.7-kb) was missing in both lines. These differences may be correlated with the presence of nuclear fertility restorer genes in the nuclear restored rice lines. Further investigations of the possible reorganization of *cox*II sequences in WA vs fertile cytoplasm using the polymerase chain reaction are in progress.



1. Electrophoretic pattern of *Bam*HI digests of total mtDNA from WA CMS lines V41 A (lane A), IR54752 A (lane B), and nuclear restored lines IR54752 A/IR21916-128-2-2-2-3 R (lane C), IR54752 A/IR46 R (lane D), and IR54752 A/IR54 R (lane E). Molecular size markers (M) are given by a *Hin*dIII digest of phage lambda DNA.



2. Hybridization of the maize cytochrome oxidase subunit I (*cox*I) gene probe to a Southern blot of *Bam*HI-digested mtDNA of WA CMS lines and nuclear restored lines. Lane designations are the same as in Figure 1. Differences in the hybridization signal intensity are due to inconsistencies in amount of mtDNA loaded in each lane.



3. Hybridization of the maize cytochrome oxidase subunit II (*cox*II) gene probe to the Southern blot of *Bam*HI-digested mtDNA of WA CMS lines and nuclear restored lines. Lane designations are the same as in Figure 1.

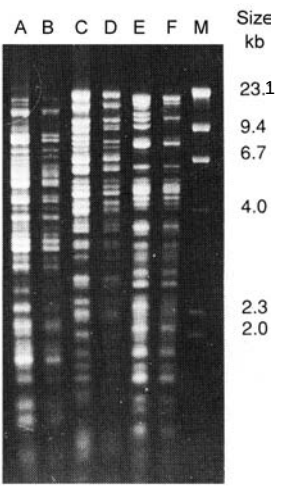
Stability of rice mitochondrial DNA in tissue culture

It seems easier to isolate mtDNA from tissue culture cells of rice than from whole plants or leaves. Gentler homogenization procedures are generally required for callus or suspension culture, with higher yields of "cleaner mtDNA" being obtained from these sources than from leaves. However, assessment of possible effects of tissue culture on the organization of the rice mitochondrial genome is necessary, since tissue culture-induced changes in mtDNA sequence organization have been well documented in other plant species.

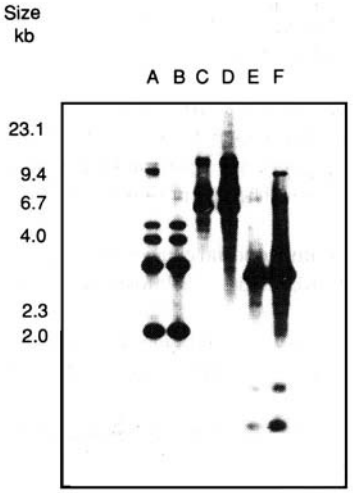
Generally, in vitro cultures generate somaclonal (somatic cell) or protoclonal (protoplast culture) variations. In rice, phenotypic variants have been observed as a result of the tissue culture process (Henke et al 1978, Nakano and Maeda 1979, Nishi et al 1968, Sun et al 1983). Molecular investigation of plant nuclear genomes reveals quantitative and qualitative changes during tissue culture (De Paepe et al l981, Durante et al 1983). These changes perhaps reflect recombination due to the presence of repetitive DNA sequences in the plant genome or the activity of transposable elements.

The organization of mtDNA is also affected by the tissue culture process. Rearrangement and amplification of mtDNA has been observed in *Brassica campestris* (Shirzadegan et al 1989). In maize CMS-T (Umbeck and Gengenbach 1983) and CMS-S (Small et al 1988), tissue-culture-induced rearrangements of mtDNAs have resulted in reversion to fertility. Transposition of plasmid-like molecules (S1 and S2) has also been observed in tissue-cultured cells of maize CMS-S (Chourney and Kemble 1982). In addition, the embryogenicity of callus culture and regeneration ability of hexaploid wheat from somatic tissue have been correlated with the organization of the mitochondrial genome (Hartman et al 1989, Rode et al 1988).

In rice, Chowdhury et al (1988) reported the instability of mtDNA in long-term (7-yr-old) rice suspension culture. The stability of other rice (Taipei 309) mitochondrial genomes was also investigated (Saleh et al 1990). Restriction analysis of Taipei 309 mtDNAs isolated from leaves, young suspension cultures (cell line T309MS2/A, 2 mo old), a totipotent cell suspension (T3MS, 8 mo old), and older cell suspensions that had lost their protoplast regeneration ability (LB3, 19 mo old; and LB 1,30 mo old) showed identical restriction patterns, with the exception of LB1. Mitochondrial DNA of LB1 showed stoichiometric differences in the restriction fragments compared with mtDNA from LB3, revealed by restriction endonucleases *Bam*HI, *Hind*III, and *Eco*RI



4. Electrophoretic patterns of total mtDNA of Taipei 309 leaf base cell suspensions (LB1 and LR3) that have lost their protoplast regeneration capabilities. Lanes A, C, and E show LB3 digested with *Hind*III, *Bam*HI, and *Eco*RI, respectively . Lanes B, D, and F show LB1 digested with *Hind*III, *Bam*HI, and *Eco*RI. respectively. Molecular markers (M) are given by a *Hind*III digest of phage lambda DNA.



5. Hybridization of the maize cytochrome oxidase subunit II(*cox*II) gene probe to a Southern blot of *Hin*dIII , *Bam*HI, and *Eco*RI-digested mtDNAs of Taipei 309 (as in Figure 4). Lanes A, C, and E show hybridization to LB3 mtDNA digested with *Hin*dIII, *Bam*HI, and *Eco*RI. respectively. Lanes B, D, and F show hybridization to LB1 mtDNA digested with *Hin*dIII, *Bam*HI, and *Eco*RI, respectively.

(Fig. 4). Hybridization with a cloned maize *cox*II gene probe (Fox and Leaver 1981) showed identical sets of prominent hybridization signals in each digest of LB1 and LB3 mtDNAs (Fig. 5). Several minor bands, however, showed differences in relative intensity. Overall, these data suggest that the Taipei 309 mitochondrial genome shows some instability in long-term tissue-cultured cells.

The lability of rice mtDNAs in tissue culture is possibly due to recombination between repeated sequences present in the mitochondrial genome, similar to those reported in other higher plants (Lonsdale 1984, Palmer and Shields 1984, Stern and Palmer 1984). Whether mtDNA recombination has any phenotypic consequences in rice plants regenerated from tissue culture remains to be determined.

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Genetic analysis of nuclear DNAs homologous to small mitochondrial plasmid-like DNAs in cultivated rice

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B1 and B2 are small, circular mitochondrial plasmid-like DNAs first found in CMS-Bo cytoplasm (derived from Chinsurah Boro II rice). We examined the distribution of these plasmids in cultivated rice mitochondria by Southern blot analysis using B1 and B2 clones as probes. The results suggest that the presence or absence of B1 and B2 in rice mitochondria is polymorphic and correlated with rice varietal group. These plasmid-like DNAs characteristically share no homology with the mitochondrial main genome, but rather with the nuclear genome. To examine whether nuclear sequences homologous to these plasmids are also polymorphic, total DNA was isolated from five rice cultivars and analyzed. Nuclear B1- and B2-homologous sequences were detected in all cultivars examined, regardless of the presence or absence of plasmids in the mitochondria. These sequences are likely to be common in the nuclear genome of rice. Southern blot analysis showed restriction fragment length polymorphism (RFLP) among them, and suggested that nuclear B1- and B2-homologous sequences are also polymorphic in rice cultivars. To further examine these nuclear homologues genetically, we carried out segregation analysis of RFLP in the F_2 progeny of an indica/japonica hybrid.

Small linear and circular DNAs called plasmid-like DNAs have been observed in the mitochondria of many higher plants by agarose gel electrophoresis and electron microscopy (for reviews, see Newton 1988, Pring and Lonsdale 1985). Although various male sterile cytoplasms contain characteristic arrays of plasmid-like DNAs, it is unclear whether they are associated with cytoplasmic male sterility.

Small, circular plasmid-like DNAs were observed in the mitochondria of cytoplasmic male sterile rice by Mignouna et al (1987), Shikanai et al (1987), and Yamaguchi and Kakiuchi (1983). At present four plasmid-like DNAs—B1, B2, B3, and B4—have been identified and characterized in rice (Shikanai et al 1987, 1989; Shikanai and Yamada 1988). They share homology with the nuclear genome but not with the mitochondrial main genome (Sakamoto et al 1990, Shikanai et al 1987). They contain a sequentially conserved region in common with maize 1.9-kb and 1.4-kb plasmids that have similar characteristics (Shikanai et al 1989), suggesting that they have a common

origin. Kadowaki et al (1988) examined the distribution of plasmid-like DNAs in cultivated rice and suggested that their presence or absence is polymorphic and related to rice varietal group.

Analysis of the nuclear homologous sequence of these plasmid-like DNAs can shed light on their origin and function. However, little is known about their homologous region in the nuclear genome. We examined the presence of sequences homologous to B1 and B2, as well as the distribution of the plasmids themselves in rice cultivars, and detected restriction fragment length polymorphism (RFLP) among them. Segregation analysis of the F_2 population of an indica/japonica hybrid was carried out. The origin of the plasmid-like DNAs is discussed.

Materials and methods

After purification of mitochondrial DNA (mtDNA) from various rice strains, homology was examined by Southern hybridization.

Plant materials

Rice seeds of Ginbozu (japonica), Nipponbare (japonica), IR24 (indica), Jamuna (indica), and OK21 (*Oryza glaberrima*) were germinated and grown at 28°C for 2 wk. Green seedlings were used for isolating total and chloroplast DNAs, and etiolated seedlings were used for isolating mtDNA.

For segregation analysis, we used F_2 leaves of the hybrid generated by the cross of Kasalath (indica) with F1134 (mutant japonica).

Isolation of DNA

Total DNA was isolated from green leaves according to the method of Murray and Thompson (1980) with some modifications. Approximately 10 g of seedlings was blended in a kitchen blender with liquid N_2. Further blending was done by a Polytron for 5 min with liquid N_2; then the rice powder was removed to a new tube. Ten milliliters of recently boiled 2x E buffer (2% wt/vol cetyltrimethylammonium bromide [CTAB], 0.1 M Tris-HCl [pH 8.0], 20 mM ethylene diamine tetraacetic acid [EDTA], 1.4 M NaCl) was added, and the tube was incubated at 55°C for 15 min with shaking. An equal volume of chloroform-isoamylalcohol mixture (24:1 vol/vol) was added. After incubation at room temperature for 30 min with gentle shaking, the suspension was centrifuged at 2800 rpm for 15 min. Extraction by chloroform-isoamylalcohol was repeated, and one-tenth volume of 10% CTAB (wt/vol) plus 0.7 M NaCl was added to the final aqueous phase. An equal volume of precipitation buffer (1% wt/vol CTAB, 50 mM Tris-HCl [pH 8.0], 10 mM EDTA) was added and mixed gently. After 30 min of incubation at room temperature, DNA was recovered by centrifugation at 2800 rpm for 15 min. The precipitate was dissolved in 3 ml of TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) containing 1 M NaCl, and an equal volume of isopropanol was added and mixed. After 30 min of incubation at room temperature, DNA was recovered by centrifugation and dissolved in TE buffer. RNase A (Sigma) was added to a final

concentration of 20 µg/ml, and the solution was incubated at 55°C for 1 h. Then the DNA solution was stored at 4°C for use in this study.

Mitochondrial DNA and chloroplast DNA (ctDNA) were isolated from seedlings as described by Kadowaki et al (1986).

Southern blot analysis

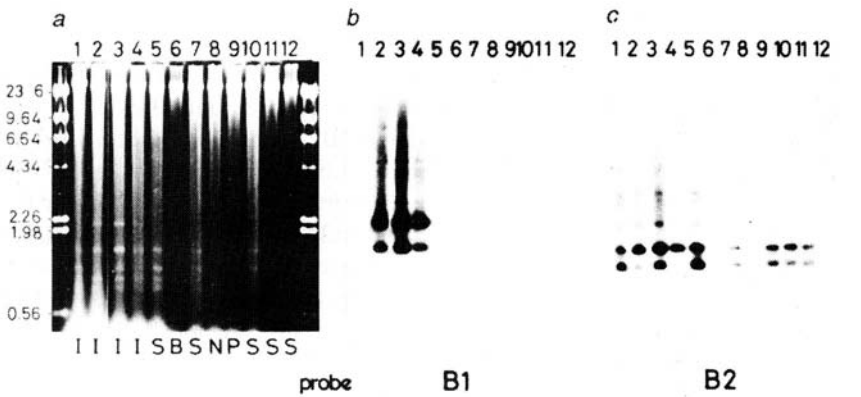
Electrophoresis and blotting of DNA onto nitrocellulose paper were carried out according to Maniatis et al (1982). Probes were prepared from plasmid-like DNAs of B1 (2.1 kb) and B2 (1.5 kb) DNA, which were previously cloned from male sterile cytoplasm of Chinsurah Boro rice (CMS-Bo) (Sakamoto et al 1989). The B1 and B2 clones were labeled using [α-^{32}P] dCTP (Amersham, >3000 Ci/mol) by the multiprime DNA labeling system (Amersham). Other protocols were the same as described by Sakamoto et al (1990).

Results and discussion

Not only was plasmid-like DNA found in rice mitochondria, but sequences in the nuclear genome homologous to these plasmids turned out to be polymorphic by RFLP analysis.

Distribution of mitochondrial plasmid-like DNAs B1 and B2

We previously reported the cloning of small, circular plasmid-like DNAs B1 and B2 from CMS-Bo cytoplasm (Sakamoto et al 1989). In this study, we used these clones as probes. We first examined the distribution of mitochondrial plasmid-like DNAs B1 and B2 in rice cultivars by Southern blot analysis. Mitochondrial DNAs were isolated from



1. Example of Southern blot analysis of mtDNAs with B1 and B2: mtDNAs from rice cultivars (lanes 1–12) were electrophoresed in 0.7% agarose gel (panel *a*) and probed with either B1 (*b*) or B2 (*c*). Molecular weights are given in kb. Derivations of rice cultivars are indicated under panel *a:* I = India, S = South China, B = Brazil, N = North China, P =Philippines.

Table 1. Presence of mitochondrial plasmid-like DNAs B1 and B2 and their relationship with varietal groups.

Varietal group[a]	Cultivars (no.) that have				
	B1	Both B1 and B2	B2	Neither B1 nor B2	Total
A	0	0	1	17	18
B	0	0	0	16	16
C	1	9	25	4	39
D	0	1	13	3	17

[a]Varietal groups were categorized by isozyme analysis, distribution of gametophyte genes, degree of hybrid sterility, morphological characteristics, etc. by Nakagahra (1978, 1986). A = javanica rice collected from hilly areas of Southeast Asia and tropical islands; B =japonica rice collected from Japan and North China; C = indica rice collected from India, Sri Lanka, and Bangladesh; D = sinica (Hsien) rice collected from South China and Vietnam.

90 rice cultivars and hybridized with B1 and B2. B1 and B2 were hybridized with multimeric bands because of their circular forms (Fig. 1). In some lanes B1 did not hybridize in spite of the existence of the B1 band (1.6 kb) by ethidium bromide-stained gel (e.g., lane 5). Perhaps the mobility of closed circular B1 is essentially the same as that of open circular B2, and open circular B2 was taken for B1.

Since Kadowaki et al (1988) reported that the distribution of plasmid-like DNA was associated with rice varietal groups, we investigated the relationship between the presence or absence of B1 and B2, and rice varietal group (Table 1). While both B1 and B2 were absent from japonica and javanica cultivars with one exception, they were present in indica cultivars; and B2, not B1, was present in sinica cultivars. These results are consistent with those of Kadowaki et al (1988) and suggest that the distribution of B1 and B2 is related to rice varietal group.

RFLP of homologous sequence to B1 and B2 in nuclear genome

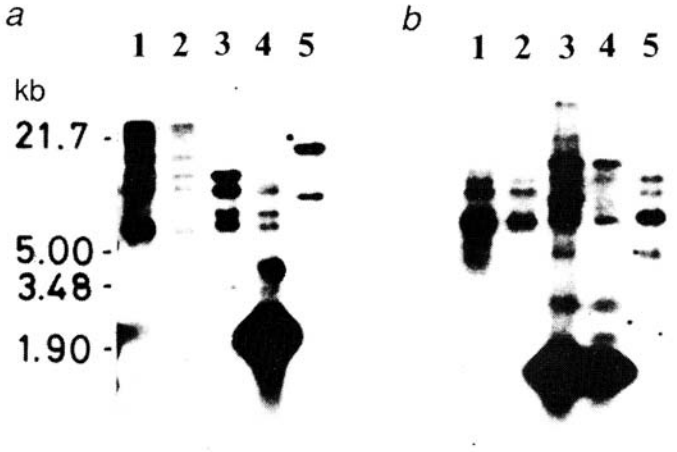
Since the presence or absence of mitochondrial plasmids was shown to be polymorphic, we considered that the homologous nuclear sequence would also be polymorphic in rice cultivars. To examine the extent of B1- and B2-homologous sequences and their RFLP in the nuclear genome, we used five rice cultivars (Table 2). First, mtDNAs were isolated from the five cultivars, and the presence or absence of plasmid B1 and B2 was analyzed by Southern hybridization. Indicas IR24 and Jamuna had the plasmids, whereas japonicas Ginbozu and Nipponbare, and *O. glaberrima* (OK21) did not. Second, ctDNAs were isolated from these cultivars and hybridized with B1 and B2. No homology between ctDNA and plasmids was detected (data not shown).

Total DNAs were isolated from the cultivars, digested with restriction enzymes, and hybridized with B1 and B2 (Fig. 2). Signals were detected in all varieties. Strongly hybridizing signals in Jamuna probed with B1 and B2, and in IR24 probed with B2 were considered due to contamination of the mitochondria with plasmid-like DNAs. Since we used a restriction enzyme that cut B1 and B2 at more than one site, and the plasmids were linearized, the possibility was excluded that the plasmids produced multimeric

Table 2. Presence of plasmid-like DNA in mitochondria and its homology with nuclear genome in rice cultivars.[a]

Cultivar	Varietal group	Mitochondrial plasmid-like DNA		Homology with nuclear genome	
		B1	B2	B1	B2
Ginbozu	Japonica	0	0	+	+
Nipponbare	Japonica	0	0	+	+
IR24	Indica	0	+	+	+
Jamuna	India	+	+	+	+
OK21	*Oryza glaberrima*	0	0	+	+

[a] + = present, 0 = absent.



2. Southern blot analysis of total DNAs from Ginbozu (lane 1), Nipponbare (2), IR24 (3), Jamuna (4), and OK21 (5) probed with either B1 (panel *a*) or B2 (*b*). Total DNAs were digested with *Stu*I.

bands with various conformations. Therefore, these homologous bands, except for those of the plasmids, may be in the nuclear genome. All varieties had sequences in the nuclear genome homologous to both B1 and B2, regardless of the presence or absence of plasmid-like DNAs in the mitochondria.

As shown in Figure 2, nuclear sequences homologous to B1 and B2 appeared to show RFLP. The degree of polymorphism was estimated. While RFLP could not be detected between japonicas Ginbozu and Nipponbare with B1 and B2 as probes, there was slight polymorphism between indicas IR24 and Jamuna. The polymorphic degree of B2-homologous sequences among subspecies was nearly the same. On the other hand, the polymorphic degree of B1-homologous sequences was different, and

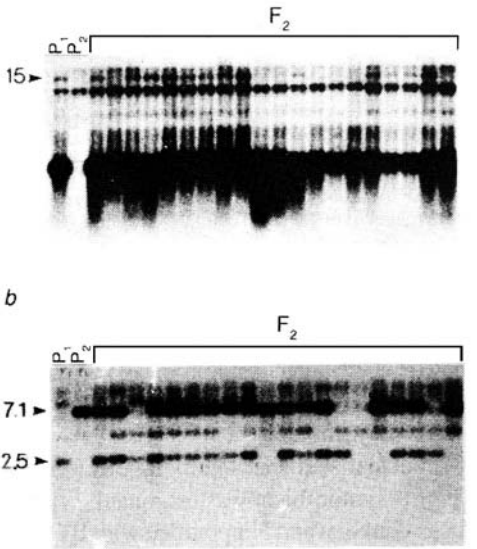
remarkable polymorphisms were observed between the indicas and *O. glaberrima,* and between the japonicas and *O. glaberrima.* These results suggest that not only the presence or absence of plasmid-like DNAs B1 and B2 in the mitochondria, but also nuclear homologous sequences to B1 and B2 are divergent and correlate with rice subspecies.

F_2 segregation analysis of nuclear sequences homologous to B1 and B2

Since nuclear homologues to B1 and B2 showed RFLP among rice subspecies, genetic analysis was subsequently carried out to characterize the homologues in the F_2 population of an indica/japonica hybrid. We used 143 F_2 progenies derived from the cross of Kasalath (indica) with F1134 (japonica).

Figure 3 shows examples of Southern blot analysis. Strong hybridizing signals of 1.5 kb were detected in all F_2 individuals when B1 was probed. This band also appeared in the P_1 (Kasalath), but not in the P_2 (F1134), so was obviously maternally inherited. Therefore this band was considered to be a plasmid-like DNA sequence present in mitochondria.

When B2 was probed, a 15-kb fragment was present in the P_1 but not the P_2. When B1 was probed, 2 fragments of 2.5 kb and 7.1 kb were detected, characteristic of the P_1 and P_2, respectively. The 2.5-kb fragment in the P_1 and 7.1-kb fragment in the P_2 were possibly allelic. However, the F_2 population contained individuals that lost both fragments, suggesting that these two fragments are independent.



3. Examples of RFLP analysis of sequences homologous to B1 and B2 in the F_2 population. *Eco*RV-digested total DNAs from maternal parent Kasalath (P_1), paternal parent F1134 (P_2). and F_2 individuals were probed with either B2 (panel *a*) or B1 (*b*).

Table 3. Segregation analysis of fragments homologous to B1 and B2 in F_2 population.

Fragment	Size (kb)	P1[a]	P2[b]	F_2 population		χ^2 (3:1)[c]
				Present	Absent	
B2	15	Present	Absent	103	40	0.0
B1	7.1	Absent	Present	129	154	16.3**
	2.5	Present	Absent	108	35	0.0

[a]Maternal parent Kasalath (indica). [b]Paternal parent F1134 (japonica).[c]**= significant at the 1% level.

The presence-to-absence ratios of the three fragments were estimated in the F_2 population (Table 3). The observed ratios of 2 fragments, the B2-homologous 15-kb fragment and the B1-homologous 2.5-kb fragment, were consistent with a 3:1 ratio and thus showed normal Mendelian inheritance. However, the segregation of the B1-homologous 7.1-kb fragment deviated from 3:1. The deviation might be explained by the fact that a gametophyte gene (Nakagahra 1972), which affected pollen fertility, was linked to the B1-homologous 7.1-kb fragment, because RFLP probes linked to it also showed deviated segregation (unpubl. data).

All three fragments were null alleles, i.e., present only in either the indica or japonica parent. Two B1-homologous sequences were revealed to be independent alleles by segregation analysis, and probably occupy different map positions in indicas and japonicas.

The origin of the sequences of these plasmid-like DNAs is unknown. If nuclear sequences homologous to the plasmids were derived from mitochondrial plasmids, our results might be explained by the fact that transmission event of the plasmids into the nuclear genome had occurred independently in indicas and japonicas. Alternatively, sequence transposition could have occurred during the course of rice differentiation into subspecies. Further mapping of these sequences is currently being done.

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Notes

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Discussion

Session 7: Molecular genetics of cytoplasmic genomes

Q—*André:* Do you know the sizes of the four subgenomic circles of rice mitochondrial DNA?

A—*Hirai:* No, but we can estimate them.

Q—*Chaudhuri:* You mentioned in your presentation the integration of chloroplast DNA (ctDNA) into mitochondrial DNA (mtDNA). Can you identify the loci where such recombination can take place?

A—*Hirai:* We have located the place only by Southern hybridization.

Q—*André:* Have you estimated the relative frequencies of the different circular mtDNAs by probing Southern blots of total mtDNA (restricted with different enzymes) with probes that cover the divergence points on your restriction map?

A—*Hirai:* Not yet. We will check this soon.

Q—*Wang:* How can you exclude the possibility that your mtDNA preparation was contaminated by ctDNA and other plasmid DNA? Why do you think the mtDNA molecular weight differs from that of different reporters?

A—*Hirai:* We know the complete sequence and physical map of ctDNA. So we would know if we had plasmid DNA contamination. We estimate the master circle to be 528 kbp. No one has reported the master circle of rice mtDNA.

Q—*Brar:* Earlier reports indicate that DNA is promiscuous, and DNA sequences between the chloroplast and mitochondrial genomes are quite similar and possibly interchangeable. What is your observation based on the map of the rice chloroplast genome?

A—*Hirai:* We found a small fragment of a ctDNA sequence in mtDNA by Southern hybridization, but it is not long enough to detect by physical mapping.

Q—*Ishii:* About cob-1 and cob-2–did you try Southern hybridization using the DNA probe from a divergent region (= probe C), and did you find any repeated sequences near the divergent region between *cob-1* and *cob-2*?

A—*André:* When we probed a Southern blot with either probe B (*cob-1*- specific) or probe C (*cob-2*- specific), we saw only one of the two bands seen with probe A. We found no large repeated sequence near the divergent region, but we cannot rule out the possibility of a small region of homology.

C—*Shimamoto:* Southern blots using contour-clamped homogeneous electric field (CHEF) gel seem to suggest the presence of a subgenomic molecule (18 S, 26 S rDNA hybridization).

C—*André:* I agree that several probes hybridizing to more than one band suggests several subgenomic circles. Also, the fact that the bands are of different intensities suggests that the circles exist in different stoichiometries.

Q—*Ranjekar:* Are there any variations in the size of mtDNA between indica and japonica rice and within indica and japonica varieties?

A—*André:* Our results are preliminary, and we have not examined many varieties. So far, we have seen a few differences between indicas and japonicas, but no differences between Calmochi 101 and M202, the two japonica varieties we examined. Also, we saw differences in the japonica suspension line (Yamabiko) and the other japonica varieties.

Q—*Gupta:* How old was the suspension culture you used for Yamabiko?

A—*André:* The cell line was obtained from Dr. Datta, who at the time was in Dr. Lucas' laboratory at the University of California at Davis. I believe the line was about 1-1/2 yr old, but I would have to check.

Q—*Sakamoto:* What were the conditions of the CHEF gel electrophoresis?

A—*André:* We used Bio-rad's CHEF DRII system. The gel was 1.4% and the pulse time was from 0.2 to 1.5 s over a run time of 24 h at 180 V.

Q—*Schaeffer:* Are there recombination hot spots for mtDNA rearrangement or subcycle formation? What is the evidence for mtDNA changes associated with plant regenerability?

A—*André:* We suspect that there are hot spots for recombination, but we have not yet defined any. The evidence for mtDNA changes associated with regenerability comes from studies in wheat and maize, where mtDNA changes were closely associated with loss of ability to regenerate from callus. This work was done in other laboratories.

Q—*André:* In maize and some other plant species, there is a region of homology between the *cox-2* upstream region and the ATP-6 coding region. Do you think a recombination between these two sequences could be involved in your cytoplasmic male sterile (CMS) lines, especially given that they seem to be the only genes that showed altered patterns on Southern blots?

A—*Saleh:* I am not aware of such recombination between ATP-6 and the *cox-2* upstream region in rice. But the reason for the slide showing hybridization of mtDNA with ATP-6 is to show the differences between the fertile maintainer and the corresponding lines. I have also used other mitochondrial probes to look at their difference (such as ATP a , 26 S, *cox-1,* and 18 S, 5 S), and all of them showed different hybridization patterns.

Q—*Shimamoto:* Three bands show up in CMS/Rf. What happens with the enzymes?

A—*Saleh:* I have also used other restriction enzymes, but there were differences in the restriction pattern. However, no hybridization using mitochondrial gene probes was used to detect differences in hybridization signals.

Q—*Zhang Qifa:* Why did you use *cox-l* and *cox-2* as probes? Have you ever considered using other genes as probes?

A—*Saleh:* In addition to *cox-1* and *cox-2,* I have used 18 S, 5 S RNA, but detected no differences in hybridization signals. Here I used two cytochrome oxidase mitochondrial probes to show the similarity and differences between WA-CMS lines and their corresponding nuclear restored lines. With *cox-2* we see

an obvious difference between the CMS and restored lines. Further analyses are in progress to study this difference.

Q—*Chaudhuri:* It would be very good if you could pinpoint your B1 and B2 probes on the chromosomes. Did you try any correlation of your probes with Tanksley's restriction fragment length polymorphism (RFLP) map?

A—*Sakamoto:* I have not examined the homology of the B1 and B2 with Tanksley's probe. McCouch et al (1988)(Theor. Appl. Genet. 76:815-829) reported one RFLP probe, RG229, which showed similar polymorphism in indica and javanica.

SESSION 8

Molecular Genetics of Nuclear Genomes

A rice DNA sequence that resembles the maize Mu 1 transposable element

Yong Xie, Yixin Wang, and Ray Wu

We report the characterization of a rice *Oryza sativa* DNA sequence that shows structural features similar to those of the Mu 1 transposable element in maize *Zea mays.* The nucleotide of the rice element contains long terminal inverted repeat sequences, 218 and 220 bp in length and flanked by 9-bp direct repeats. The G+C content of the internal portion of this element is unusually high. The putative Mu 1-like transposable element is present in all 18 rices analyzed so far, including wild species. However, the copy number varies between species. In addition, the copy number of the Mu 1-like sequence is four times higher in IR36 grown for 10 seasons in Texas than in IR36 obtained directly from the International Rice Research Institute.

Rice *Oryza sativa* L. can be classified into three ecospecies: indica, japonica, and javanica (Rutger and Brandon 1981, Takahashi 1984). Most cultivated rice grown today contains the AA genome. Wild rice has been classified according to its chromosome number, genetic characteristics, and geographical distribution as of either BBCC, CC, CCDD, EE, or FF (Chang 1976, Morishima 1984). The importance of rice has made it the subject of an increasing number of molecular studies.

Transposable elements were first discovered in maize by McClintock (1950). These elements play an important role in generating mutations and have proved useful in facilitating genetic analysis of the plant genome as well as tagging unknown genes (Doring 1989, Shepherd et al 1988). Over the last 8 yr, molecular cloning of transposable elements in plants has been extensively described in maize (Fedoroff 1984, Freeling 1984, Lillis and Freeling 1986, Nevers et al 1986) and in *Antirrhinum majus* (Robbins et al 1989). Putative transposable elements have also been reported in wheat (Harberd et al 1987), *Arabidopsis* (Voytas and Ausubel 1988), and tobacco (Grandbastein et al 1989).

In analyzing the 5' flanking sequence of a rice alcohol dehydrogenase gene (Xie and Wu 1989), we discovered a sequence displaying all the structural features characteristic of the maize Mu 1 transposable element. The Mu 1 element in maize contains 215-bp terminal inverted repeats flanked by 9-bp direct repeats. The internal region of the

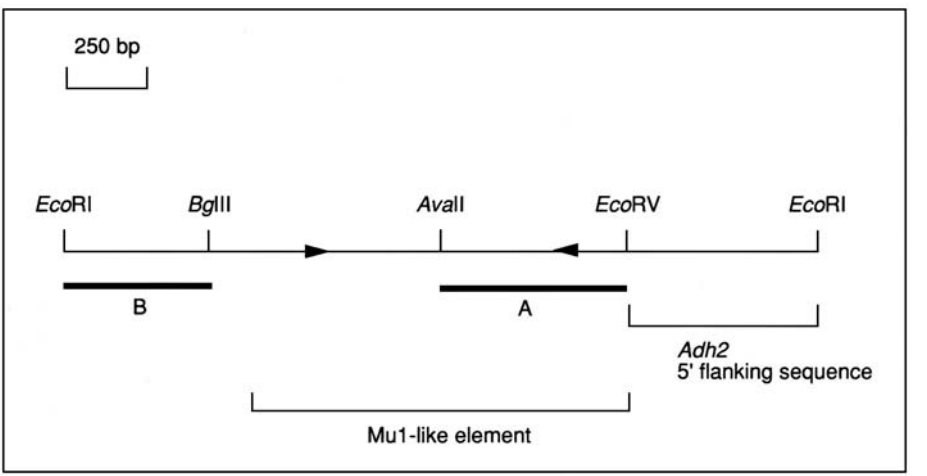
element has a very high G+C content except in two short regions (Freeling 1984, Lillis and Freeling 1986).

Materials and methods

Variety IR26 from the International Rice Research Institute (IRRI), Philippines, was used as a source of DNA for a rice genomic library (Xie and Wu 1989). Most other rices used for genomic analysis were also obtained from IRRI. A sample of IR36 was also obtained from C.N. Bollich, Rice Research Station, United States Department of Agriculture, Beaumont, Texas, USA; it originally came from IRRI but had been grown in Beaumont for approximately 10 seasons between 1978 and 1988. This sample of IR36 is referred to as IR36 (Texas) in this paper. Variety Labelle was also obtained from C.N. Bollich.

A 2.2-kb *Eco*RI-*Eco*RI fragment (Fig. 1), which includes the 5' flanking sequence of the rice alcohol dehydrogenase 2 (*Adh2*) gene, was subcloned into pUC13. This subclone, termed pR 2.2, was used for nucleotide sequence determinations.

All sequencing reactions were performed using the dideoxynucleotide chain termination procedure. Both strands of the double-stranded DNA were sequenced several times. The sequences were analyzed using the Microgenie program developed by Queen and Korn (1984) on an IBM PCXT computer. The techniques for rice genomic DNA purification from 30- to 60-d-old plants and genomic blot hybridization analysis are given by Xie and Wu (1989). Quantitative slot-blot hybridization was carried out according to Zhao et al (1989).



1. Restriction map of clone pR2.2 showing position and length of DNA probes. Probe A was a 0.49-kb *Ava*II-*Eco*RV fragment. Probe B was a 0.43-kb *Eco*RI- *Bg*III fragment. Arrows and adjacent heavy lines represent inverted repeats of rice Mu l-like element. Restriction sites indicated along top.

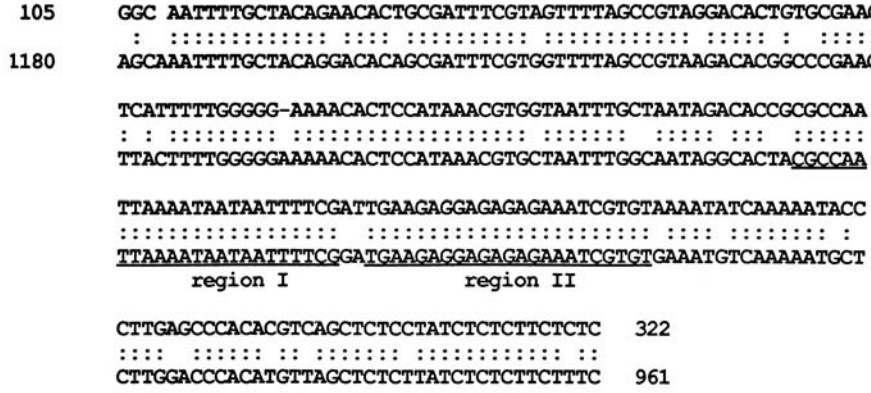
Results and discussion

The putative Mu 1-like transposable element was characterized using three approaches.

Nucleotide sequence of a putative Mu1-like transposable element in rice

We cloned and sequenced a 2.2-kb *Eco*RI fragment (pR 2.2) that includes the 5' flanking sequence of the rice *Adh2* gene (Fig. 1). The nucleotide sequence of 1.26 kb

2. Nucleotide sequence of 1.26-kb fragment that contains a putative Mu 1-like transposable element in rice. Double solid lines beneath sequences show location of 9-bp direct repeats flanking the 1076-bp putative Mu 1-like element. Single solid lines beneath sequences show location of direct repeats (e.g., sequences in 3A and 3B are almost identical). Dashed lines above sequence mark regions corresponding to long terminal inverted repeats.



3. Nucleotide sequence alignment of long terminal inverted repeats in putative Mu1-like element in rice. The 5' terminal inverted repeat (105-322) and the complementary sequence of the 3' terminal inverted repeat (1180-961) are aligned to maximize homology. Within inverted repeats are 188 matched nucleotides, 30 mismatched nucleotides, and 2 unmatched nucleotides. Percentage of identical bases is 87%. Solid lines beneath sequences show location of 2 longest stretches of perfect sequence identity between inverted repeats (e.g., region I and region II).

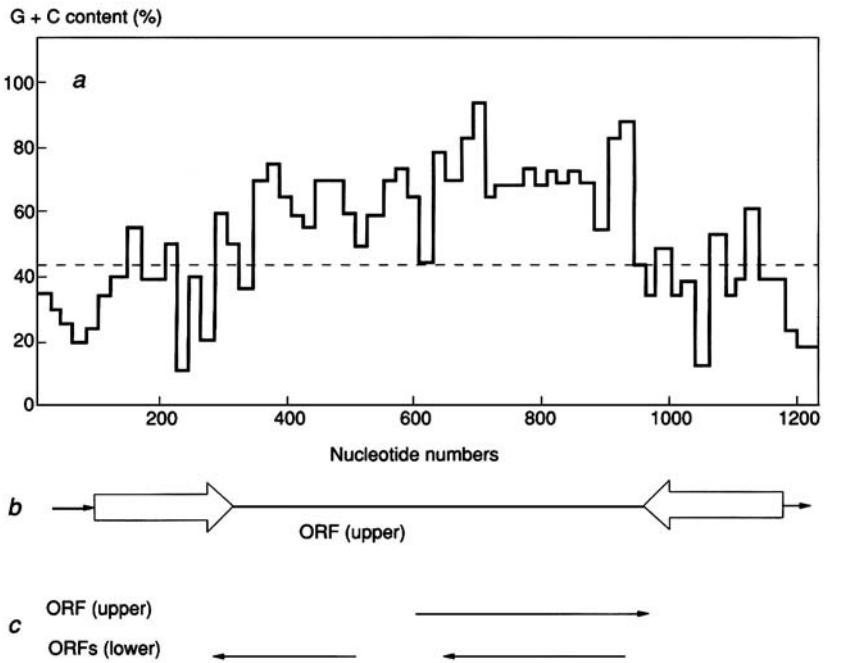
of the fragment is shown in Figure 2. Within this fragment, long, nearly perfect inverted repeats extending for 218 bp and 220 bp, respectively, were found (Fig. 2, positions 105-322 and 961-1180, the regions with dotted lines on top of the sequence). In Figure 3, the inverted repeats are aligned. They show 87% sequence identity. The longest stretch of perfect sequence identity is 24 bp (region I), which includes 18 consecutive A:T base pairs. The second longest stretch of perfect sequence identity is 23 bp (region II), which includes 16 consecutive purines. The total length of the long inverted repeats and the internal sequence is 1076 bp. A 9-bp direct repeat sequence, TAATCATTA (Fig. 2, positions 96-104 and 1181-1189), flanks the long inverted repeats. Most transposable elements in plants terminate in inverted repeats, and all of them possess a short target-site duplication as direct repeats flanking the element (Federoff 1984, Freeling 1984, Nevers et al 1986). By these criteria, the 1076-bp rice sequence described here (Fig. 2, from nucleotides 105-1180) fits the general structural characteristics of a transposon, and we refer to it as a putative Mu 1-like element.

The rice putative Mu 1-like transposable element also includes several short direct repeats within the terminal inverted repeats. As shown in Figure 2, sequences 1A and 1B are almost identical; this is also true for 2A and 2B, and so on. This feature is relatively rare among the plant transposable elements described to date.

To determine if this rice putative transposable element shares any sequence identity with other transposable elements reported to date, comparisons were made with all the transposable element sequences in the data bank of the Microgenie program. The rice element shared 38% sequence identity in the terminal repeats with the maize Mu 1 transposable element. Although the degree of sequence identity is low, the structural feature of the rice sequence is identical to that of the Mu 1 transposable element in maize, which includes 215-bp inverted terminal repeats flanked by 9-bp direct repeats,

Table 1. G+C percentage distribution in the putative Mu 1-like transposable element in rice and the Mu 1 transposable element in maize (Freeling 1984).

Plant	Structural feature	5' inverted repeat	3' inverted repeat	Internal sequence	Overall length	Average G+C (%) in the genome
Rice	Length (bp)	218	220	634	1067	
	G+C%	41	41	67	56	44
Maize	Length (bp)	215	213	950	1378	
	G+C%	54	50	67	61	50



4. Structural features found in the putative Mu 1-like element in rice. a. Percentage of G+C content for every 20 nucleotides in the putative Mu 1-like element. Dotted line indicates average G+C content (44%) in the rice genome. b. Position of inverted repeats (long and open arrows) flanked by short direct repeats (solid arrows). c. Positions of open reading frames (ORFs) found in the putative Mu 1-like element. Each line represents a different ORF.

and a very high G+C content of the internal sequence. The common features are summarized in Table 1 and Figure 4. The G+C content of the internal region of the maize Mu 1 transposable element is 67%. That for the rice element is also 67%, which is much higher than the average G+C content of 44% for the rice genome (Iyengar et al 1979). Both the maize Mu 1 transposable element (Freeling 1984, Lillis and Freeling 1986) and the rice putative Mu 1-like element include several open reading frames (Fig.

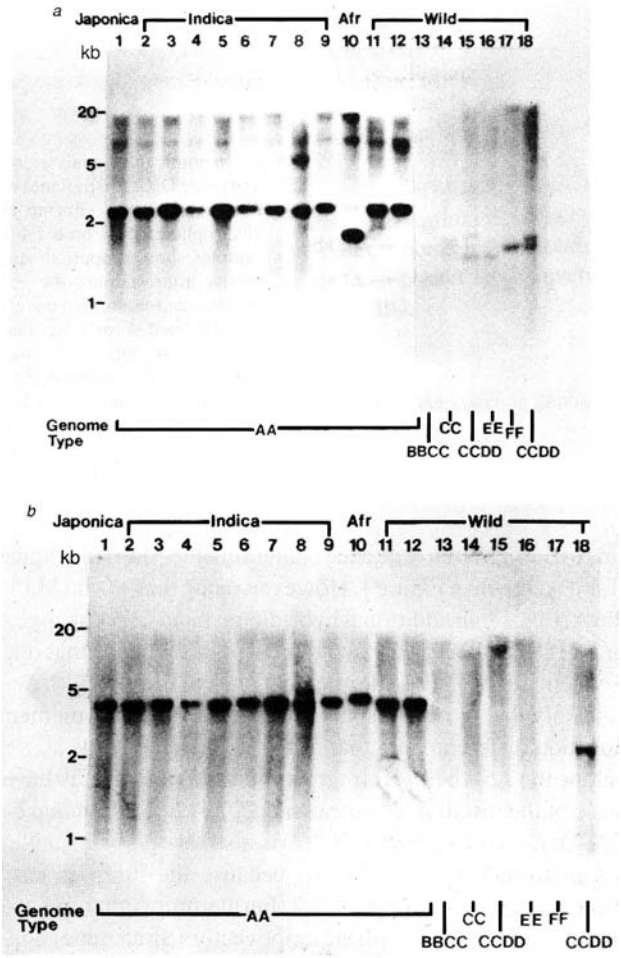
4c). However, no messenger RNA can be detected by RNA blot hybridization using the rice putative Mu 1-like element as a probe (data not shown).

Putative Mu 1-like element in 18 rices

The putative Mu 1-like transposable element described above was first found in rice variety IR26. To determine if this element exists in other rices, including wild species, genomic blot hybridization analysis was carried out on 18 rices using a 490-bp probe containing a portion of the internal region and 1 inverted repeat of the rice Mu 1-like element (Fig. 1, probe A). All rices contained this element (Fig. 5). All rices with the AA genome gave the same major hybridization bands (approximately 2.2 kb) except the African cultivated species (Afr) *O. glaberrima* (Fig. 5a, lane 10). which has a shorter major band (1.7 kb). Most rices also showed weaker hybridizing bands at approximately 8 kb. IR36 (lane 8) also had a 5.4-kb band. Wild species with other genomes also possessed the putative Mu 1-like sequences, but they showed different hybridization patterns and gave lighter bands than did those with the AA genome. Two accessions of the CCDD genome showed different hybridization patterns (lanes 15 and 18). The intensity of the major bands (2.2 kb) in the autoradiogram of Figure 5 was measured by a densitometer (BioRad, Model 620). A gene-specific probe from the rice actin 3 gene (from D. McElroy) was used as an internal control to correct for variations in the amount of DNA loaded in each lane (Fig. Sb). Since the specific activities of the Mu 1-like probe and the actin probe were the same, we conclude that variety M202 (lane 4) contains one copy of the element. Eight rices (Fujisaka, IR24, IR8, IR26, IR36, Cina, *O. rufipugon*, *O. nivara*) have two copies of the element, *O. glaberrima* (lane 10) has three copies, and Tadukan (lane 3) and TKM6 (lane 5) have four copies of the element. These numbers are minimum numbers, because we did not take into account the other weaker hybridizing bands.

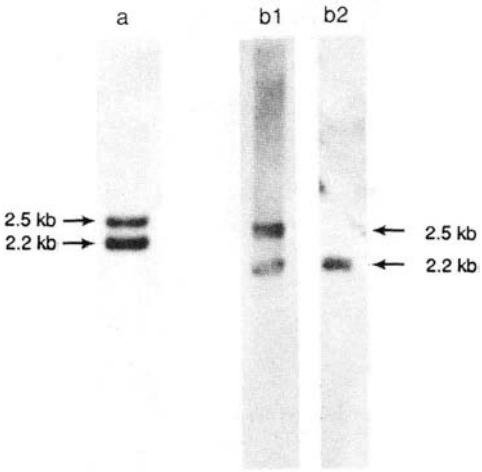
TKM6, Tadukan, IR8, and Cina are parents of IR26 and IR36. The position of the most intense hybridization band (Fig. 5a), which hybridizes to the element, is identical in all four parental rice species, as well as in the IR26 genome. Since TKM6 and Tadukan have higher copy numbers of the element, these results suggest that some of the putative Mu 1-like transposable elements in the genome of these two rices may have been lost during breeding of IR26. It is likely that at one time the putative elements in these rices were active. Experimental results showing that the putative Mu 1-like element is perhaps a transposable element are described below.

More recently, we started to use IR36 (Texas). The IR36 (Texas) plants looked uniform in the field according to C.N. Bollich (pers. comm.). DNA isolated from IR36 (Texas) showed a different genomic blot hybridization pattern than did IR36 from IRRI using the rice Mu 1-like sequence as a probe. As shown in Figure 6, IR36 (Texas) showed two strong hybridizing bands using the 490-bp probe including a portion of the Mu 1-like element (probe A, Fig. 1). The 2.2-kb lower band was the same as in Figure 5a (lane 8, strongest band). However, there is an additional strong hybridizing band (2.5 kb) shown in Figure 6a. To determine whether this 2.5-kb band represents a putative Mu 1-like element located in the same or different chromosomal location



5. Genomic blot hybridization analysis of 18 rices. a) DNA samples (15 µg) from 18 rices were digested with EcoRI, fractionated in 1% agarose gel, and transferred to a Nytran filter. The filters were hybridized with a 32P-labeled putative Mu 1-like transposable element-specific probe (probe A shown in Figure 1, which is a 490-bp fragment containing nucleotides from position 708-1198) and then washed extensively at 60 ºC in 0.02 x SSC (1 x SSC is 0.15 M sodium chloride and 0.015 M sodium citrate) and 0.5% sodium dodecyl sulfate. Genomes of the 18 rices are shown under each lane. I = Fujisaka 5, 2 = IR24,3 = Tadukan, 4 = M202.5 = TKM6, 6 = IR8,7 = IR26, 8 = IR36 (IRRI), 9 = Cina, 10= *O. glaberrina,* 11 = *O. rufipogon,* 12 = *O. nivara,* 13= *O. punctata,* 14= *O. officinalis,* 15= *O. alta,* 16= *O. australiensis,* 17= *O. brachyantha,* 18= *O. latifolia.* Molecular weight markers (in kb) are given on the left side of the genomic blot. b) The filter was boiled to remove the labeled probe and reprobed with ^{32}P-labeled 3' untranslated fragment of the rice actin 3 gene (the actin 3 gene is a single copy gene: D. McElroy, pers. comm.) usinp the same experimental conditions.

compared with the 2.2-kb band, the 5' flanking sequence of the Mu 1-like element was used as a probe. This probe (probe B, Fig. 1) represents the DNA sequence upstream of the Mu 1-like element but does not include the rice Mu 1-like sequence. As shown in Figure 6b, the 5' flanking sequence probe (lane 2) showed only the 2.2-kb band. This



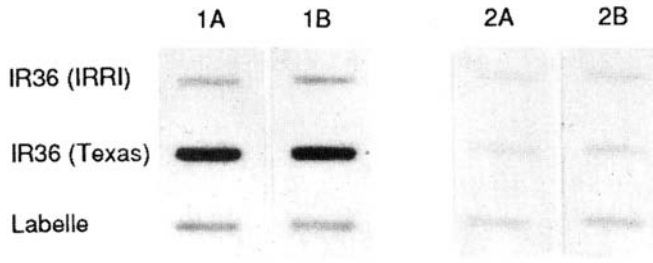
6. Genomic blot analysis of IR36 (Texas). Total rice DNA (10 µg/lane) was digested with EcoRI restriction enzyme and fractionated electrophoretically on a 1% agarose gel. All samples showed approximately the same intensity after staining the gel with ethidium bromide and examining under ultraviolet light, *Hin*dIII- and *Eco*Ri-digested lambda DNA samples were run as size markers. The DNA samples were transferred to a Nytran filter for hybridization using a ^{32}P-labeled rice Mu-like element and its flanking region as probes. a = with probe A (Fig. 1); b = with probe A in the left lane and probe B (Fig. 1) in the right lane.

band corresponds to the genomic sequence containing the Mu 1-like sequence that was cloned (pR 2.2) and is shown in Figure 1. However, using the 490-bp Mu 1-like element as a probe (probe A, Fig. 1), an additional hybridizing band (2.5 kb) appeared as shown in Figure 6b (lane 1). Thus the 2.5-kb band represents a sequence that does not include the 5' flanking sequence of the Mu 1-like element present in the 2.2-kb *Eco*RI fragment (Fig. l), and it is likely that the 2.5-kb band includes a Mu 1-like element located at a chromosomal location different from that of the 2.2-kb fragment.

Attempts to clone the 2.5-kb *Eco*RI fragment (Fig. 6a) into pUCI9 have not yet been successful because of the instability of the cloned DNA in *Escherichia coli* strain XL-1 (recA-). The 2.2-kb *Eco*RI fragment (pR 2.2) is also relatively unstable when cloned in pUC13 and transformed *E. coli* JM101, because the insert is easily lost upon subculturing of the bacteria harboring pR 2.2. Furthermore, attempts to subclone the Mu 1-like fragment in pR 2.2 in the pBluescript vector (Strategene) and transformed *E. coli* JM109 or XL- 1, with the hope of making DNA sequencing easier, have not been successful. Thus, pR 2.2 was sequenced as double-stranded DNA using several synthetic primers. In the future, cloning of the 2.5-kb *Eco*RI fragment will be attempted by using a low-copy-number plasmid.

Copy number of the Mu 1-like element in IR36 (IRRI) and IR36 (Texas)

The genomic blot hybridization shown in Figure 6 showed that IR36 (Texas) has an additional strong hybridizing band. We next examined whether the copy number of the rice Mu 1-like element had also increased in IR36 (Texas). Copy-number determination was carried out by quantitative slot-blot hybridization using duplicate samples of rice genomic DNA from IR36 (IRRI) and IR36 (Texas). DNA from Labelle was used for comparison, because this rice variety had also been grown in Texas for over 10 yr.



7. Slot-blot analysis of DNA from 3 rices. Duplicate samples of 1.1 µg or 2 µg each of DNA from IR36 (IRRI), IR36 (Texas), and Labelle were applied to nitrocellulose papers using a slot-blot template. For experiments 1A and 1B, the filters contained 1.1 µg of genomic DNA in each slot and were hybridized with probe A (Fig. 1). For experiments 2A and 2B, the filters contained 2 µg of DNA in each slot and were hybridized with the rice actin 1 gene probe.

Table 2. Copy-number determination of Mu 1-like element in 3 rices.[a]

Variety	Size of each peak							
	1A	1B	Av value	Copy number	2A	2B	Av value	Copy number
IR36 (IRRI)	8.0	9.6	8.8	2.7	5.6	6.0	5.8	1.0
IR36 (Texas)	40.0	40.8	40.4	11.2	5.8	7.2	6.5	1.0
Labelle	11.8	13.6	12.7	3.4	6.3	7.3	6.8	1.0

[a]The x-ray film shown in Figure 7 was scanned with a densitometer. The tracing paper was photocopied, and the peaks were cut out and weighed. The size of each peak (expressed as milligrams of paper) is proportional to the amount of probe hybridized. The copy number of a specific sequence in the rice genome was calculated by assuming that the actin probe detected the only copy of the actin gene in the rice genome of each of the rice varieties. The values for copy number of the Mu1-like element in samples 1A and 1B were calculated by dividing the "average value" of the peak size by that of samples 2A and 2B, and multiplying by 1.8 (2.0 ÷ 1.1).

Six identical nitrocellulose filters were prepared, and two each were hybridized separately to each of three probes: 1) probe A, which contained a rice Mu 1-like sequence including both a portion of the internal Mu 1-like sequence and one inverted repeat (Fig. 1), 2) an actin 1 probe (from D. McElroy) that is unique to the rice actin 1 gene and is present in only one copy in the rice genome (McElroy et al 1990), and 3) the RC48 rice repetitive DNA (Wu and Wu 1987, Zhao et al 1989). Figure 7 shows the radioautogram of slot-blot experiments using the first two probes. The X-ray film was then traced with a densitometer, and the density of each band was quantified. Results from tracing duplicate samples are shown in Table 2, and the numbers for each experiment were averaged. Assuming that the actin 1 gene is present in one copy in the rice genome, the calculated copy number of the Mu 1-like element using probe A was 2.7 for IR36 (IRRI), which agrees with the genomic blot result (Fig. 5). However, IR36 (Texas) gave approximately 11 copies of the Mu 1-like sequence, which is 4 times higher than that of IR36 (IRRI). Another way to compare the copy number of the Mu 1-like element is to use the primary data for IR36 (Texas): IR36 (IRRI), 40.4:8.8.

This gives a ratio of 4.6, which is similar to the other calculation. Since the copy number of the actin 1 sequence in all samples and of the Mu 1-like elements in Labelle and IR36 (IRRI) did not change (Fig. 7), and the RC48 repetitive DNA copy number in IR36 (IRRI) and IR36 (Texas) also did not change (data not shown), the fourfold increase in the copy number in IR36 (Texas) suggests that duplication of the rice Mu 1-like element occurred. This conclusion is consistent with the detection of the new 2.5-kb hybridizing band in the genomic blot experiment in IR36 (Texas) (Fig. 6). Although the rice Mu 1-like sequence may be a transposable element, our results are preliminary. More work is needed to follow up the observations.

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Notes

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Genetic variation in tissue culture-derived rice plants

P.T.H. Brown, E. Müller, K. Shimamoto, and H. Lörz

To determine whether tissue culture of rice results in an increase in DNA polymorphism, callus cultures, plants regenerated from callus cultures, and the progeny of protoplast-derived plants were examined by restriction fragment length polymorphism analysis. The results demonstrate that, in comparison with nontissue culture-derived control plants, significant levels of DNA polymorphism are produced as a result of tissue culture. The results also indicate that, while the level of DNA variation between callus- and protoplast-derived plants is not significantly different, the amount and degree of polymorphism are greatly increased in plants derived from callus cultures maintained for longer culture periods (67 d vs 28 d). Analysis of plants with gene sequences representative of different domains and functions, in conjunction with different restriction enzymes, indicates that there is no single underlying mechanism for these DNA changes, the polymorphism being random. The consequences of this variation are discussed.

Regeneration of plants by tissue culture should result in clones that are phenotypically and genetically identical to the plant from which they were derived. In many cases, however, some regenerants exhibit deviations from the parental type, a phenomenon known as somaclonal variation (Larkin and Scowcroft 1981).

Previous descriptions of somaclonal variation in rice have been predominantly in terms of phenotypic change. Oono (1981) demonstrated that 72% of rice plants regenerated from callus cultures showed morphological mutations. A similar level of phenotypic disturbance was reported by Sun et al (1983), who found that only 24.4% of more than 2,000 regenerated rice plants were normal for each of 5 traits examined. Fukui (1983) found that, although R_o generation rice plants were normal for such characteristics as flag leaf length, width, and color, mutations were observed in subsequent generations, presumably reflecting a change from recessive heterozygosity to homozygosity. As it can be assumed that regeneration of plants from protoplast cultures would be more stressful than that from callus cultures, variation levels are expected to be higher in protoplast-derived plants than in callus-derived plants. However, Ogura et al (1987) found that in the R_o of protoplast-derived plants,

morphological distortions were present in only approximately 20% of the regenerants. Such levels of phenotypic change cannot always be easily correlated with karyological differences. For example, karyological abnormalities could be found in only 10% of these regenerants.

One factor of apparent importance in the subsequent genetic fidelity of regenerants is the time cultures are maintained as callus (Meins 1983). McCoy et al (1982) found that the frequency of chromosomal abnormality in oat plants regenerated from callus cultures rose from 49% after 4 mo in culture to 88% after 20 mo. Analyses of callus cultures and their progeny have indicated that such cultures should be regarded as genetically heterogeneous. Fukui (1983) examined the progeny of 12 plants derived from a single callus and speculated that the callus had a heterogeneous constitution.

Discussion has focused on whether this variation is due to genetic heterogeneity of the explant or is the result of tissue culture itself (see review by Morrish et al 1987). In work with callus-derived maize plants (unpubl. data), we were able to demonstrate that one possible factor in this increased level of variation is a significant alteration in the level of gene methylation. Although the role of methylation is the subject of intense discussion, changes in the level of methylated base 5-methylcytosine are clearly intimately associated with alterations in the transcriptional activity of the gene. Results of work with *Escherichia coli* (Coulondre et al 1978) and the human genome (Barker et al 1984) have shown that methylated sequences are also mutational "hot spots."

Although somaclonal variation may represent a valuable source of novel genetic variation, this variation may act as an impediment to the efficient application of modem methods of gene transfer as part of cereal breeding programs. A greater understanding of the mechanisms of tissue culture-induced variation may lead to a means of control and to subsequent elimination of the problem.

Materials and methods

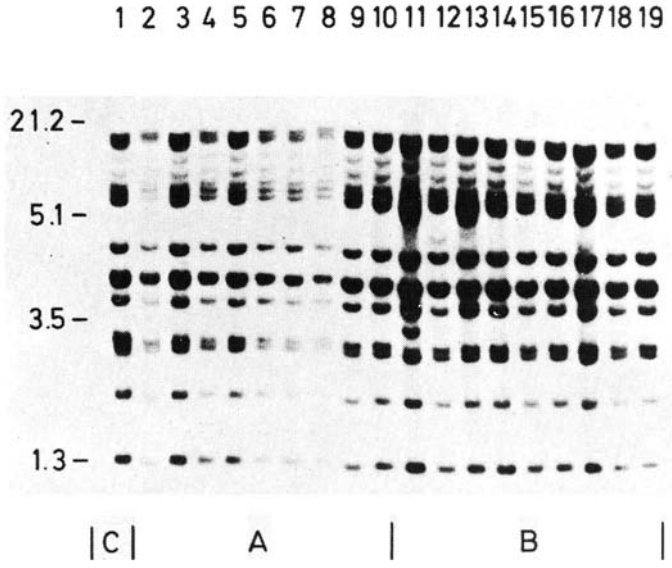
Callus cultures and plants regenerated from them were of the *Oryza sativa* L. indica type IR40 (courtesy of G.S. Khush, IRRI, Philippines). Culture and regeneration protocols were as described by Hartke and Lörz (1989). Progeny of protoplast-derived rice plants were of the japonica line Norin N10. Culture and regeneration protocols were as previously described by Kyozuka et al (1987).

Methods for restriction fragment length polymorphism (RFLP) analysis including DNA isolation were as described by Brown (1989).

Results

Analysis of callus-derived plants

Digestion of genomic DNA with a number of restriction enzymes and subsequent Southern analysis using cloned, characterized gene sequences showed that RFLP analysis based on *Hin*dIII digestion of genomic DNA and subsequent probing with the 3-kb *Hin*dIII fragment of the soybean actin gene (Shah et al 1982) was the most suitable

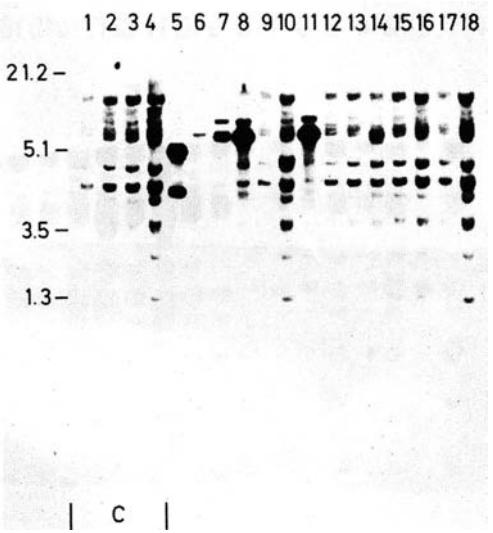


1. Genomic DNA was isolated from rice plants regenerated from calli grown for 28 d (A) or 67 d (B). The DNA was digested with *Hin*dIII and Southern analyzed with the 3-kb *Hin*dIII fragment of the actin gene. The results demonstrate that regenerants from short-term cultures have less DNA polymorphism than those repenerants produced from long-term callus cultures. C = control.

combination for RFLP analysis, with a number of strongly hybridizing fragments and the ability to clearly differentiate between rice lines. Using this system, our first analysis was to determine whether DNA polymorphism exists in nontissue culture-derived control plants. Analysis of more than 40 such plants failed to reveal any such variation. To determine whether DNA polymorphism was induced in tissue culture-derived plants, phenotypically normal and abnormal regenerants (R_0) derived from different calli, as well as siblings from the same callus, were analyzed. We were able to show not only that plants derived from different calli exhibited differences in the actin gene, but also that plants from the same callus also showed polymorphism.

To determine the basis for this variation, we investigated sibling plants derived from callus material maintained for two growth periods: a short-term growth phase of 28 d and a longer period of 67 d. A representative analysis (Fig. 1) of phenotypically normal and deviant plants from these two culture periods revealed that plants regenerated from short-term callus cultures infrequently showed DNA polymorphism, Conversely, long-term callus culture resulted in a significantly larger number of plants (tracks 11, 12, 13, and 17) with DNA variation.

The overall percentage of regenerants exhibiting DNA polymorphism was low: of all the plants subjected to RFLP analysis, approximately 19% showed such variation (compared with 5% showing phenotypic variation). However, 23% of the plants regenerated from 67-d-old callus cultures showed RFLP changes compared with only



2. Genomic DNA from rice plants regenerated from callus cultures maintained for long periods (67 d) reveals not only more DNA polymorphism but significant DNA rearrangement. Genomic DNA was digested with *Hin*dIII and Southern analyzed with the 3-kb *Hin*dIII fragment of the actin gene. C = control.

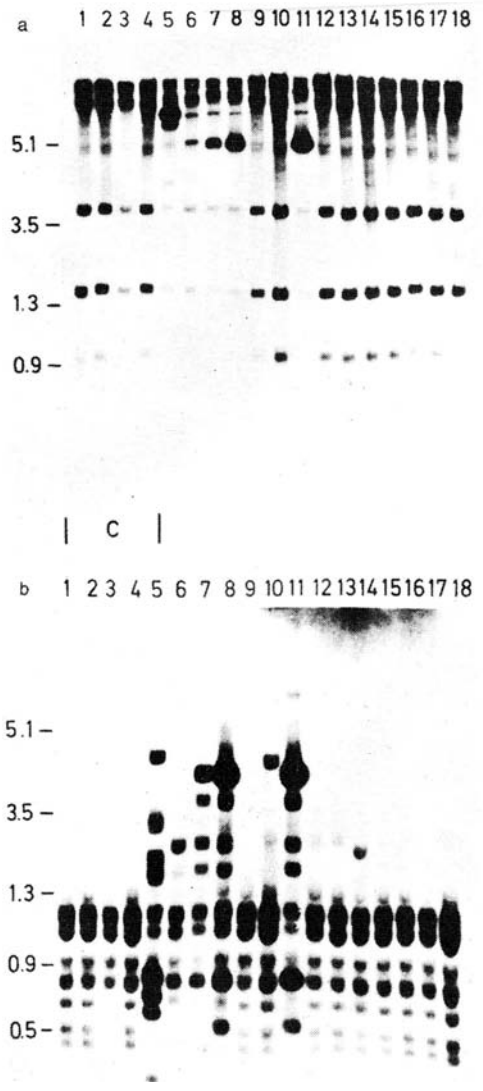
6.3% of those regenerated from 28-d-old cultures. Therefore, approximate doubling of the culture period (from 28 to 67 d) increased the level of detectable variation by nearly 4 times. The results also confirmed that no correlation can be made between those plants with a deviant phenotype and the level of DNA polymorphism.

Subsequent analysis revealed that, in addition to exhibiting higher levels of polymorphism, plants regenerated from longer culture periods showed considerable range of DNA changes. Figure 2 shows not only that differences existed between sibling plants, but that the form of polymorphism varied extensively, ranging from plants with an apparent loss of one restriction site (track 14) to those plants where there was extensive sequence rearrangement (tracks 5 and 11).

Further analyses of plants with different restriction enzymes including *Bam*HI (Fig. 3a) or the methylation-insensitive enzyme *Rsa*I (Fig. 3b). as well as with different genes (Fig. 4), strongly indicate that a large proportion of these regenerants had wide-ranging alterations throughout the genomes. A very close correlation existed between plants exhibiting DNA polymorphism and those with apparent alterations in gene methylation.

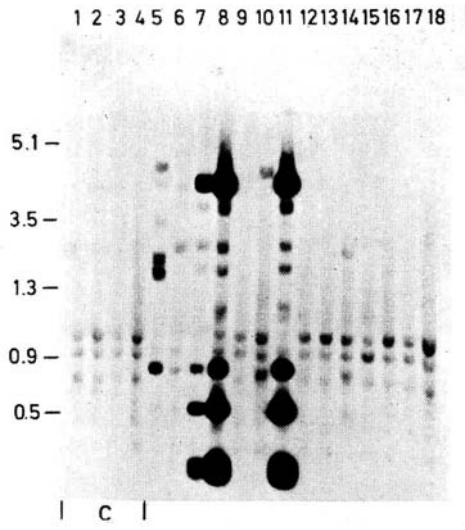
Analysis of protoplast-derived progeny

To determine whether the level of DNA polymorphism was higher in plants regenerated from protoplasts than in those regenerated from callus cultures, the progeny plants (R1 generation) of protoplast-derived regenerants from the japonica line Norin N10 were analyzed.



3. Genomic DNA from regenerated rice plants digested with *Bam* HI (a) or the methylation-independent enzyme *Rsa*I (b) probed with the 3-kb actin gene sequence reveals that a number of plants had wide-ranging DNA variation. C = control.

Ten seeds from each of 10 independent protoplast-derived plants were sown for analysis. Table 1 shows that significant differences occurred in germination frequency: in 4 lines germination was 100%; in line 55, only 20% of the seeds germinated. The level of phenotypic polymorphism was also extremely high: 58% of the plants showed some form of change, which ranged from slight chlorosis to severe stunting and male



4. Further analysis of the same DNA as in Figure 3 with probes representative of other genes such as the 1.2-kb ATP/ADP translocator gene indicates that these plants have undergone significant DNA rearrangement throughout their genomes. C = control.

Table 1. Germination levels for R_1 generation protoplast-derived japonica rice plants.

Plant line[a]	Germination[b] (%)	Plants (no.)
64	100	10
32	100	10
19	100	10
31	100	10
42	90	9
3	80	8
33	80	8
76	60	6
110	30	3
55	20	2

[a] Derived from Norin N10. [b] Av = 76%.

sterility. Subsequent analyses of a series of genes representing promoters, 3' ends, or a comparison between structural (Table 2) and housekeeping (Table 3) genes demonstrated that variation could be found regardless of the gene domain represented. Similarly, although variation rates were often higher in the methylation-dependent digestions, the levels cannot be regarded as indicative of a major role for methylation in inducing DNA mutation.

Genomic DNA from each of the protoplast-derived plants was Southern analyzed after restriction digestion with the enzymes *Rsa*I or the methylation-sensitive isoschizomers *Msp*I and *Hpa*II. To determine stability differences between plant lines, the mean polymorphism rate per line was determined by summing the number of

Table 2. RFLP analysis of rice plants for structural genes.

Plant line	Enzyme *R*sal							Enzyme *Msp*I/*Hpa*II						
	Polymorphism in given gene[a]						Mean	Polymorphism in given gene[a]						Mean
	1	2	3	4	5	6		1	2	3	4	5	6	
64	0	0	0	1	0	1	3.3	1	0	0	1	0	1	5
32	1	0	0	0	0	0	1.6	0	0	0	0	0	0	0
19	1	3	3	2	1	0	16.6	1	3	3	4	0	1	20.0
31	4	2	0	0	0	3	15.0	4	2	0	1	3	3	21.6
42	4	2	2	3	5	1	31.4	3	2	2	2	5	1	27.7
3	3	2	2	0	0	0	14.5	3	2	2	1	0	0	16.6
33	4	2	1	0	2	3	25.0	4	2	1	0	2	3	25.0
76	5	3	3	3	3	3	55.5	5	3	3	3	3	4	58.3
110	3	3	0	3	0	0	50.0	3	3	0	3	0	0	50.0
55	2	2	2	2	2	2	100.0	2	2	2	2	2	2	100.0
Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Polymorphism per gene	35.5	25.0	17.0	18.4	17.1		17.1	34.2	25.0	17.1	22.3	19.7		19.7

[a]Gene 1 = sucrose synthase gene from maize (Werr, Köln, pers. comm.), Gene 2 = actin gene from soybean (Shah et al 1982), Gene 3 = waxy gene from maize (Schwarz-Sommer, Köln. pers. comm.), Gene 4 = bronzy gene from maize (Federoff et al 1984), Gene 5 = *Adh* gene from maize (Dennis et al 1984), Gene 6 = dihydroquercetin-4-reductase gene from maize (Schwarz-Sommer et al 1987).

Table 3. RFLP analysis of rice plants for housekeeping genes.

Plant line	Enzyme *R*sal				Enzyme *Msp*I/*Hpa*II			
	Polymorphism in given gene[a]			Mean	Polymorphism in given gene[a]			Mean
	1	2	3		1	2	3	
64	0	0	0	0	0	0	0	0
32	0	0	1	3.3	0	0	0	0
19	0	0	0	0	0	0	0	0
31	0	0	0	0	0	0	0	0
42	0	0	0	0	0	0	0	0
3	0	1	0	4.1	0	2	0	8.2
33	2	0	3	20.8	2	0	2	16.6
76	1	3	0	22.2	1	3	0	22.0
110	2	2	1	55.5	2	2	1	55.5
55	2	2	2	100.0	2	2	2	100.0
Control	0	0	0	0	0	0	0	0
Polymorphism per gene	9.2	10.5	9.2		9.2	11.8	9.2	

[a]Gene 1 = ATP/ADP translocator gene from maize (Baker and Leaver 1985), Gene 2 = cytosolic glyceraldehyde-3-Pdehydrogenase gene from maize (Brinkman et al 1987), Gene 3 = ribosomal gene from bean (Yakura et al 1984).

detected polymorphisms for each gene and dividing by the number of plants in each line. Stability differences between genes was determined by summing the number of detected polymorphisms for each gene between lines and dividing by the total number of plants examined to produce a mean polymorphism per gene.

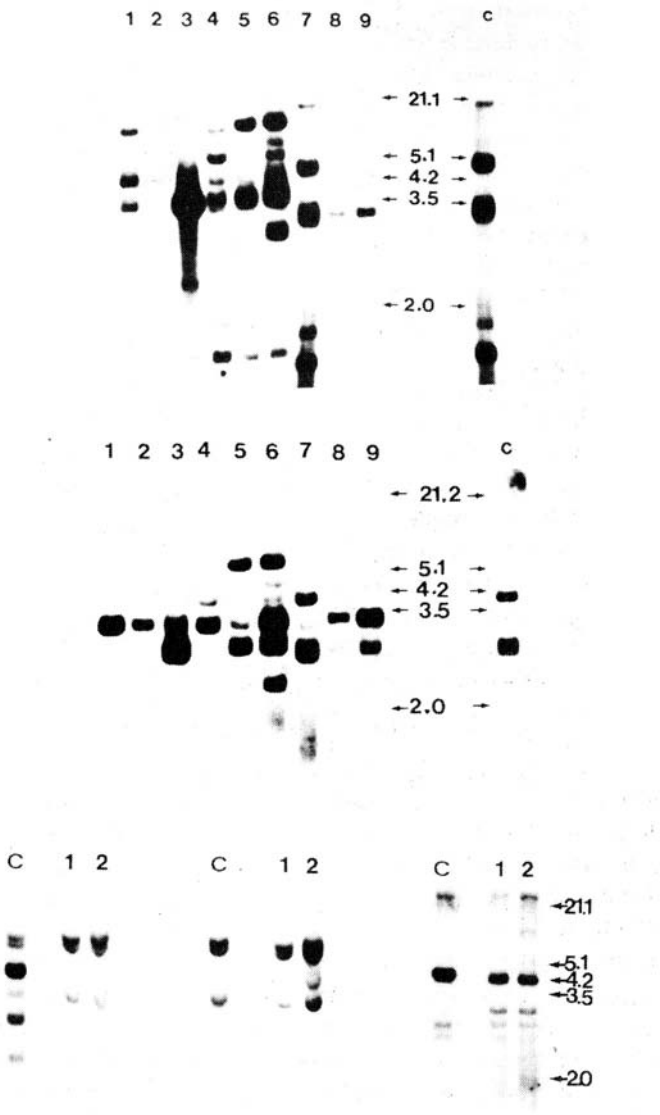
Comparative analysis of rice and maize callus

To determine when such DNA variation occurs in cereal tissue culture, we analyzed both rice and maize callus cultures. Analyses of the sucrose synthase gene (Fig. 5a) or the ATP/ADP translocator gene (Fig. 5b) in cultures of maize inbred line A188 revealed a total reorganization of these genes. Further analysis demonstrated that this high variability was apparently independent of such factors as media and hormone concentration. Conversely, callus cultures of rice lines IR40, IR54, and IR45 (Fig. 5c) were considerably more genetically stable than those of maize. Although there were obvious differences in the control plant genotype, the changes were predominantly stoichiometric rather than indicative of substantial reorganization, as in maize.

Discussion

Our results show that some regenerated plants exhibit DNA polymorphism, presumably induced by the stress of tissue culture. Surprisingly, the level of variation is not significantly different between plants regenerated from protoplasts (via callus culture) or directly from callus. The average frequency of detected polymorphism in protoplast-derived plants was 29%. In plants derived from callus cultures without a protoplast phase, the level of DNA variation ranged between 6.3 and 23%. depending on the time that cultures were maintained as undifferentiated callus. Therefore, even though the two sets of experiments are not strictly comparable, the results indicate that the crucial phase in the induction of tissue culture-associated DNA variation is during callus growth, and that the longer the material is maintained in this form, the greater the level of DNA variation, both quantitative and qualitative, subsequently found in the whole plant. During our analysis of callus-derived indica rice plants. we found that the stress of tissue culture was sufficient to cause not only an increase in the level of DNA polymorphism but a significant increase in the level of methylated bases. There was a strong correlation between those plants that showed RFLP changes and those with apparent methylation changes. Therefore, in conjunction with results from previous work, we proposed a theory to link these tissue culture-induced changes (Brown 1989). As methylated sequences are known to be mutational “hot spots” (Barker et al 1984, Coulondre et al 1978), culture stress may be responsible for significant methylation changes, with a consequent rise in the level of DNA polymorphism, as revealed here. Conversely, culture stress may be responsible for direct DNA variation in genes that subsequently have their methylation status altered to transcriptionally inhibit potentially lethal sequences.

Significant differences exist between genes with regard to the relevance of methylation, methylation of promoter and 5' sequences being considerably more important



5. Genomic DNA was isolated from callus produced from maize inbred line A188, and probed with the 4.5-kb waxy gene fragment (a) or the 1.2-kb ATP/ADP translocator gene (b). The results reveal that callus growth in maize leads to significant DNA rearrangement. Conversely, Southern analysis of genomic DNA from callus produced from indica lines IR54, IR40, and IR45 (c) reveals that DNA in rice callus is more stable than DNA in maize in response to tissue culture. C =control.

than that of coding or 3' domains (Brown 1989). Similarly, differences occur between housekeeping and structural genes. Consequently, if methylation is to play an important role in tissue culture-induced DNA change, then probing regenerated plants with cloned sequences representative of different gene domains should reveal a higher level of polymorphism in methylation-controlled structural genes than in nonmethylated housekeeping sequences. The results reveal that, although the level of gene polymorphism detected is higher in DNA subjected to methylation analysis, the level cannot be regarded as significantly different and certainly is not as high as would be expected if methylation were an essential factor in culture-induced DNA variation.

If methylation changes did not play a significant role in inducing DNA polymorphism, is there any single unifying mechanism that can be responsible? Our analysis of genetic changes directly in maize callus cultures demonstrated that a complete reorganization of some genes could be detected. Similar analyses of three indica rice callus cultures revealed that, despite some differences from control plant RFLP patterns, they corresponded essentially to controls.

One difference between the genomes of rice and maize is in the way that the repetitive and single-copy sequences are organized. Hake and Walbot (1980) demonstrated that 20% of the genome of *Zea mays* is highly repetitive DNA, and 40% is middle repetitive. Interspaced within this middle repetitive DNA is up to one-third unique sequences. Conversely, studies on repetitive sequences in the rice genome (Dhar et al 1988) showed that this DNA occurs as long stretches of up to 20 kb uninterrupted by single-copy sequences. Flavell (1985) reported that, in contrast to conserved coding sequences, repetitive DNA evolves and has a higher turnover, which suggests that it may be predominantly affected by tissue culture. Therefore, due to the close proximity of single-copy sequences to repetitive DNA in the maize genome, high levels of DNA change can be detected, whereas due to the dispersed nature of the rice genome, changes in such sequences are mitigated. Caboche and Lark (1981) isolated nuclei from soybean suspension cultures and demonstrated that repetitive sequences are preferentially replicated. They suggested that replication of single-copy DNA and that of repetitive DNA are controlled differently, the DNA produced under in vitro conditions being "biased" toward replication of repetitive sequences.

Thus, no single unifying mechanism for the induction of tissue culture-associated mutation is likely; rather there is a range of both direct DNA rearrangements and paramutational events such as methylation changes responsible for a broad spectrum of genetic alteration. If this is true, then techniques to reduce this tissue culture mutation are probably not feasible in the immediate future.

The most important result of DNA variation will be the consequences for the application of direct gene transfer methods to cereal breeding programs. If regenerant plants are phenotypically and genetically altered to a significant extent, then a considerable amount of backcrossing will be necessary to remove this variation. Similarly, transformation of plants may be limited if the desired traits are transcriptionally inactivated by alterations in methylation or changes in the DNA sequence.

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Characterization of repeated DNA sequences specific to different rice genomes

F. Cordesse, A.S. Reddy, A. De Kochko, M.C. Kiefer, and M. Delseny

Repeated DNA sequences diverge very rapidly and often become species specific. We adopted two strategies to pick up such sequences in the AA and CC genomes of rice. In the first one, prominent bands on ethidium bromide-stained gels were cut out after preparative electrophoresis of restriction enzyme digests and cloned into plasmid vectors. For the AA genome, we isolated a tandemly repeated 352-bp fragment that shows the same general organization in all Oryza sativa accessions. Various rearrangements were observed in *O. rufipogon* and *O. longistaminata.* The presence of an additional Sau 3A site and a higher copy number distinguish indica types from japonicas. The sequence was not detectable in other genomes. Three tandem repeats (374, 367, and 193 bp long) were isolated from *O. officinalis* and were shown to be specific to the CC genome. These sequences permit analysis of the phylogenetic relationships between the CC species. In the second strategy, the intergenic spacer of a cloned ribosomal DNA sequence was dissected, and short sequences with various genome specificity were identified. All these specific sequences should be useful in analyzing AA/CC interspecific crosses.

The *Oryzae* tribe comprises several distinct genome types that have been defined mostly by their capacity to pair during meiosis. The cultivated species (*O. sativa* and *O. glaberrima*) belong to the AA type, as do a few related wild species (*O. rufipogon, O. longistaminata,* and *O. breviligulata*). Four other diploid genomes exist in wild species: BB (*O. minuta*), CC (*O. officinalis, O. collina,* and *O. eichingeri*), EE (*O. australiensis*), and FF (*O. brachyantha*). In addition, some species are allotetraploids with combinations of these genomes (BBCC: *O. malampuzhaensis* and *O. coarctata*) or of one of them with another genome that has not yet been identified at the diploid level (CCDD: *O. latifolia* and *O. alta*).

Besides elucidation of the phylogenetic relationships among these genomes, there is much interest in the study of wild species because rice breeders use them in interspecific crosses to introgress new favorable agronomic traits into cultivated varieties. Examples of characters that are possessed by the CC genome are resistance to blast, planthoppers, and drought.

The discovery that some repeated DNA sequences are highly species-specific (Grellet et al 1986) prompted us to search systematically for repeated sequences in the AA and CC genomes.

Materials and methods

A list of plant material used in this study as well as a description of growth conditions has recently been published (Cordesse et al 1990).

DNA was prepared from leaves by standard methods. It was digested with various restriction enzymes, and the resulting fragments were separated by agarose gel electrophoresis. Then the DNA was transferred from the gel to a nylon membrane and hybridized with the appropriate probe.

With several restriction enzymes, prominent DNA bands were readily detected on ethidium bromide-stained gels. These DNA fragments were recovered from the gel and used as hybridization probes after labeling by nick translation. Following this preliminary characterization, the fragments were ligated in vitro with the appropriate vector and amplified by cloning in *Escherichia coli.* Resulting colonies were screened by hybridization with the initial fragment eluted from a gel, and positives were further characterized by minipreparation of plasmids, restriction mapping, and sequencing (Maniatis et al 1982).

Results

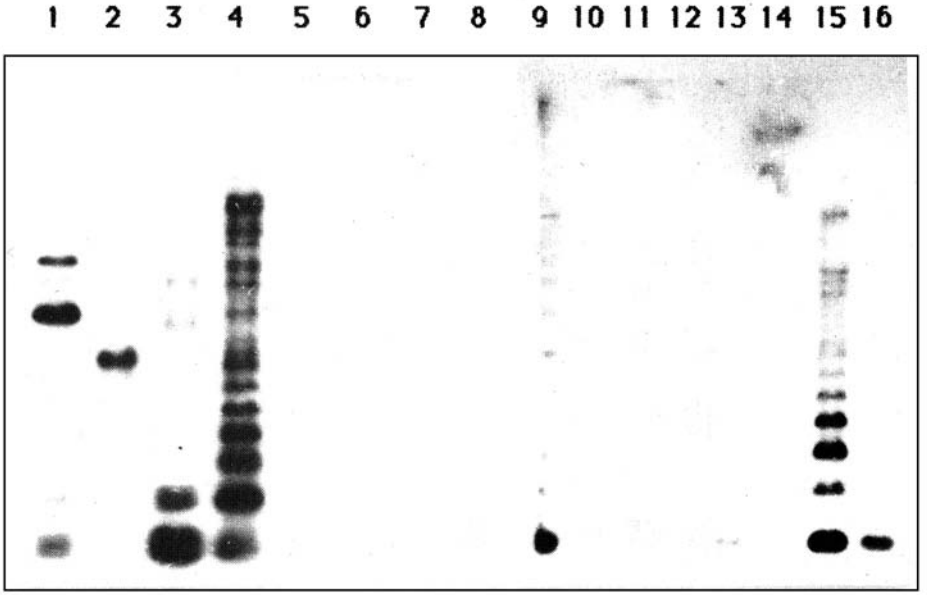
In this report we describe the isolation and preliminary characterization of one AA- and three CC-specific repeated sequences. We also demonstrate that species-specific repeated sequences are found in the intergenic spacer sequence between adjacent ribosomal RNA genes.

A 352-bp tandem repeat specific to the AA genome

From the cultivar Cigalon, a 360-bp *Eco*RI fragment was cloned and sequenced (De Kochko et al 1991). The sequence is 94% homologous to that isolated from the cultivar Labelle (Wu and Wu 1987). This cloned fragment was used as a hybridization probe to look for identical or related sequences in various *Oryzae* having different genomes.

An example of theresults is shown in Figure 1. Very clearly, the sequence is specific to the AA genome, being undetectable in the BB, CC, BBCC, CCDD, and EE genomes. As indicated by the typical ladder pattern, the sequence is generally organized as blocks of tandem repeats. However, during our survey of the AA accessions we observed a number of interesting variations both in copy number and sequence organization.

On the basis of copy number, the AA accessions can be separated into two groups. One comprises indica subtypes of *O. sativa, O. longistaminata,* and a fraction of *O. rufipogon* with a few thousand copies. The other group has a much lower copy number (a few hundred or less) and is composed of the japonica subtypes of *O. sativa, O. glaberrima, O. breviligulata,* plus the remaining *O. rufipogon.* Examples of reampli-



1. Hybridization with AA-specific 358-bp repeated fragment. DNA from various rice accessions was digested with *Eco*RI, blot-transferred, and hybridized with plasmid containing 358-bp AA fragment as an insert. Lane 1 = *O. rufipogon* (DN41), 2 = *O. rufipogon* (100968), 3 = *O. rufipogon* (W1655), 4 = *O. longistaminata* (EL34), 5 = *O. officinalis* (W65), 6 = *O. officinalis* (DO 4), 7 = *O. alta* (W17), 8 = *O. coarctata* (W551), 9 = *O. sativa* (58881), 10 = *O. officinalis* (101314), 11 = *O. minuta* (W1344), 12 = *O. officinalis* (W1306). 13 = *O. breviligulata* (WB35). 14 = *O. malampuzhaensis* (W1159), 15 = *O. longistaminata* (EL 15-17), 16 = *O. sativa* (Cigalon).

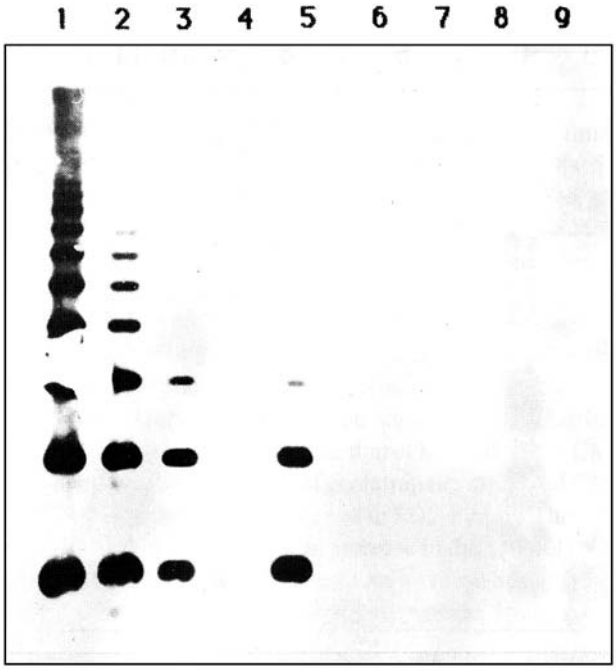
fication of multimers were observed in several high-copy-number *O. rufipogon*, while several *O. longistaminata* have a double ladder pattern, indicating the presence of several subfamilies within these species.

The use of 4-bp cutter enzymes allowed us to recognize two basic patterns discriminating between the japonica and indica types. These two patterns were also observed in different accessions of *O. rufipogon.* This observation lends additional support to the hypothesis that present-day *O. sativa* have a biphyletic origin (Second 1982).

Three tandemly repeated sequences specific to the CC genome

A similar strategy was used to isolate tandemly repeated sequences from *O. officinalis* accession W1278. Two distinct repeats of 374 and 367 bp as well as another of 193 bp were isolated by molecular cloning and were sequenced (unpubl. data). All these clones, when used as probes on the original *O. officinalis* DNA digested with the enzyme used for cloning, revealed typical ladder patterns (Fig. 2).

The 374- and 367-bp clones cross-hybridize, and sequencing revealed extensive homology (85%). These two sequences are also related to the *O. officinalis*-specific sequence recently described by Zhao et al (1989). Although the two repeated units



2. Hybridization with CC genome-specific 367-bp repeated fragment. DNA from different rice genomes was digested with SphI, electrophoresed, blot-transferred, and hybridized with in vitro-synthesized, labeled probe corresponding to 367-bp insert. Lane 1 = *O. officinalis* (CC), 2 = *O. eichingeri* (CC), 3 = *O. collina* (CC), 4 = *O. punctata* (BB), 5 = *O. minuta* (BBCC), 6 = *O. grandiglumis* (CCDD), 7 = *O. sativa* (AA), 8 = *O. australiensis* (EE), 9 = *O. brachyantha* (FF). Genome type is in parentheses.

clearly belong to two distinct subfamilies, it is not yet clear whether they are physically independent or interspersed in the genome.

The 193-bp repeat is a distinct one and does not cross-hybridize with the 2 others.

When the various genome types were analyzed with these three probes, it became clear that the three sequences are highly specific to the CC genome, since they do not hybridize with any of the others (Fig. 2). However, hybridization with the 193-bp probe is detected with the AA genome when less stringent conditions are used.

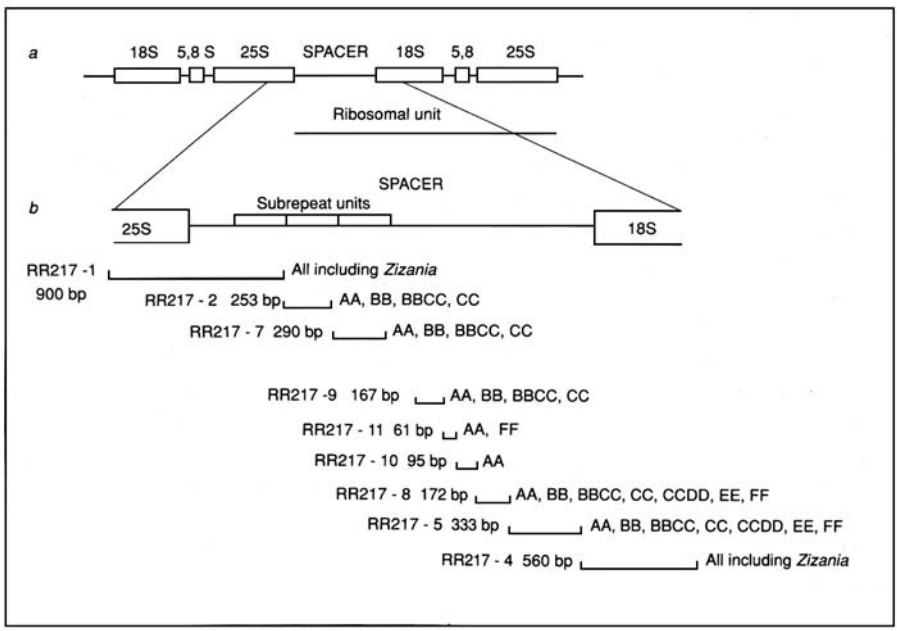
The copy numbers of the repeated elements were determined in W1278, from which they were isolated. The 374- and 367-bp repeats are present in 164,000 and 237,000 copies, respectively, and the 193-bp element is repeated approximately 200,000 times. Due to cross-hybridization, the copy numbers of the 374- and 367-bp repeats might be overestimated. Altogether, these 3 repeats account for 10–15% of the *O. officinalis* genome.

A survey of several CC genome accessions also revealed variations in copy number. We found few *O. officinalis* accessions in which both the 374- and 367-bp sequences were absent. We also obtained evidence of rearrangement. One of the most conspicuous situations is with the 193-bp repeat. In all the *O. officinalis* the typical ladder of 193-bp multimers was observed, but in *O. collina* only multimers of 750-bp (tetramers)

were observed, suggesting that a tetramer block has been amplified in this species. In *O. eichingeri,* a complex and irregular pattern was observed, suggesting that blocks of repeats are now dispersed in the genome.

A short sequence within the *O. sativa* ribosomal DNA spacer specific to the AA genome

Since we previously demonstrated that short repeats within the ribosomal DNA (rDNA) spacer are frequently species-specific (Tremousaygue et al 1988), we looked for a similar sequence in rice. Clone RR 217 (Takaiwa et al 1984; provided by F. Takaiwa) contains a full-length rDNA unit. A fragment containing all the intergenic spacer was subcloned and further dissected into nine subclones. Each was used in Southern blot hybridization experiments with DNA representatives of the various *Oryza* genomes and of that of the related genus *Zizania.* As shown in Figure 3, the genomes for these fragments showed a variable degree of homology. In the rDNA unit cloned in RR 217, there are three 260-bp short subrepeats, which account for size variation in the *O. sativa* rDNA (Cordesse et al 1989). These subrepeats hybridize with DNA from the AA, BB, BBCC, and CC genomes but not with DNA from the EE or CCDD genomes. Downstream from this repeat is a short, 95-bp sequence that hybridizes only with the AA genome. It hybridizes equally well with DNA from japonica or indica types.



3. Genome specificity of various rDNA spacer regions: a) general organization of rDNA units, b) enlargement and genome specificity of fragments subcloned from pRR217.

Fate of repeated sequences in allotetraploid genomes

The presence of the CC repeated sequences was examined in the allotetraploids BBCC and CCDD.

So far, the three CC-specific sequences (374, 367, and 193 bp) have been observed in all the BBCC accessions we have examined. Similarly, the 253-bp repeated fragment from the rDNA spacer, although not CC specific, recognizes a homologous sequence in the BBCC rDNA.

As far as the CCDD genome is concerned, the situation is completely different. None of the CC-specific repeated elements was detected in the CCDD genome. Neither did the subrepeat in the rDNA spacer that cross-hybridized with CC DNA recognize a fragment in the CCDD genome.

Discussion

We have isolated a set of repeated sequences from the AA and CC genomes. Hybridization of these cloned sequences to DNA from various cultivated and wild rices revealed that most of the sequences are highly species-specific. From a limited survey of accessions, we obtained evidence for rapid evolution of these elements involving several independent rounds of amplification and divergence as well as homogenization of each subfamily. The 352-bp AA sequence gave us further evidence for a biphyletic origin of *O. sativa* with an early separation of the indica and japonica subtypes in the presumed *O. rufipogon* ancestral population. This adds to the number of molecular methods that discriminate between these subtypes.

The CC-specific sequences should prove invaluable to trace CC introgressions into the AA genome. They have already revealed some diversity among *O. officinalis, O. eichingeri,* and *O. collina,* which are the three major CC species, and even within *O. officinalis.* We have also recently isolated a specific dispersed repeat from *O. officinalis* that reveals similar and additional variability. By using these sequences as probes, we can evaluate more precisely the phylogenetic relationships within the CC genome and elucidate the origin of allotetraploids. A surprising result from our study was the complete absence of any of the currently identified specific sequences from the CCDD genome. Several explanations can account for this observation. The CC genome might derive from a CC genome that did not contain the sequences we isolated. Part of the CC genome, and particularly repeated specific sequences, might have been completely excluded from the allotetraploid. Finally, early cytogenetic experiments might have been misleading, and the CCDD genome might result from rapid rearrangement of other rice genomes following their introduction into America, as suggested by Second (1990).

Information on the AA-specific 352-bp tandemly repeated sequence will be published soon (De Kochko et al 1991).

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Citation information: International Rice Research Institute (1991) Rice genetics II. P.O. Box 933, Manila, Philippines.

Species-specific repetitive DNA sequences as markers in rice backcross breeding programs

H. Aswidinnoor, J.F. Dallas, C.L. McIntyre, and J.P. Gustafson

The aim of this project was to isolate genomic DNA sequences specific to wild species of rice that could be used to study the nature and extent of alien chromatin introgression into progeny of interspecific crosses with cultivated rice (*Oryza sativa* L.). The results suggest that extensive divergence in repetitive DNA sequences has occurred between rice species belonging to different genome groups. A repetitive DNA sequence (pOm6) isolated from *O. minuta,* a tetraploid wild rice (BBCC genome), showed no detectable hybridization with *O. sativa* (AA genome). In addition, it hybridized with other wild rice species possessing the CC genome. The potential use of such species-specific sequences in backcross breeding programs is discussed.

Wild species of the genus *Oryza* represent a rich source of agronomic traits for rice (*O. sativa* L.) improvement, including genes for disease and insect resistance; for tolerance for abiotic stresses such as drought, excess water, and problem soils; and for increased biomass (Sitch 1990). The transfer of such traits to cultivated rice is becoming an important part of current breeding programs.

A typical cultivar possesses many desirable properties but may lack specific traits known to reside in wild relatives. In such cases, backcross breeding has been the method of choice for introduction of desirable traits from wild species to cultivated varieties. The inherent problem in such crosses is that many undesirable characteristics of the wild species are transferred along with the desirable genes. In rice, this problem is coupled with other constraints: routine cytogenetic procedures have insufficient resolution to characterize wild cross progeny for the presence of alien inserts; and both the desirable and unwanted traits may be difficult to score, especially at the seedling stage. Therefore, other methods for scoring the presence of alien inserts are needed.

New markers could be used in the selection procedures, in addition and complementary to existing conventional selection based on phenotypic performance. DNA markers can be used together with isozymes for this purpose. One advantage of using molecular markers in the selection process is that they are free from interaction with the environment. For example, the use of restriction fragment length polymorphism

markers may facilitate selection during backcross breeding (Young and Tanksley 1989). A second potential method for monitoring alien introgression is the use of DNA sequences dispersed throughout the alien genome. Dispersed species-specific repetitive sequences could fill this need. In this context, species-specific DNA sequences are defined as those present in the wild species (donor parent) but absent from the cultivar (recurrent parent). Such sequences can be used as hybridization probes to examine the nature and extent of chromatin introgression (Appels and Moran 1984).

A substantial fraction of the genome of higher eukaryotes is known to be composed of moderately and highly repetitive DNA sequences. The occurrence of such sequences in several cereal species has been studied and reported (Appels and Moran 1984; Flavell 1980; McIntyre et al 1988; Rayburn and Gill 1986; Rimpau et al 1978, 1980). Some repetitive sequences are unique to a species, while others occur in two or more species. Recent evidence has established that species-specific repetitive DNA sequences also occur in rice (Zhao et al 1989).

O. minuta, a tetraploid wild rice (BBCC genome), is resistant to brown planthopper, whitebacked planthopper, green leafhopper, blast, and bacterial blight (Sitch 1990). All these pests and pathogens cause serious problems in rice production. *O. minuta* is currently being crossed with cultivated rice to transfer these valuable resistance traits. The present study describes one method for isolating species-specific DNA sequences, illustrates an example of such a sequence from *O. minuta*, and discusses possible uses of that sequence as a marker in backcross breeding programs.

Materials and methods

Seed of the rice species used in this study was obtained from the International Rice Research Institute, Philippines (Table 1). Plants were grown in a growth chamber (21/29 °C, 12-h cycle, 70% relative humidity) and under similar conditions in a greenhouse. Leaf material was harvested, lyophilized for 3-4 d in a Labconco freeze-drier, and powdered using a Tectator Cyclotec sample mill (Fisher Scientific).

Genomic DNA was extracted according to the method of Saghai-Maroof et al (1984). Restriction enzyme digests, gel electrophoresis, Southern blotting, hybridization, and autoradiography were done using standard methods (Maniatis et al 1982), except that 25 mM $NaPO_4$ (pH 6.5) was used as the Southern transfer buffer. Hybridization probes were prepared by labeling DNA fragments with ^{32}P-dCTP using the random-primer method (Feinberg and Vogelstein 1983).

Candidate species-specific DNA sequences from *O. minuta* accession #101141 were identified by restriction enzyme digestion and parallel gel electrophoresis of genomic DNA samples of *O. sativa* and *O. minuta*. Distinct bands present in the *O. minuta* lane and absent from the *O. sativa* lane were cut from the gel and isolated by electroelution in dialysis tubing (Maniatis et al 1982). To assess their species-specificity, these crude band preparations were used as hybridization probes onto dot blots of genomic DNA from *O. minuta* and *O. sativa*. Those that hybridized strongly to *O. minuta* but weakly to *O. sativa* were either cloned directly into the plasmid vector

Table 1. Rice species used in the study.

Species	Cultivar or accession	Chromosomes[a] (no.)	Genome group[a]
Oryza sativa	IR36, IR54, IR64	24	AA
O. nivara	103839	24	AA
O. rufipogon	100907	24	AA
O. perennis	104453	24	AA
O. punctata	103888, 103906	24	BB
O. punctata	101409	48	BBCC
O. officinalis	100896	24	CC
O. eichingeri	101422, 101425	24	CC
O. minuta	101089, 101125, 101141	48[b]	BBCC
O. malampuzhaensis	100957	48[b]	BBCC[b]
O. latifolia	100963, 100966	48	CCDD
O. alta	101395	48	CCDD
O. grandiglumis	101405	48	CCDD
O. australiensis	100882	24	EE
O. brachyantha	101231	24	FF

[a]Sitch (1990).[b] Nayar (1973)

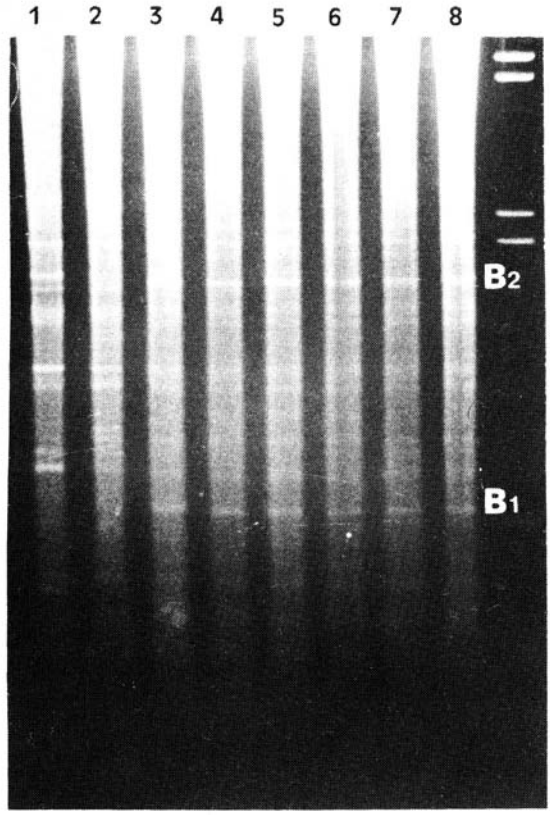
pUC 19, which had been linearized with the restriction enzyme BamHI and treated with alkaline phosphatase, or used as hybridization probes to select clones from a pUC19 library. The latter contained size-selected (<1 kb) *Taq*I fragments of genomic DNA from *O. minuta* inserted into the A*cc*I cloning site. The size was selected by glycerol gradient centrifugation, and the library was screened using Grunstein-Hogness filters (Maniatis et al 1982).

Results

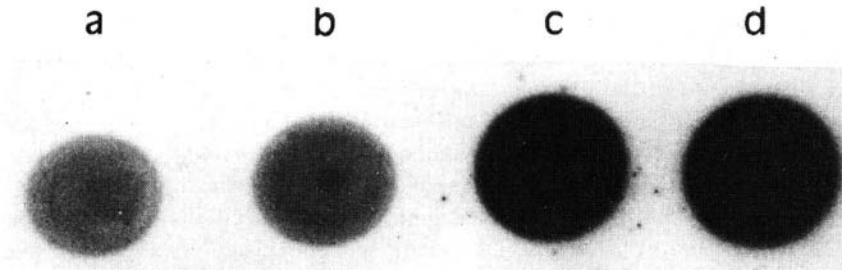
When genomic DNA of *O. minuta* was digested with a restriction enzyme, electrophoresed on an agarose gel, and stained with ethidium bromide, the resulting smear pattern showed many strong bands of various lengths. These strong bands most likely represented repetitive DNA families, because they consisted of large numbers of DNA fragments of similar size migrating to the same position on the gel.

Figure 1 shows genomic DNA of different rice species digested with *Bam*HI. Two distinct bands, B1 and B2, were considered as candidate *minuta*-specific sequences, since they appeared in the *O. minuta* DNA (lanes 3-8) but not in that of *O. sativa* (lane 1) or *O. officinalis* (lane 2). These bands were isolated and tested for specificity by using them as probes to dot blots of genomic DNA of *O. minuta* and *O. sativa* B1 hybridized more strongly with *O. minuta* and *O. officinalis* than it did with *O. sativa* (Fig. 2). The band showing the highest degree of species specificity (B1) was selected and cloned to obtain a purified species-specific sequence. The cloned sequence pOm6 was obtained by screening a plasmid library containing *Taq*I-digested genomic DNA fragments using DNA from band B1 as a probe. pOm6 contains a 350-bp insert.

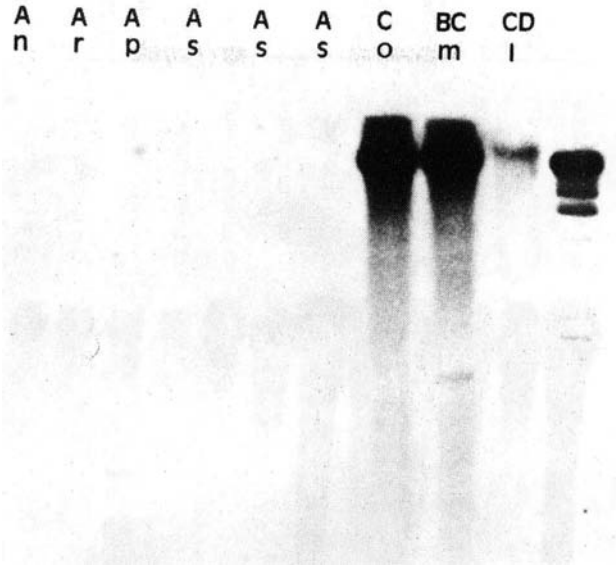
Figure 3 shows the extent of cross-hybridization between pOm6 and genomic DNA of *O. minuta, O. officinalis, O. latifolia,* and four other species containing the A genome



1. Genomic DNA of rice species digested with *Bam*HI enzyme, electrophoresed on 1% agarose gel, and stained with ethidium bromide. Lane 1 = *O. sativa* cultivar IR36 (A genome). 2 = *O. officinalis* #100896 (C genome), 3-8 = *O. minuta* #101141 (BC genome). Size marker is lambda DNA digested with *Hind*III. B1 and B2 are distinct bands present in *O. minuta* but not in *O. sativa.* These distinct bands were then cut out from gel, eluted, and analyzed further as described in text.



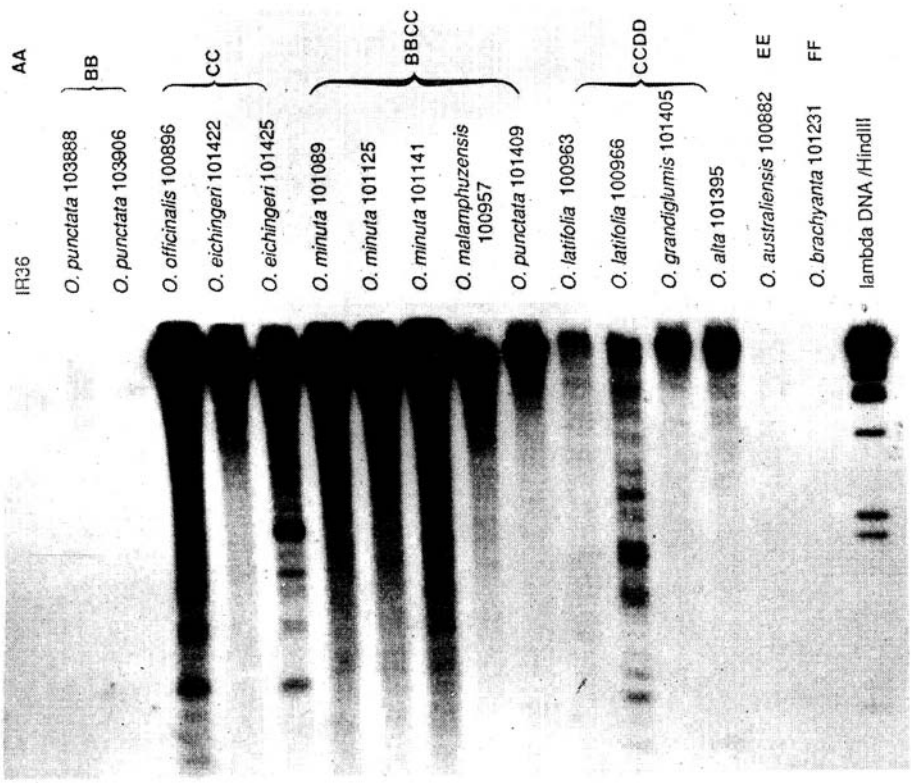
2. Hybridization of eluted DNA from B1 band to dot blot of genornic DNA of rice. a = *O. sativa* cultivar IR36 (A genome), b = *O. punctata* #10388 (B genome), c = *O. officinalis* #l00896 (C genome), d = *O. minuta* #l01141 (BC genome). 1 µg genomic DNA was spotted in each dot. Hybridization was carried out at 5 × salt-sodium citrate (SSC) at 65 °C, and washed at 0.1 × SSC at 65 °C.



3. Hybridization of pOm6 to *Eco*RI-digested genomic DNA of rice. Left to right: Rice accessions from A-genome group (n = *O. nivara* #103839; r = *O. rufipogon* #1 100907; p = *O. perennis* #104453; s = *O. sativa* cultivars IR36, IR54, IR64, respectively, and 3 wild species (o = *O. officinalis* #l00896 [C genome], m = *O. minuta* #l01141 [BC genome], 1 = *O. latifolia* #l00963 [CD genome]). 6 µg of digested genomic DNA was loaded onto each lane, electrophoresed on 1% agarose gel, and blotted onto nylon filter as described in text. Lambda DNA digested with *Hind* III was run as marker. Hybridization was carried out in 5 × SSC at 65 °C for 18 h, followed by washing in 2 × SSC at 65 °C.

(*O. sativa, O. nivara, O. rufipogon,* and *O. perennis).* The specificity of pOm6 for *O. minuta* and *O. officinalis,* and more weakly for *O. latifolia,* can be seen. There was no detectable hybridization to any of the A-genome species, including *O. sativa* (cultivated rice). The pOm6 sequence was also hybridized to genomic DNA from different accessions of each of the genome groups (Fig. 4). Again, the specificity of the probe for species possessing the C genome can be seen. Since pOm6 hybridizes to more than one wild species containing the C genome, this sequence will be useful for analyzing progeny from wide crosses involving *O. officinalis, O. eichingeri, O. malampuzhaensis, O. punctata, O. latifolia, O. grandiglumis,* and *O. alta,* in addition to *O. minuta.*

The pOm6 probe has been hybridized to pOa4, pOo2, and pOb1 as reported by Zhao et al (1989) and does not show cross hybridization utilizing 0.1 × salt-sodium citrate (SSC) at 65 °C wash conditions. Furthermore, it does not cross-hybridize to the mitochondrial and chloroplast DNA isolated from IR36 (provided by V. Walbot of Stanford University and K. Tsunewaki of Kyoto University, Japan, respectively).



4. Hybridization of pOm6 to *Eco*RI-digested genomic DNA from different species of rice. Hybridization and wash conditions were the same as in Figure 3.

Discussion

There are two requirements for the use of DNA markers in wide-cross breeding programs. First, isolating plant DNA requires rapid and reliable methods that do not inhibit plant growth and development. Second, rapid, inexpensive, and large-scale methods are needed for analyzing the introgression of alien chromatin from wild species into cultivated varieties.

Numerous procedures for isolating DNA from plant material have been published. Most of them were developed to overcome specific difficulties or to satisfy certain objectives associated with the particular program and type of plant being analyzed. Clarke et al (1989) reported a simplified procedure for isolating plant DNA that utilizes a sap extractor. They reported that approximately 1 g of fresh leaves yielded enough DNA for 10-20 analyses, and approximately 500 samples could be processed in 1 wk. Large numbers of samples from segregating populations and advanced backcross lines could be analyzed using this method, which could fit in well with a plant breeding program when suitable DNA markers are available. We intend to adapt this procedure

for analyzing alien introgression in segregating populations and advanced backcross lines derived from wide crosses in rice.

The dot blot procedure, in conjunction with species-specific probes, could be utilized to analyze the progeny of backcross materials. The isolated DNA is spotted onto a filter, fixed, and hybridized to a species-specific probe. Complementary to phenotypic determination and selection, the presence of alien chromatin could be identified by the positive signal of hybridization on the dot blot after washing at determined stringency. Individuals or lines that show positive hybridization could be further analyzed.

The potential value of wild rice species as sources of molecular markers for plant breeding purposes will be increased if DNA markers are dispersed over as much of the wild genome as possible. This can be accomplished using a single dispersed probe or a combination of several probes that show specificity to the wild species in question.

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Organization, structure, and expression of the rice a-amylase multigene family

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E. Karrer, and J. Litts

To better understand rice seed germination, we cloned and characterized the a-amylase genes. Ten distinct genes were identified and classified into three subfamilies: *RAmy1A-1C, RAmy2A,* and *RAmy3A-3F.* Trisomic mapping placed the genes on chromosomes 1, 2, 6, 8, and 9. Several genes were found to be linked as a result of duplication (e.g., *RAmy3D* and *RAmy3E*) and triplication (e.g., *RAmy3A, 3B,* and *3C*) events. DNA sequence analysis of the genes revealed the presence of either two or three introns. The positions of the introns are conserved in all cereal a-amylase genes examined. Amino acid sequence comparisons indicated that the a-amylase genes are conserved, with homology values ranging from 60 to 95%. RNA studies indicated that *RAmy3D* is moderately expressed during early germination in the scutellar epithelium, while *RAmy1A* is highly expressed at later times in the aleurone layer.

In cereals, seed germination and subsequent seedling growth require the hydrolytic breakdown of the starchy endosperm into metabolizable sugars such as maltose and glucose. Although a number of enzymes are involved in this process, a-amylase is the enzyme primarily responsible for the endoglycolytic cleavage of amylose and amylopectin (see Akazawa and Hara-Nishimura 1985 for a review). Previous studies have shown that a-amylase activity is the result of several isozymes and that these isozymes are encoded by multigene families in barley and wheat (Gale et al 1983, Huttly et al 1988, Khursheed and Rogers 1988. Knox et al 1987). In barley, a-amylases are classified into types A (or low pI) and B (high pI), with each type consisting of multiple isozymes (Jacobsen and Higgins 1982). In germinating wheat seeds, 27 isozymes have been identified (Gale et al 1983). These isozymes are encoded by various members of three a-amylase gene subfamilies, designated Amy1, Amy2, and Amy3 (Baulcombe et al 1987).

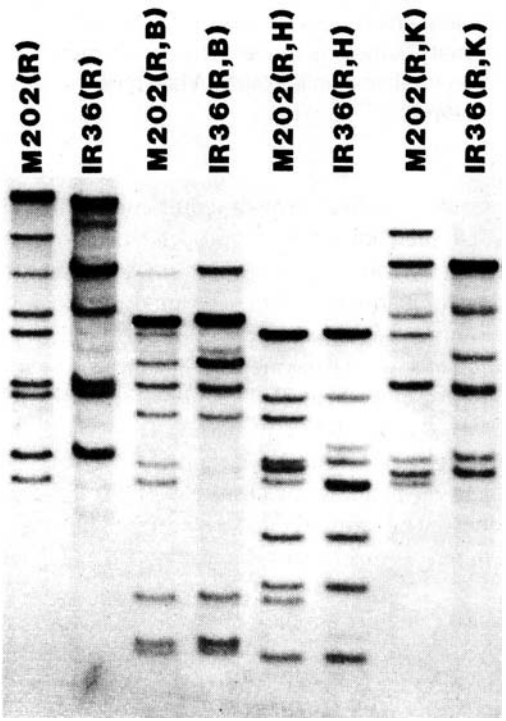
Isoelectric focusing gels reveal at least three a-amylase isozymes in extracts of germinating rice seeds (Daussant et al 1983, Miyata and Akazawa 1982), while four rice a-amylase isozymes can be detected immunologically after in vitro translation of poly $(A)^+$mRNA isolated from germinating seeds (Miyata and Akazawa 1982). It is

still unclear how many isozymes are produced during rice seed germination, or whether they correspond to the two isozyme groups found in germinated barley and wheat seeds.

The short-term objective of this research is to elucidate the structure, function, and regulation of the rice α–amylase multigene family. Our long-term objective is to use this information to manipulate and eventually enhance the germination characteristics of rice. Toward these ends, we have defined the size, structure, and organization of the rice α-amylase genes. Nucleotide sequence analysis of the rice α-amylase genes has enabled us to compare them with other cereal α–amylase genes. The results of these analyses indicate that the cereal amylases are highly conserved proteins that fall into three gene subfamilies. We hope that this research will provide the information needed to establish a consistent classification scheme for the cereal α amylases and to develop successful strategies for genetically manipulating the process of rice seed germination.

Number and genomic organization of the rice α-amylase genes

The Southern blot shown in Figure 1 provides an estimate of the size and complexity of the rice a-amylase gene family. The blot was constructed using genomic DNA



1. Genomic Southern blot comparing cultivars M202 and IR36. Genomic DNA was digested with restriction enzymes in single or double digests as indicated above their respective lanes. R = *Eco*RI, B = *Bam*HI. H = *Hin*dIII, K = *Kpn*I. Digested DNAs were electrophoresed on agarose gels pairwise such that restriction fragment length polymorphism could be identified between the two varieties. The blot was then probed at low stringency (T_m-30) with the rice a-amylase cDNA, pOS103 (O'Neill et al 1990).

isolated from rice varieties IR36 and M202. M202 is a medium-grain, short-statured, photoperiod-insensitive rice. As Figure 1 shows, 8–10 bands were observed for both genomic DNAs digested with 1 or with pairs of restriction enzymes. This suggests that rice contains about 10 α-amylase genes, and that M202 and IR36 are monomorphic for some restriction fragments containing α-amylase genes. This is particularly evident in the lanes containing *Eco*RI/*Hin*dIII double-digested DNA. Of the 18 bands visible in these 2 lanes, M202 and IR36 are polymorphic for only 4.

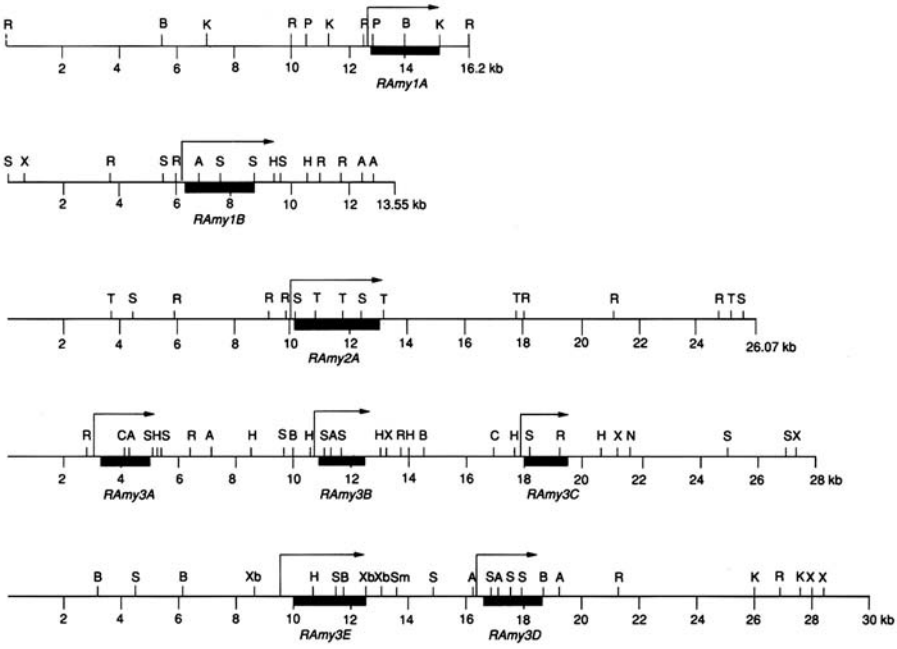
Gene cloning and classification

To clone all rice αa-amylase genes, we isolated 83 clones from 3 independent rice genomic libraries. These libraries were constructed in phage vectors EMBL3 and EMBL4 using total M202 DNA, and in Charon 35 using DNA from the rice cultivar M201/M101. DNA from 30 of these genomic clones, (including 1 from Charon 35) was prepared for further study. The 30 clones were then subjected to slot-blot analysis using different rice α-amylase probes and a regimen of low-to-high stringency hybridization to sort the clones into 5 distinct hybridization groups (Huang et al 1990b). Because of the high probability that many of the 30 genomic clones overlapped on their 5' and 3' ends, organizing them into 5 hybridization groups was a critical step in the gene classification process. α-amylase genomic clones were not evenly distributed among the five hybridization groups: Group 1 contained three clones; Group 2, five clones; Group 3, fourteen clones (1 clone was found to be rearranged during cloning; Sutliff et al 1990); Group 4, seven clones; and Group 5, one clone. The members of each group were further analyzed by Southern blotting and restriction enzyme mapping to produce the combined physical and genetic map shown in Figure 2.

Physical and genetic map of the rice α-amylase multigene family

Figure 2 shows that rice contains eight α-amylase genes that occupy five chromosomal loci. As will be discussed later, these genes have been divided into three gene subfamilies and given the designations *RAmy1A* and *B, RAmy2A,* and *RAmy3A-E.* Subsequent studies have provided evidence for two additional α-amylase genes. Huang et al (1990a) have shown by Southern blot analysis the presence of a *RAmy1C* gene on a 15.5-kb *Eco*RI (or 5.7-kb *Kpn*I) restriction fragment. This gene can be seen in the first lane of Figure 1, second band from the top of the gel. In the course of mapping the rice α-amylase genes using Southern blot analysis of primary trisomic DNA from IR36, Ranjhan et al (1991) identified a *RAmy3F* gene. Whether or not *RAmy3F* is in our collection of 83 clones is being investigated; we have not yet found *RAmy1C* among them.

One of the most interesting features of the rice α-amylase genes is their clustering in the RAmy3 subfamily. *RAmy3D* and *3E* are located only 4 kb apart, while *RAmy3A, B,* and *C* are separated by 6 kb and 5 kb, respectively. Preliminary mapping data indicate that the *RAmy3D* and *E* genes are on chromosome 8, the *RAmy3A-C* genes are on chromosome 9 (Table 1). The clustering of these genes appears to be the result of well-documented gene duplication events promoted by unequal crossing over at



2. Restriction map of 5 rice chromosomal segments containing 8 a -amylase genes derived from overlapping rice genomic clones and presented as contiguous maps. Restriction enzymes are abbreviated (A = *Apa*I, B = *Bam*HI, C = *Cla*I, H = *Hind* III, K = *Kpn*I, P = *Sph*I, R = *Eco*RI, S = *Sal* I, Sm = *Sma*I, T = *Sst*I, N = *Nru*I, X = *Xho*I, Xb = *Xba*I). a -amylase genes are denoted by black boxes with arrows showing both direction and start of transcription. Gene name shown below each gene is derived from subfamily assignment (*RAmyl, RAmy2,* or *RAmy3*) followed by a letter for each gene. The relationship between this nomenclature and original genomic clones (Huang et al 1990b) is as follows: *RAmy1A* from lambda OSg2, *RAmy1B* from lambda OSg3A, *RAmy2A* from lambda OSg9C, *RAmy3A* from lambda OSg7D. *RAm3B* from lambda OSgl, *RAmy3C* from lambda OSg1, *RAmy3D* from lambda OSg1A, and *RAmy3E* from lambda OSg7F.

Table 1. Ratios of *RAmy 1, 2,* and 3 a-amylase genes to *Em* gene.[a]

Trisomic	*RAmy1*			*RAmy2*	*RAmy3*	
	A	*B*	*C*	*A*	*A* and *B*	*C*
T-1	1.70	1.40	0.74	1.24	1.00	1.00
T-2	2.50	1.00	1.47	1.55	1.15	0.88
T-3	1.60	1.20	1.18	1.85	0.92	0.84
T-4	2.25	1.20	0.86	1.12	0.73	1.08
T-5	0.95	0.70	0.71	1.06	0.62	0.40
T-6	1.30	1.00	0.86	1.50	1.62	0.64
T-7	1.70	1.10	1.10	1.40	3.10	1.00
T-8	1.70	1.27	1.15	1.21	1.00	0.56
T-9	1.70	1.10	0.95	1.63	3.76	1.72
T-10	1.65	1.15	1.00	1.54	3.00	1.00
T-11	1.70	1.16	0.86	1.16	3.90	0.92
T-12	1.45	1.12	0.99	1.60	3.18	1.00

[a]Boldface indicates highest and lowest values.

repetitive DNA sequences (Sutliff et al 1991). Since clustering has been reported for several genes of different plant species, we expect that clustering of α-amylase genes in other monocot species will be found when more genes are cloned.

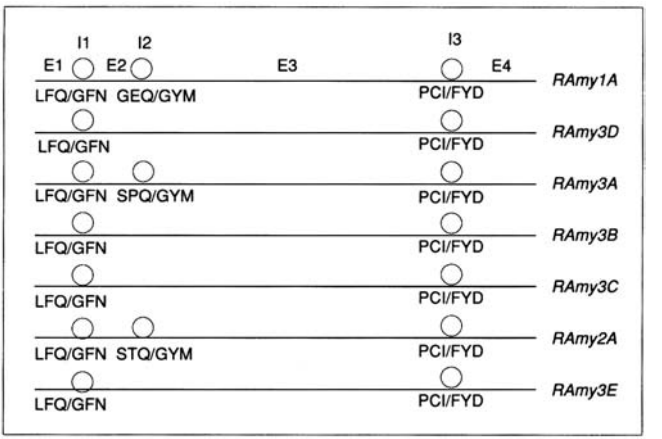
Nucleotide and amino acid sequence analysis

Using standard M13 and plasmid DNA sequencing methods, all rice α-amylase genes shown in Figure 2 were completely (Huang et al 1990a, b; Sutliff et al 1991) or partially (unpubl. data) sequenced. These sequence data, in combination with those available for other cereal α-amylase genes, have been extremely helpful in developing a better understanding of α-amylase gene structure and evolution.

Intron and exon organization

The number and location of introns in seven rice α-amylase genes are shown schematically in Figure 3. Although the number of introns is either two or three, the insertion sites are fixed. Analysis of eight additional α-amylase genes from wheat and barley suggests that this intron and exon organization is conserved among the cereal α-amylases. In the rice α-amylases, introns range from a minimum of 76 bp for the first intron of *RAmy3B* to a maximum of at least 1200 bp for the second intron of *RAmy2A* (Huang et al 1990b; unpubl. data). It is likely that the ancestral α-amylase gene contained three introns, and that during the course of evolution, one intron was lost in some genes. This interpretation is consistent with the current model for intron evolution (Doolittle 1987).

An interesting consequence of highly conserved intron insertion sites is the conservation of amino acids flanking these sites. Consistent with the invariant

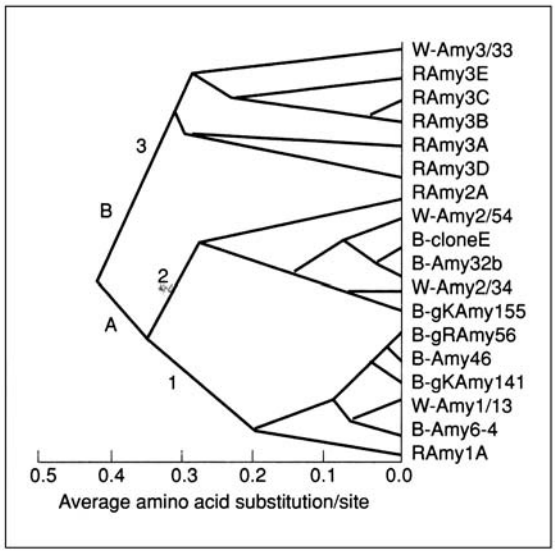


3. Schematic representation of intron (I) and exon (E) positions in *RAmy1, RAmy2, and RAmy3* genes. Letters below horizontal lines correspond to one-letter amino acid residues translated from the DNA sequence surrounding the intron splice sites. Slash (/) indicates intron position in the DNA sequence.

Table 2. Genetic distance of 17 a-amylase genes.[a]

Clone	Clone															
	46	56	6-4	E	141	155	3D	3B	3C	1A	3A	3E	2A	1/13	2/54	3/33
B-Amy32b	72.27	70.85	72.99	96.68	69.67	87.44	67.54	67.30	66.11	73.46	64.22	66.82	76.30	71.80	92.89	63.04
B-Amy46		97.90	94.86	72.43	96.50	68.69	70.09	69.63	68.93	83.18	66.59	68.93	68.93	95.07	71.26	64.73
B-gRAmy56			93.22	71.16	96.51	67.91	68.84	68.37	67.91	81.86	65.35	68.14	67.67	93.90	70.00	64.49
B-Amy6-4				73.13	92.52	69.39	69.39	69.63	68.69	83.41	65.65	67.52	69.63	93.90	71.96	64.73
B-cloneE					70.00	88.13	66.74	66.97	65.98	72.64	63.78	66.89	76.99	71.83	94.52	65.46
B-gKAmy141						66.98	67.21	67.67	66.51	80.70	63.95	66.74	66.74	92.72	68.84	62.56
B-gKAmy155							62.61	63.01	62.56	68.51	68.90	61.64	70.55	68.08	84.70	61.59
R-RAmy3D								75.23	74.77	67.13	73.62	72.71	64.68	68.54	65.60	67.87
R-RAmy3B									95.21	67.13	75.63	77.85	64.24	67.37	66.67	73.91
R-RAmy3C										65.75	74.20	78.54	63.93	66.43	65.30	73.67
R-RAmy1A											62.99	66.21	68.28	82.16	71.26	64.01
R-RAmy3A												72.15	60.77	65.02	63.70	69.81
R-RAmy3E													61.64	67.61	65.75	76.09
R-RAmy2A														68.31	75.11	58.45
W-Amy1/13															71.13	63.29
W-Amy2/154																64.49
W-Amy3/33																

[a]Horizontal clones are abbreviated. See Figure 4 caption for sequence sources. B = barley, R = rice, W = wheat.



4. Cereal α-amylase phylogenic tree constructed from comparison of 17 α-amylase amino acid sequences (Table 2). Relationship between Amy2/34 (Huttly et al 1988) and gKAmyl55 was calculated based on partial DNA and amino acid sequence. Sources of sequences used are as follows: Amy1/13, Amy2/54 and Amy3/33 (Baulcombe et al 1987): *RAmy3E* and 3D (Huang et al 1990a); *RAmy3A*, *3B*, and *3C* (Sutliff et al 1990); *RAmy2A* (unpubl. data); clone E (Rogers and Milliman 1983); RAmy32b (Whittier et al 1987); gKAmy155 and gKAmyl41 (Knox et al 1987); gRAmy56 (Rahmatullah et al 1989): Amy46 and Amy6-4 (Khursheed and Rogers 1988); *RAmy1A* (Huang et al 1990b). B = barley, W = wheat, R = rice.

positions of introns I1 and I3 is the conservation of six amino acids that flank these sites. Amino acids flanking the site for the second intron (I2), on the other hand, vary on the 5' side of the splice site (Fig. 3). The conservation of amino acids in and around intron splice sites is an interesting example of enzyme structure being determined by the need for RNA processing as well as by catalytic requirements. If introns I1 and I3 are essential for enzyme structure and function, this might explain why cereal α-amylase genes with only one intron have not yet been found. Whether or not these intron splice sites are separating functionally important domains (Branden et al 1984) remains to be seen.

Protein similarity

By comparing the amino acid sequences of 7 rice α-amylases and 10 α-amylases from wheat and barley, we were able to approximate the phylogenetic relationship between the rice, barley, and wheat α-amylases and their respective subfamilies. A pair-wise comparison of 17 amino acid sequences was performed, and protein similarity values are summarized in Table 2. These values were calculated using the Sequence Analysis Software Package (Devereux et a1 1984), and the corresponding phylogenetic tree (Fig. 4) was generated by average linkage cluster analysis using the Statistical Analysis System statistical package (SAS Institute, Inc., Cary, North Carolina, USA). As Figure 4 shows, the cereal α-amylases probably evolved from a single progenitor gene with three introns and, by gene duplication, diverged into the A and B gene classes and eventually into the current Amyl, Amy2, and Amy3 subfamilies. Most of the α-amylase genes in rice fall into the Amy3 subfamily, while only one rice gene, *RAmy2A*, is found in the Amy2 subfamily. In barley (Khursheed and Rogers 1988) and wheat

(Huttly et al 1988), the Amy2 subfamily contains 3-4 and 10-11 genes, respectively. This leads us to speculate that selection pressure (e.g., vigorous germination) is not equal among the cereals and results in the expansion of a particular a-amylase gene subfamily. The availability of a-amylase clones and the ability to transform rice should make it possible to test whether the number of a-amylase genes in a subfamily has any influence on cereal seed germination.

a-amylase "signatures"

Visual inspection of 17 aligned a-amylase amino acid sequences revealed that the cereal a-amylases are highly conserved proteins. They range from 430 to 440 amino acids in length and show significant divergence at only three locations: the signal peptide, the carboxy terminus, and a small region around consensus residue 280. When the variable region at position 280 was compared and ordered into groups, the a-amylase genes fell into the same Amy subfamilies predicted by the full amino acid sequence comparisons (Table 3). We call these variations "a-amylase signatures," because we believe that inspection of this region alone may indicate the subfamily to which a cereal a-amylase gene belongs. The validity of this speculation will be tested as more cereal a-amylase genes are sequenced. Whether or not a-amylase signatures can be used to classify genes outside the cereals is currently being investigated.

Trisomic mapping of the a-amylase genes

Although nulli-tetrasomics and addition lines are not available in rice, mapping of genes to chromosomes has been accomplished using Southern blot analysis of genomic DNA from primary trisomics (McCouch et al 1988). The rationale for this approach is based on the assumption that the critical trisomic (*AAA*), having three copies of the chromosome from which the restriction fragment originates, will exhibit gene dosage effects for that fragment. These effects can be detected as an increase in hybridization signal for the fragment of unknown location in Southern blots of DNA from the critical trisomic. Trisomics that are not critical (*AA*) will behave as disomics and will not show increased dosage of the restriction fragment in their DNA blots (Young et al 1987).

We have used inbred primary trisomics to map known genetic sequences on their respective chromosomes (Ranjhan et al 1991). Trisomics developed from indica rice IR36 were identified cytologically and morphologically by Khush et a1 (1984). We used one of two types of internal standard: another single-copy genomic fragment detected by a different probe, or another restriction fragment of known location detected by the same probe (for multicopy fragments or genes). Although any fragment can be used to make intralane comparisons, single-copy genomic sequences are preferable. In this way, two or more genes can be mapped simultaneously if they are on different chromosomes. No dosage effect is apparent if both genes are on the same chromosome. In this study, *Em,* an abscisic acid-regulated gene expressed late in embryogenesis (Litts et al 1987, Williamson and Quatrano 1988), was used as an internal control. Table 1 shows the a-amylase-to-*Em* ratios for 7 a-amylase genes in each of the 12 trisomic lines. The highest ratio indicates the chromosomal location of

Table 3. Alignment of 17 α-amylase protein sequences showing α-amylase signature region around amino acid 280.[a]

Gene	280 region	Subfamily
B-Amy46	N K V G G S G P A T T . F D F T T K G I	
B-gRAmy 56	N K V G G S G P A T T . F D F T T K G I	Amy1
B-Amy 6-4	D K V G G K G P A T T . F D F T T K G I	
B-gKAmy141	N K V G G S G P A T T . F D F T T K G I	
W-Amy 1/13	N K V G G S G P G T T . F D F T T K G I	
R-RAmy1A	D R V G G A N S N G T A F D F T T K G I	
B-Amy 32b	D K V G G A A S A G M V F D F T T K G I	
B-cloneE	D K V G G A A S A G M V F D F T T K G I	
B-gKAmy 155	D K V G G A A S A G M V F D F T T K G I	Amy2
R-RAmy 2A	G Q G G W D A S P G M V F D F T T R G I	
W-Amy 2/54	D K V G G A A S A G M V F D F T T K G I	
R-RAmy 3D	N A V G G . . P A M T F D F T T K G L	
R-RAmy 3B	Q A V G G . . P A S A F D F T T K G E	
R-RAmy 3C	Q A V G G . . P A S A F D F T T K G E	Amy3
R-RAmy 3A	K Q V G G . . P A T A F D F T T K G I	
R-RAmy 3E	E G V G K . . P A T A F D F T T K G I	
W-AMy 3/33	R G V G G . . P A T A F D F P T K G V	

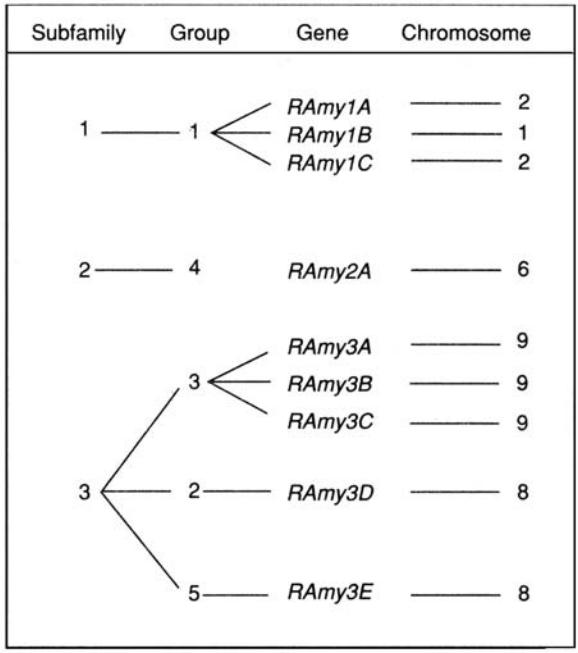
[a] See Figure 4 for sequence sources.

the α-amylase gene, while the lowest ratio indicates the location of the *Em* gene. *T*-test analysis showed that the highest and the lowest values (indicated by boldface in Table 1) were statistically different from the next nearest values in the trisomic population. The nine α-amylase genes have been mapped and localized on chromosomes 1, 2, 6, 8, and 9 (Fig. 5). In the process of mapping the α-amylase genes Ranjhan et al (1991) also determined the chromosomal location of the *Em* gene (chromosome 5) and identified a second locus for the rice alcohol dehydrogenase gene, *Adh-2* (chromosome 9).

α-amylase gene expression during rice seed germination

Any attempt to manipulate or enhance the expression of α-amylase in germinating rice seeds will require a thorough understanding of the structure and organization of the α-amylase multigene family and a knowledge of when and where each α-amylase gene is expressed during germination. Although isozyme (Miyata and Akazawa 1982) and histochemical (Okamoto and Akazawa 1979) studies on rice α-amylase have been performed, studies on α-amylase gene activity (i.e., transcription) have not.

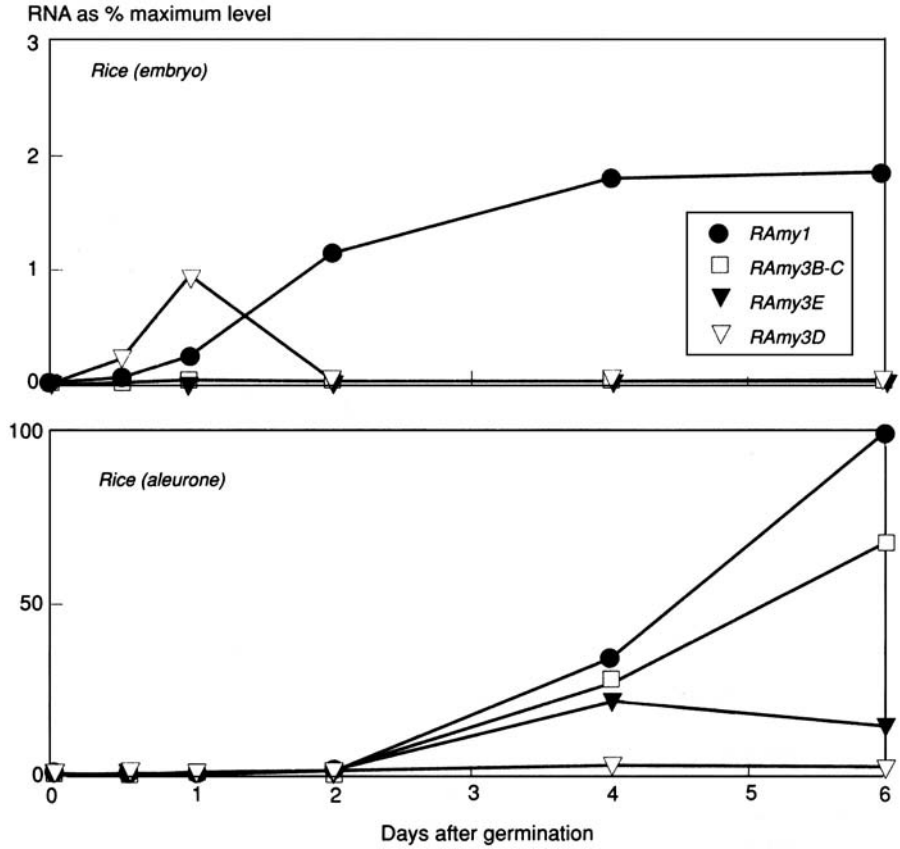
Using northern blot analysis and gene-specific and group-specific probes, we examined the steady-state levels of rice α-amylase genes in embryo and aleurone tissues during germination (Karrer et al 1991). Figure 6 shows that α-amylase messenger RNAs (mRNAs) first appeared in the embryo tissues at 0.5-1 d and were not detected in the aleurone half of the seed until 2 d after germination. In these



5. Summary of rice a-amylase genes and their chromosomal assignments. Relationships between a-amylase gene subfamilies and hybridization groups, using nomenclature adopted at this symposium, are also shown. Mapping data for *RAmy3D* and *3E* are reported by Ranjhan et al (1991).

experiments, hybridization conditions were not sufficiently stringent to discriminate among *RAmy1A, 1B,* and *1C* transcripts or between *RAmy3B* and *3C* transcripts.

Messenger RNA corresponding to *RAmy1A-C* and *RAmy3D* first appeared in embryo tissue at 0.5 d. After this time, a-amylase mRNA accumulated rapidly in the aleurone, with *RAmy1A-C, RAmy3B-C,* and *RAmy3E* appearing at 2 d, followed by *RAmy3D* at 4 d. *RAmy1A-C* genes are expressed in both tissues and exhibit similar patterns of mRNA accumulation in each. Three a-amylase genes, *RAmy3B-C* and *RAmy3E,* were preferentially expressed in the aleurone tissue. *RAmy3E* gene expression was unique in that this mRNA reached a peak of accumulation at 4 d in the aleurone layer, while levels of mRNA from the other genes continued to increase at 6 d in this tissue. *RAmy3D* was expressed in each tissue at different stages of germination; *RAmy3D* mRNA levels peaked at 1 d in embryos and were still increasing at 6 d in aleurone tissue. No hybridization signals for *RAmy2A* or *RAmy3A* transcripts were observed on rice northern blots. However, recent experiments using the RNA polymerase chain reaction (PCR) have demonstrated that these genes are transcriptionally active at very low levels in germinating seeds (unpubl. data). Additional studies using RNAPCR demonstrate that many of the a-amylase genes are expressed in other tissues such as roots, leaves, immature seeds, and callus (Huang et al 1990a).



6. Slot-blot quantification of rice a-amylase mRNA. RNA accumulation is represented as percent maximum expression of *RAmy1* at 6 d after germination. Levels of *RAmy3B-C* and *RAmy3E* mRNA were too low in embryo tissues to be detected by slot-blot analysis.

Conclusions

The rice a-amylase gene family is composed of 10 members classified into 3 subfamilies related to those previously described in barley and wheat. The relative size of each subfamily seems to vary between species. Based on current information, the largest rice subfamily appears to be underrepresented in barley and wheat. Rice a-amylase genes have been mapped on five chromosomes. Those genes that map on a single chromosome are very closely linked in two of three cases. In these two cases it appears that gene duplication arose from unequal crossing over.

The primary structures of the cereal a-amylase genes and the proteins they encode are highly conserved. Some a-amylase genes contain two introns, others three. The introns are always found at the same locations in all the cereals, although the second

intron is not always present. One particular region of the amylase protein, called the "signature" region, contains a sequence that appears to be diagnostic of the subfamily to which the gene belongs.

There are distinct patterns of mRNA accumulation for these genes with respect to tissue specificity, level of accumulation, and timing of accumulation. Genes within a subfamily appear to be more similarly regulated than are members of different subfamilies.

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Notes

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Discussion

Session 8: Molecular genetics of nuclear genomes

Q—*Janoria:* From their evolutionary relationships, would not there be a greater chance of finding diverse transposable elements in wild rather than in domesticated *Oryza* species?

A—*Ray Wu:* Yes, there may be a greater chance of finding MU-like sequences in wild rice varieties. However, the element may or may not be more active in transposition in wild rice varieties.

Q—*Reddy:* (a) Could one of the two bands (2.5 or 2.2 kb) be due to the occurrence of the same element in circular form? (b) Did you rule out the possibility that this could be due to a cytoplasmic element?

A—*Ray Wu:* (a) It is not likely, because the 2.5-kb band was absent when we probed even the upstream sequence of the MU-1-like element. (b) Yes, but only to the extent that a chloroplast-enriched DNA did not hybridize with the MU-1-like probe.

Q—*André:* In the MU transposon of maize, mutator activity is correlated with unmethylated DNA, and inactive lines show methylation of the MU sequences. Have you checked your rice MU for methylation?

A—Ray *Wu:* Yes. Preliminary work using two pairs of restriction enzymes (one methylation sensitive, one methylation insensitive for each pair) showed that the rice MU-1-like element was fairly extensively methylated.

Q—*Brar:* Did you notice any phenotypic variation in IR36 plants showing the MU-transposable-like element? What was the behavior of the progeny of the plants with high copy number (11-1)?

A—*Ray Wu:* We did not observe any phenotypic variation in IR36 (Texas) plants showing increased copy number of the MU-1-like elements. However, we did not look too hard for subtle differences.

Q—*Ranjekar:* Was there any specific reason for using an actin gene as a probe to detect DNA variation?

A—*Brown:* The *Hind*III and actin gene combination allowed us to easily determine the levels of DNA polymorphism in regenerants. We also used several other genes as well as other restriction enzymes.

Q—*Ray* Wu: You showed variations in restriction pattern in different lines derived from rice culture samples, using an actin probe. Did you also detect copy-number changes?

A—*Brown:* One of the major effects of tissue culture we found is significant differences in gene copy number in callus in maize, and also in rice. These changes are often much greater than we would expect, and the differences between maize and rice copy-number changes are the subject of further investigation.

Q—*Zhang Qifa:* Did you try to observe the morphology of the plants to correlate morphological differences with restriction fragment length polymorphism (RFLP) changes?

A—*Brown:* Possession of a normal phenotype in tissue culture-derived plants is absolutely no guarantee that DNA variation has not been induced. Some of the highest levels of DNA polymorphism we have found have been in plants that appear phenotypically and karyologically normal.

Q—*Shao Qiguan:* What is the genetic meaning of RFLP variation, and what is your understanding of the difference in frequency in phenotypical genetic variation?

A—*Brown:* We are trying to determine the level of DNA variation associated with tissue culture. Our results show little or no correlation between what we see as a RFLP difference and phenotypic change. This is probably a regression of a lack of probes relative to phenotypic change. There is no guarantee that possession of a normal phenotype means no somaclonal variation or RFLP difference.

C—*Wang Xiangmin:* The work of Dr. Brown and his colleagues is very interesting and has manifold applications. Many new varieties have been obtained in China by haploid breeding in rice, wheat, tobacco, etc. But it has been questioned whether improved genotypes originate from new variants during in vitro culture or are merely the product of recombination of varietal genes. Now Dr. Brown has shown us that, among lines derived from a single callus DNA, changes have been detected that could provide an explanation of genetic variation.

Q—*Ranjekar:* CC genome-specific DNA sequences are not observed in the CCDD genome. This is confusing. Can you explain?

A—*Delseny:* This has been confusing for us too. The origin of the CCDD genome is not yet known, although there are a few hypotheses (c.f. work of Dr. Second). A possibility is that the source of the CC genome in CCDD did not contain the CC repeated sequences that we isolated. We have observed that some *O. officinalis* accessions do not contain any of the four sequences. A second possibility is that some repeated sequences are just eliminated in the progeny of interspecific crosses. The isolation of CCDD-specific sequences should help resolve this problem.

Q—*Chaudhuri:* In your CC genomes, the tandem repeats change the pattern and become dispersed. Can you elaborate? What is the percentage of homology between the original and new sequences?

A—*Delseny:* We do not know how these changes occur, but they have also been observed in other plants such as *Allium.* We did not make any comparison of the sequences in the tandem organization or in the dispersed ones. This awaits cloning and sequencing of the dispersed repeat. However, from the high stringency of the hybridization conditions, they are most likely closely related.

Q—*Ranjekar:* a-amylase is a multigene family. Are all the genes active at one stage or another?

A—*Rodriguez:* We have performed northern blot analyses and primer-extension studies using various rice a-amylase probes and find gene activity for 4 of the 12 genes: *Amy1A* (NOSg2), *3D* (NOSglA); *Amy3B* and *3C* (NOSg1B and C). *Amy1A* produces most of the a-amylase in RNA in the germinating seed.

Q—*Chaudhuri:* Can you identify the different amylase groups in different chromosomes of rice?

A—*Rodriguez:* DNA sequence analysis of 10 of the 12 amylase genes has enabled us to establish group-specific or in some cases gene-specific hybridization conditions. Southern blots of genomic DNA from the 12 trisomic lines and bands showing a 50% increase in hybridization signal are correlated with the restriction maps for each group.

Q—*Zhang Qifa:* What is the criterion for placing the probe into a group?

A—*Rodriguez:* We started with two cDNA clones for the rice amylase to establish groups 1 and 2. After that, we used subfragments for the remaining clones, and by process of elimination, to sort them into groups 3, 4, and 5.

C—*Chang:* Different authors are using seeds provided largely by the International Rice Germplasm Center at IRRI, but species names and genome composition are sometimes inconsistent with well-established nomenclature and genome designation. Some seeds have obviously changed hands, which may have introduced error. The same problem surfaced at the 1963 Rice Genetics and Cytogenetics Symposium. I suggest uniform nomenclature and checking (re-identification) of plants being used.

SESSION 9

RFLP Analysis of Rice Genomes

RFLP mapping of the rice genome

S.D. Tanksley, N. Ahn, M. Causse, R. Coffman, T. Fulton, S.R. McCouch, G. Second, T. Tai, Z. Wang, K. Wu, and Z. Yu

A restriction fragment length polymorphism (RFLP) linkage map has been constructed for rice based on a cross between an indica and a javanica cultivar. The current number of markers in the map corresponds to a density of approximately 1 marker for every 10 map units. This level of coverage is sufficient for many plant breeding applications, including gene tagging and detection of genes underlying quantitative traits. A map based on doubled haploids, also under construction, will allow distribution of progeny seed for collaborative work on rice. This population (derived from an anther-cultured indica/japonica F_1) also segregates for a number of previously mapped loci and will allow merging of the isozyme and RFLP maps. A third population, intended for high-density mapping in which 1000 or more markers can be located, is currently being screened. This population comes from an interspecific cross between *Oryza sativa* and *O. longistaminata* (an AA genome species from Africa). The map thus far produced from this cross looks very similar to the previous two maps. The advantage of this population is that the majority of tested RFLP clones segregate using only three restriction enzymes. As a result, mapping progresses with several-fold greater efficiency. The high-density map to be derived from this cross will likely be useful for map-based gene cloning when used in conjunction with a yeast artificial chromosome library.

Compared with other monocots, rice has several attractive features that make it ideal for genetic studies. Not only is it diploid and self-pollinating, which facilitates genetic studies, but it has a genome that is approximately 10-fold smaller (0.6 pg DNA/haploid genome) than most other domesticated grasses such as maize or wheat.

In the past few years, our laboratory has been engaged in developing one aspect of genome research in rice, namely construction of a high-density restriction fragment length polymorphism (RFLP) map. There are several reasons for constructing such a map. First, the map can be used to locate genes of economic importance. Finding tight linkage between an RFLP marker and a gene of interest offers the possibility of detecting that gene in a plant or plant population by assaying for the associated RFLP

marker. This can be a welcome alternative to breeders when the genes of interest are difficult, time-consuming, or expensive to screen for directly. Examples of association of RFLP markers with genes of economic importance in rice are presented by McCouch et al (1991) and Yu et al (1991). Genes underlying quantitative traits can also be "tagged" with RFLP markers and used for selection. avoiding time-consuming progeny testing that would otherwise be required (Paterson et al 1988, Tanksley et al 1989).

Once a gene of interest has been located on an RFLP map, it becomes possible to consider cloning that gene via chromosome walking using yeast artificial chromosome libraries (Burke et al 1987). This can be an especially important contribution of RFLP maps, since it opens the door to cloning genes of unknown gene product such as those for resistance to or tolerance for diseases, insects, and environmental stresses (e.g., drought or salt tolerance).

In this paper, we discuss the strategies and current progress of a project at Cornell University for constructing a high-density RFLP map for rice.

Map construction

Several factors contribute to the success of an RFLP mapping project. Considerations regarding the type of plant population, source of clones, and selection of restriction enzymes that have proven useful are presented here.

Population choice

To construct a genetic map, one needs to obtain some type of segregating population in which linkage can be detected and measured. There are many options for mapping populations, including backcrosses, F_2s, doubled haploids, and recombinant inbreds. The ideal mapping population has two attributes:

- It is derived from two homozygous parents which, when compared with one another, reveal polymorphism with every probe tested (using as few restriction enzymes as possible).
- The progeny from this cross are homozygous so that they can be propagated in perpetuity, thus allowing accumulation of large numbers of mapped markers by many researchers.

In reality, it is seldom possible to find such an ideal population. In rice, we initially made several compromises in population choice for the sake of expediency.

Indica/javanica F_2 population. The population from which we constructed our first map was an F_2 derived from a cross between javanica cultivar Bulu Dalam and indica breeding line IR34583-19-3-3. Using 11 restriction enzymes, it was possible to detect more than 70% polymorphism with random *Pst*I genomic clones (McCouch et al 1988). Two hundred and thirty-five clones were thus mapped onto this population to produce a rice RFLP map.

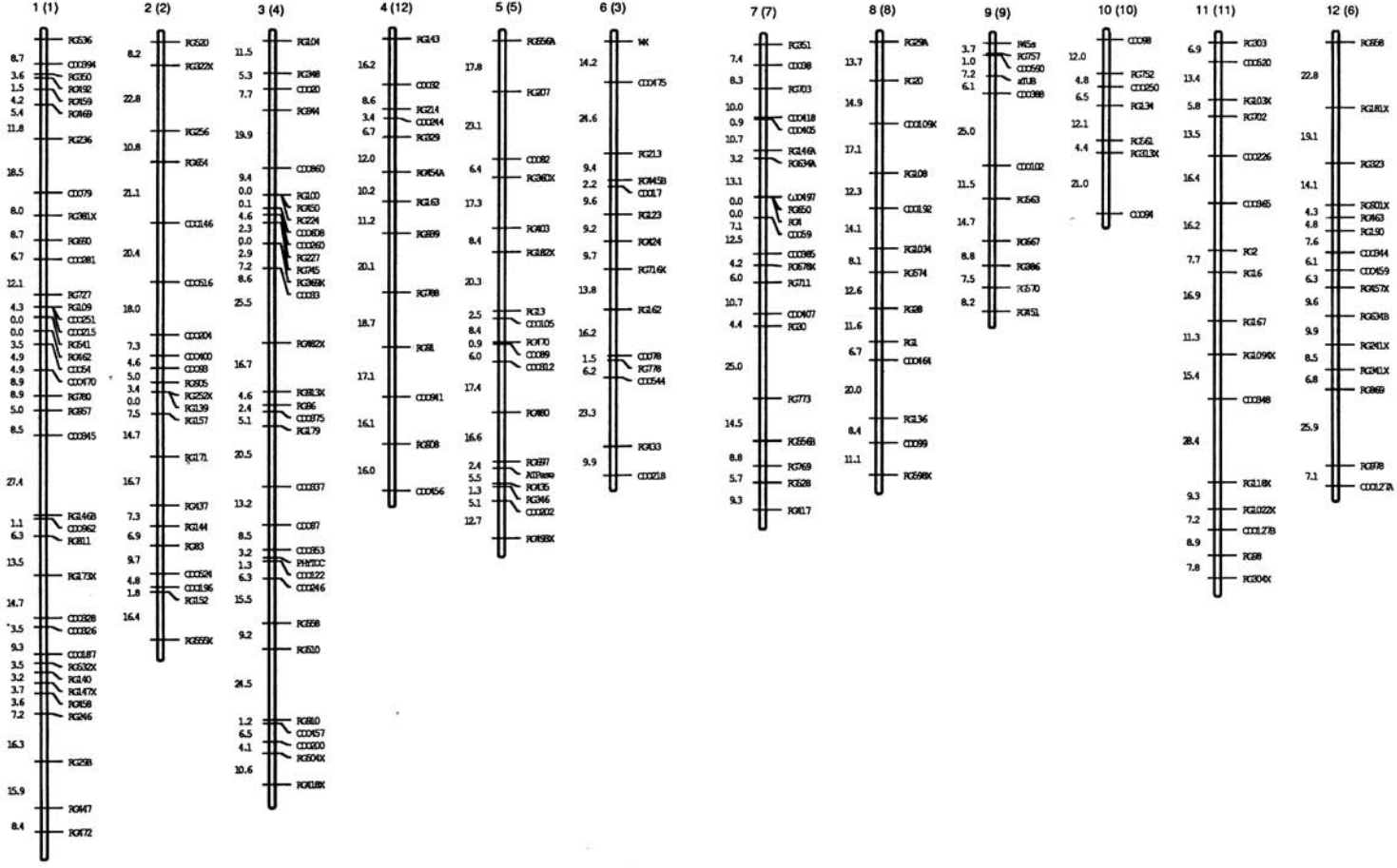
One of the first things we observed about the rice RFLP map derived from this population was that there were more total map units than had previously been described

for plant species with comparable genome sizes. Tomato and potato, for example, both have the same basic chromosome number (x = 12) and similar DNA contents as rice. Yet these two species have 1200 and 800 cM, respectively, in their total genome (based on RFLP mapping) as compared with the estimated 2000 cM for rice. As a result, whereas the tomato and potato genomes were well-covered when the number of RFLP markers reached 200, there are several gaps remaining in the rice map (Fig. 1).

The gaps in the rice map might be due to several causes. First, since there are so many map units in the rice genome, it is likely that the current number of markers is insufficient to give complete coverage. Second, the gaps in the map represent segments of the two parental genomes that are common by descent. Clones corresponding to such regions would be monomorphic and thus could not be mapped in this cross. Even though there is no documented common parentage between the indica and javanica parents used in constructing this F_2, the records do not go back far enough to rule out this possibility. If gaps in the map are due to this factor, mapping more clones on this same population would not eliminate them. For this reason and others, we chose to continue the map using two additional populations.

Indica/japonica doubled haploids. F_2 populations, and other populations containing heterozygous individuals, cannot be propagated as pure genetic stocks by seed because the alleles continue to segregate. Populations composed of recombinant inbreds (single seed descent $>F_6$) or doubled haploid lines, however, can be propagated as pure seed lines and thus multiplied, maintained, and distributed to other researchers (Burr et al 1988). Techniques have been established for producing doubled haploid populations from cultured rice anthers, and the availability of one such population, derived from a cross of a tropical japonica (IRAT177) and an indica (Apura) selected for anther culture, provided an opportunity for us to establish the first doubled haploid mapping population for rice (Guiderdoni et al 1990). Although the polymorphism detected between these two parents is less than that observed in our original mapping population, it was sufficient (about 50%) to allow construction of a map. Currently, 100 markers have been mapped onto this population, and this number is expected to double in the next 12 mo. It is too early to determine whether all the gaps found in the first map will be filled, but results indicate that this map will supplement the earlier one. This population also segregates for 12 isozyme markers as well as several genes for resistance to rice blast and other agronomically important traits, which can now be located on the RFLP map. Seeds and mapping data from this doubled haploid population will be available to other researchers in the near future.

O. sativa/O. longistaminata//O. sativa. O. longistaminata is a wild AA genome rice species from Africa. Although F_1 hybrids with *O. sativa* are largely male sterile, it is possible to generate seeds from backcrossing to *O. sativa,* and backcrosses previously studied for isozymic segregation show nearly normal segregation ratios (Causse and Ghesquière 1991). We screened one F_1 hybrid and the *O. sativa* parents from one such backcross and found that approximately 85% of the tested RFLP clones detected mappable polymorphism with only three restriction enzymes. We are constructing a map with this population (Fig. 1). Approximately 230 markers have been tested so far



1. RFLP "framework" map (not all mapped markers shown) of rice chromosomes, derived from linkage analysis of 53 F_2 plants from a cross between IR34583 (indica) and Bulu Dalam (japonica). Numbers at the top indicate chromosomes. Markers represented here include both single-and multiple-copy sequences (adapted from Tanksley et al 1988a).

on 113 backcross individuals, and the derived map looks very similar to the F_2 and doubled haploid maps constructed from intraspecific crosses. Because of the high levels of polymorphism and the need for using only a small number of restriction enzymes, the rate of progress in mapping in this population is severalfold greater than in the intraspecific crosses, and more than 500 markers may be mapped on this population in the next 1-2 yr. The drawback to this population is that the backcross plants must be vegetatively propagated. However, these plants are very vigorous and readily tiller, allowing for vegetative multiplication. Currently, three clonal sets of this population are maintained at Cornell University, and additional sets are being prepared for distribution to other researchers.

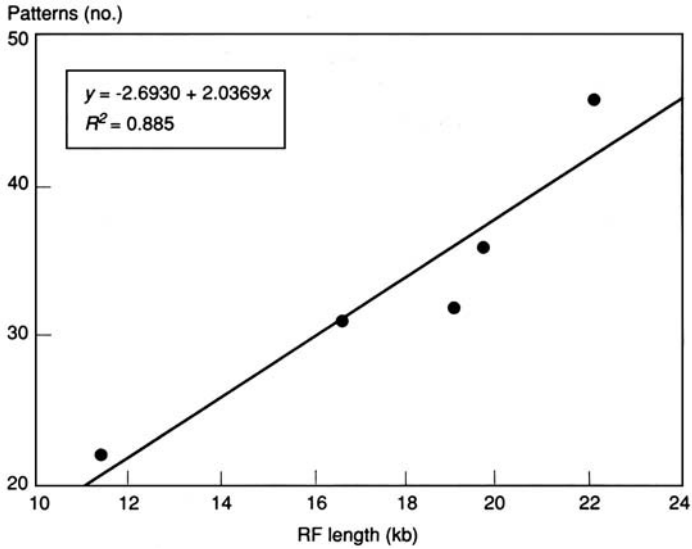
Clone source

RFLP mapping requires a source of single-copy clones. Since most plant genomes, including rice, contain repetitive elements, some selective procedure must be implemented, in either constructing or screening the library, to avoid clones containing repetitive DNA (Tanksley et al 1988b). In some plant species (e.g., tomato and maize), it is possible to generate libraries highly enriched for single-copy sequences by cloning with restriction enzymes that are methylation-sensitive and have recognition sites rich in GC (Burret al 1988, Tanksley et al 1988b). Presumably, single-copy regions are less methylated, have higher GC content, and are thus more likely to be cleaved by such enzymes. Rice, however, has a lower level of methylation and higher GC content than many other plant species, and attempts to construct single-copy enriched libraries with methylation-sensitive enzymes have been only partially successful (McCouch et al 1988; R. Messeguer, Cornell University, pers. comm.). As an alternative, we have chosen to use a complementary DNA (cDNA) library as a source of probes for further mapping of the rice genome. By using a phage library derived from size-selected cDNA, it has been possible to obtain clones with insert sizes greater than 1 kb. In the past, it has been difficult to generate cDNA libraries with large numbers of unique clones with inserts this large.

A second improvement that we have added in the preparation of probes is the use of a polymerase chain reaction (PCR) to generate purified cDNA inserts directly from phage plaques (Erlich 1989). Individual plaques are removed from the plate with a Pasteur pipet and vortexed in a microfuge tube in 20 µl H_2O; a small aliquot of this is used to start a PCR containing primers specific to the cloning site in the phage. The PCR product from this reaction can be used directly for probing onto filters. This procedure eliminates the time-consuming steps of phage or plasmid miniprepping and the need to isolate inserts directly from gels. More probe can be generated directly from the primary PCR product with another PCR reaction. Using this method, it is practical for one person to generate several hundred new probes per week.

Restriction enzymes

Not all restriction enzymes detect the same level of polymorphism in rice DNA. There is a strong, positive correlation between the amount of polymorphism an enzyme



2. Regression of total number of unique restriction patterns detected for each restriction enzyme against average total length of restriction fragments hybridizing to genomic clone in each probe/plant combination. Each point represents average value for each of 5 restriction enzymes (from Wang and Tanksley 1989).

detects and the average size of the restriction fragments generated when the enzyme is used to cleave rice DNA (Fig. 2; McCouch et al 1988, Wang and Tanksley 1989). This phenomenon has also been observed in tomato and is likely due to the fact that much polymorphism in plant DNA is due to insertions, deletions, or other types of DNA rearrangement (Roth et al 1989). This observation can be used to advantage by those working with RFLP maps. Preferential use can be made of enzymes that cleave large fragments, such as *Eco*RV or *Xba*I, to maximize the chance of detecting polymorphism (McCouch et al 1988, Wang and Tanksley 1989).

Future directions

Much of the past 5 yr has been devoted to constructing RFLP maps in a variety of crop plants including rice. While much work remains to be done in improving and expanding current maps (especially in producing a high-density map for gene cloning), we nonetheless are now in a position to begin applying this new tool for practical purposes (Tanksley et al 1989).

Technology transfer

Until now, much of the RFLP work has been conducted at universities or biotechnology companies in developed countries. For a significant impact on crop breeding, however, this technology must be transferred to facilities where the individual crops are actually bred. For rice, this means transferring the technology to developing countries where

rice is the major grain crop. To facilitate this process, it may be necessary to modify or change some of the existing methodologies. While this transition period may at first be cumbersome, when it has been accomplished successfully, we may begin to feel the real impact and potential of the technology for rice improvement.

Need for an internationally coordinated effort on the rice genome

Because rice is of great importance to so many countries, and because of its intrinsic attraction due to its well-developed genetics and small genome size, many researchers worldwide have established, or are in the process of establishing, research projects to study the rice genome. If such projects are properly funded and coordinated, rice may become the first crop plant in which the nuclear genome has been characterized in detail. The payoffs would be tremendous for rice as well as for all other crop plants, which would benefit directly or indirectly. The key to attaining this goal may be the establishment of an internationally coordinated effort for exchange of information and ideas and for fostering collaborative research on the rice genome.

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Tagging genes for disease and insect resistance via linkage to RFLP markers

S.R. McCouch, G.S. Khush, and S.D. Tanksley

Tagging genes for resistance to diseases and insects via linkage to restriction fragment length polymorphism (RFLP) markers requires a well-saturated RFLP map, reliable phenotypic screening procedures, and appropriate plant material. A subset of 130 mapped RFLP markers was used to search the rice genome for resistance genes of interest. Phenotypic screening was performed at IRRI based on traditionally accepted techniques. Two types of plant material were analyzed and compared for efficiency in locating target genes: 1) pairs of near-isogenic lines (NILs), each containing a single gene for resistance to the whitebacked planthopper or to *Xanthomonas oryzae* pv. *oryzae,* and 2) 78 F_3 families derived from a single cross (TN1/IR36) segregating for 5 single resistance genes. The locations of the resistance genes based on NIL surveys were confirmed by using RFLP markers from putative positive regions as probes onto segregating F_2 populations derived from resistant isoline/susceptible recurrent parent crosses. Two RFLP markers have been confirmed to be linked to the *Wbph-1* resistance gene, although the chromosomal location of the gene remains unclear because both linked markers are multiple-copy clones.

The availability of a restriction fragment length polymorphism (RFLP) map of rice (McCouch et al 1988) has enabled workers to investigate the genetics of important agronomic characters with a degree of resolution previously unattainable. Genes governing traits of interest can be located on the chromosomes via linkage to mapped RFLP markers in a process known as "gene tagging" (Burr et al 1988, Landry et al 1987, Young et al 1988). Both single-gene and polygenic traits are amenable to this approach. In the case of quantitative traits, the location of the various Mendelian loci that contribute to the observed polygenic character, and the relative contribution that each locus makes to phenotype, can be determined (Paterson et al 1988, 1990). Such information is useful in marker-aided selection schemes, and also as a precursor to map-based cloning.

The objective of this study was to tag single-gene resistance characters. Two approaches are discussed, and the implications of this work for future research are outlined.

Approaches to tagging

Two approaches to gene tagging were investigated in this study: 1) analyzing pairs of near-isogenic lines (NILs) for introgression of a monogene for resistance, followed by analysis of segregating populations to confirm linkage; and 2) performing linkage analysis directly on a population of 78 F_3 families derived from 1 cross in which 5 individual resistance characters segregated.

Plant material

Pairs of NILs are developed by crossing a resistant donor line with a susceptible recurrent parent line. The isoline and the recurrent parent are purported to be genetically identical except for the presence or absence of the target gene, plus a small region of flanking DNA inherited along with the gene from the resistant donor. Simply inherited genes for resistance to the whitebacked planthopper (WBPH) *Sogatella furcifera* (Horvath) and to *Xanthomonas oryzae* pv. *oryzae* (Xoo), which causes bacterial blight (BB) of rice (Ogawa et al 1988), were introgressed during construction of the NILs (Table 1). Resistant phenotypes were identified based on inoculation and subsequent counting of insects in the case of WBPH resistance genes (Romena et al 1986) or on evaluation of lesion size and number in the case of Xoo resistance genes (Yoshimura et al 1985). RFLP studies identify the chromosomal segments that have been introgressed from resistant donors into respective NILs.

The second approach undertaken as part of this study involved segregation analysis of F_3 families derived from a cross between rice varieties IR36 and TN1. These parents were selected because they are easy to cross, produce highly fertile offspring, and differ with respect to 5 monogenes for resistance (Table 2). Extensive F_3 progeny testing was required to identify plants as phenotypically resistant or susceptible to all the relevant pests and diseases, and thus a cross with a high fertility rate was essential. This imposed a limit on the genetic distance that could be incorporated, and, consequently, on the degree of polymorphism that could be expected in an inbred crop such as rice.

Table 1. Resistance characters monitored in pairs of near-isogenic lines.

Resistance gene	Donor variety	Recurrent parent	Isoline designation
Wph-1	N22	IR36	IR58034-1
Wph-2	ARC10239	IR36	IR58036-1
Wph-3	ADR52	IR36	IR58044-4
wph-4	Podiwi A-8	IR36	IR58046-2
Xa-3	Choguku 45	IR24	IR-BB3 (IS399)
Xa-4	IR20	IR24	IR-BB4 (IS417)
xa-5	IR1545-339	IR24	IR-BB5 (IS435)
Xa-7	DV85	IR24	IR-BB7 (IS471)
xa-8	PI231129	IR24	IR-BB8 (IS509)
Xa-10	CAS209	IR24	IR-BB1 (IS453)

Table 2. Summary of resistance characters monitored in segregating F_2 and F_3 families of TN1/IR36.

Resistance gene	Phenotype of parents[a]		Original donor	Avirulent biotype or strain
	IR36	TN1		
bph-2	R	S	PTB18	Bph I, II, III(?)
Glh-6	R	S	PTB18 or PTB21	Field population
Xa-4	R	S	TKM6	Race 1, 5
Blast	R	S	?	Ik81-25[b]
Gs	R	S	O. nivara	Strain 1

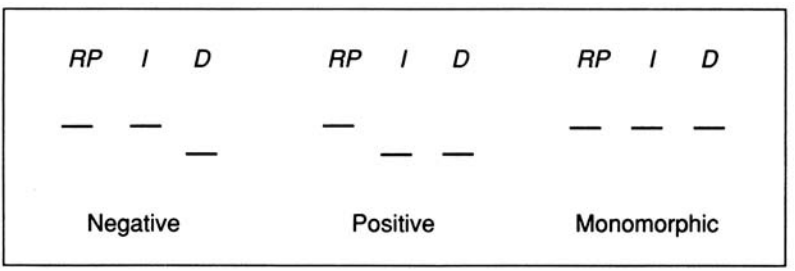
[a] R = resistant, S = susceptible. [b] IRRI's designation for this strain of *Pyricularia oryzae* (also referred to as "102" in Yu et al 1987).

BB resistance gene *Xa-4* was present in both the NIL and the F_3 family studies. This redundancy allowed us to verify the map location of this gene based on an analysis of two populations. Studies based on trisomic analysis have shown that *Xa-4* is located on chromosome 11, although it was previously reported to lie on chromosome 4 (Prasad and Tomar 1988).

Restriction fragment length polymorphism analysis

With a subset of 130 of the mapped RFLP markers, we searched the rice genome for resistance genes of interest. Selected markers were well distributed on all chromosomes at an average distance of 15 cM and, where possible, represented single- or low-copy sequences. Additional markers (bringing the total to 200) were used to probe TN1/IR36 filters because of the low level of polymorphism encountered in that cross.

Autoradiograms for the NILs were analyzed on a locus-by-locus basis (Fig. 1). Whenever NIL survey information led to putative positive results, confirmation of

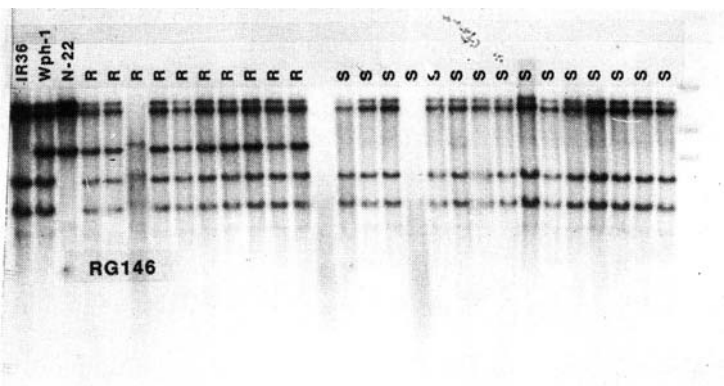


1. Three expected outcomes of RFLP survey to determine parental origin of chromosomal segments of DNA in near-isogenic lines. Lanes represent susceptible recurrent parent (RP), resistant isoline (I), and resistant donor (D). In the first triplet, banding pattern indicates recurrent parental origin of isoline DNA at locus represented by RFLP marker—a "negative" result, meaning resistance gene is not located there. In the second triplet, banding pattern indicates introgression of donor DNA—a putative "positive" result. In the third triplet, pattern is monomorphic and offers no information.

linkage between the RFLP marker(s) in question and the resistant phenotype was required. To this end, F_2 populations from crosses between each resistant isoline and its susceptible recurrent parent were constructed and screened phenotypically for resistance. Segregation of the donor-derived (resistant) or recurrent parent-derived (susceptible) "alleles" of putatively linked RFLP marker loci was monitored. If the donor-derived allele cosegregated with the resistant phenotype, linkage was confirmed. Map distance could be estimated from the number of crossovers that were observed between the linked RFLP marker and the resistant phenotype in the F_2 population.

An example of a confirmed case of markers found linked to a resistance gene is that of *Wbph-1,* where two linked loci were identified. For both markers, alleles inherited from the donor N22 were confirmed to cosegregate with the resistant phenotype in an F_2 derived from a cross between IR36 and *Whph-1* isoline, and consisting of 10 resistant and 18 susceptible individuals (Fig. 2). Cosegregation was perfect, with no crossovers separating either marker from the resistant phenotype. We therefore conclude that the *Whph-1* gene, conferring resistance to WBPH, lies between 0 and 5.2 cM (p=0.05) of marker RG146 and RG445.

The chromosomal location of *Whph-1* is not yet clear, despite clear evidence of close linkage to two RFLP markers. RG146 and RG445 are both multiple-copy sequences, as can be seen from the hybridization patterns in Figure 3. Currently, two loci corresponding to the markers have been mapped, and no linkage between RG146 and RG445 has been detected. We believe that the character is controlled by a single gene because of the phenotypic segregation ratio (3:1) in the F_2. To clarify the chromosomal location of *Whph-1,* two approaches are being pursued: 1) We look for crosses in which unmapped loci detected by RG146 and RG445 segregate and can be



2. Autoradiogram showing confirmation of linkage between RG146 and whitebacked planthopper-resistant and -susceptible phenotypes. Lane 1 = IR36, susceptible recurrent parent; lane 2 = resistant isoline containing *Wbph-1* gene; lane 3 = N22, the resistant donor. Lanes 4-13 = resistant phenotypes (R), lanes 15-29 = susceptible phenotypes (S).

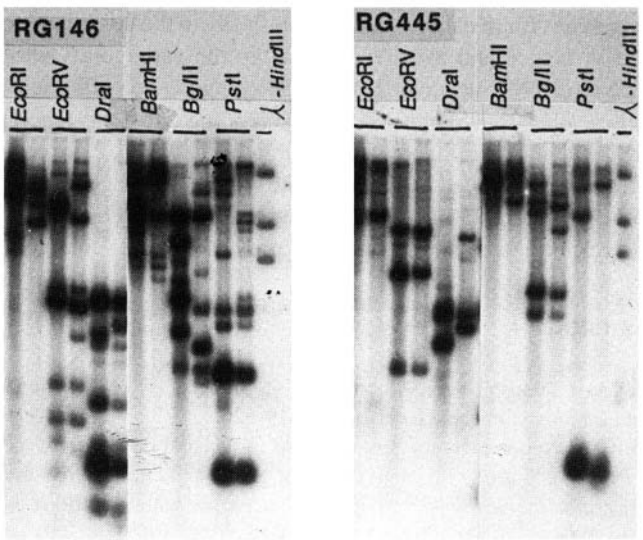
mapped. 2) We clone unique, or single-copy, sequences from regions of DNA flanking the repeats within RG146 and RG445, and confirm polymorphism on NIL surveys and cosegregation with the resistance character.

Linkage of *Xa-4* to RG53 at a distance of 17 cM was detected in the F_3 population derived from TN1/IR36. It will be confirmed on the NILs in the near future. RG53 has not been mapped because, like RG146 and RG445, it is a multiple-copy clone.

Efficiency of isolines versus segregating population approaches

In projects where the location of target genes is unknown, or the RFLP map available for the species concerned has a limited number of markers (<500), the approach involving one segregating population offers greater assurance of success than does the isoline approach (as long as the cross is genetically divergent enough that RFLP polymorphism is not a problem). In a segregating population, a range of different-size fragments containing the gene is inherited from the resistant parent, and detection of linkage does not depend on the single representative of recombination, as is the case with isolines.

In terms of initial investment in preparation of plant material, a single population that segregates for many traits simultaneously is generally more efficient than NILs, where only a single gene is targeted for each pair. Furthermore, isoline construction is



3. Autoradiograms showing multiple banding patterns of RG146 and RG445 when probed onto IR34583 and Bulu Dalam DNA, digested with several restriction enzymes. Both cloned sequences occur in multiple copies in the rice genome.

very labor- and time-intensive, since controlled crossing and phenotypic screening are required in each generation.

However, once the approximate location of a gene is known, or if a highly saturated RFLP map is available (>500 markers), isolines offer many advantages. In terms of time and effort invested in RFLP analysis, using isolines to survey RFLP markers will provide more information more quickly than will analysis of parental lines, where the segregating population must be mapped in its entirety before any information about gene location is obtained. Pairs of NILs facilitate selection of RFLP markers that are very close to the gene of interest. This is particularly valuable if the ultimate objective of the mapping and tagging work is to clone genes (Ganal et al 1989). In the random screening of large numbers of unmapped RFLP markers, isolines help identify those that are linked to the gene of interest (Young et al 1988). Useful clones can then be mapped using standard segregation analysis.

Implications for future research

Questions of basic scientific interest relating to the mechanism of host-pathogen interaction have remained unresolved for many years; we lack information about which genes and gene products actually control resistance and susceptibility in plants, and about virulence and avirulence in pathogens. An experimental system such as that represented by the relationship between the rice plant and Xoo, the agent causing BB, offers an ideal framework within which to investigate general questions about genome organization and the mechanism of plant-pathogen interaction. Locating and ultimately cloning genes for resistance and susceptibility in the host plant, to complement the cloning of virulence and avirulence genes in the pathogen (Gabriel et al 1986, Kelemu and Leach 1990, Staskawicz et al 1984), should soon offer new opportunities to approach these questions. High resolution mapping and gene-tagging are the first steps toward the eventual cloning of genes of interest in plants. Efforts in this direction promise to offer unprecedented insights into the validity of the gene-for-gene hypothesis as proposed by Flor (1956), and into the ways in which plants and microbes recognize and respond to each other in their array of complex interactions.

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RFLP tagging of blast resistance genes in rice

Zhihong Yu, D.J. Mackill, J.M. Bonman, and S.D. Tanksley

Inheritance of complete resistance to rice blast (BI) isolates (*Pyricularia oryzae* Cav.) is usually conferred by major dominant genes. After a survey of more than 140 selected random genomic clones, several putative positive markers were obtained for each of a set of near-isogenic lines of the highly susceptible indica cultivar CO 39. Linkage of BI resistance genes to these markers was verified using F_3 segregating populations of known BI reaction. Restriction fragment length polymorphism (RFLP) analysis showed that BI resistance gene *Pi-2(t),* derived from indica cultivar 5173, is closely linked to single-copy DNA clone RG64 on chromosome 6. The distance between *Pi-2(t)* and RG64 is about 4.2 + 1.7 (S.E.) cM. Another BI resistance gene, *Pi-?,* derived from indica cultivar Tetep, is significantly linked to single-copy DNA clone RG869 on chromosome 12. The distance between *Pi-?* and RG869 is about 10.6 + 3.8 (S.E.) cM. RFLP markers tightly linked to BI resistance genes may be useful in breeding programs. They may also be employed to map new genes for resistance to BI isolates. Eventually, BI resistance genes may be cloned via chromosome walking.

Blast (BI) caused by *Pyricularia oryzae* Cav. is one of the most destructive diseases of rice. It occurs in all rice culture types and can cause severe damage under favorable conditions. The use of resistant cultivars has been the most economical approach to BI control (Bonman and Mackill 1988). Knowledge of the genetics of resistance to this disease is essential for developing cultivars with durable resistance.

Inheritance of resistance to rice BI has been studied in Japan (Kiyosawa 1981) and the Philippines (Mackill et al 1985), as well as in many other countries. Complete resistance is expressed as the absence of lesions or the presence of small necrotic lesions. Complete resistance is race specific and is generally controlled by one or two dominant genes. A few recessive resistance genes (Woo 1965, Yu et al 1987) and some modifying genes (Rosero 1967) have also been reported. No classical map is available for BI resistance genes except for those identified in Japan (Kinoshita 1986). The gene products of BI resistance genes are not known.

Recently developed restriction fragment length polymorphism (RFLP) techniques offer a new tool to monitor gene transfer in breeding programs and potentially to clone genes whose products are unknown (Tanksley et al 1989). Collaborative research between IRRI and Cornell University has focused on the genetics of complete resistance, and on tagging resistance genes with RFLP markers.

The objective of this study was to tag single genes for complete resistance to BI with RFLP markers.

Materials and methods

For more systematic study of resistance genes in the host and of avirulence genes in the pathogen, a set of near-isogenic lines (NILs) was developed using the highly susceptible indica cultivar CO 39 (Mackill et al 1989). Four nonallelic genes for BI resistance were identified in these lines by allelism tests. All these NILs are the product of six backcrosses followed by three selfings (BC_6F_4). Four varieties—5173, LAC23, Pai-kan-tao, and Tetep—were the donors of resistance genes. Ten NILs were initially chosen for these tagging studies. It was later determined that the NILs represent at least four independent resistance genes (Table 1). Seeds of the five parents, corresponding NILs, and BC_6F_3 populations segregating for resistance genes were grown for leaf tissue harvest.

Five *P. oryzae* Cav. isolates collected from the Philippines—designated 101, 102, 103, 104, and 105—were used to develop the NILs. All belong to different international races (IA-125, IF-3, IB-47, ID-15, and IA-127, respectively). These isolates were used to inoculate the subset of BC_6F_3 segregating populations. The isolates are stable and are commonly used for studies at IRRI. Seedlings were inoculated at the 5- to 6-leaf stage (3 wk after seeding). They were sprayed with an inoculum suspension of 5×10^4 conidia/ml, then placed in a dew chamber at 25 °C overnight. Inoculated plants were

Table 1. Ten near-isogenic lines (NILs) for blast resistance genes.

Code	NIL	Reaction pattern group	Gene designation[a]
BR05	C103A51-24	2	*Pi2- (t)*
BR32	C101A51-2	2	*Pi-2 (t)*
BR35	C102A51-4	2	*Pi-2 (t)*
BR38	C104A51-4	2	*Pi-2 (t)*
BR41	C105A51-2	2	
BR06	C101LAC-4	1	*Pi-1 (t)*
BR11	C104PKT-2	5	*Pi-3 (t)*
BR50	C101TTP03	3	
BR60	C102TTP-20	3	
BR62	C105TTP01	3	*Pi-?*

[a]*Pi-?* is nonallelic to *Pi-2 (t),* but its relationships to *Pi-7 (t)* and *Pi-3 (t)* have not been clarified. It may possibly be a new resistance gene to BI disease. (–) = no designation has been assigned, and relationship to other genes is unclear.

then transferred to a humid greenhouse room. Disease reactions were scored about 1 wk after inoculation, when typical lesions appeared on the leaves of susceptible check plants. Plants were scored using a 0-5 scale as described by Bonman et al (1986).

A RFLP genetic map of rice chromosomes was constructed (McCouch et al 1988) and was recently augmented with additional DNA markers (unpubl. data). For the NIL survey, 140 mapped genomic clones were selected. The criteria were single copy and map position. Single copy was defined at moderate stringency (0.5 × sodium chloride-sodium citrate, 65C). Since all NILs were at a relatively early stage of backcrossing (BC_6), the theoretically introgressed segment was estimated to be 15-30 map units (Hanson 1959). All selected clones were well distributed over the rice genome, with most being less than 15 cM apart on the chromosomes.

Plant DNA was prepared from fresh-frozen leaf tissues following McCouch et al (1988). Total genomic DNA was digested with the five restriction enzymes found to be most efficient in detecting polymorphism: *Eco*RV, *Xba*1, *EcoR* I, *Hind* III, and *Dra*I (Wang and Tanksley 1989). For the verification filters, only those enzymes giving positive results were used. Electrophoresis and Southern analysis followed McCouch et al (1988).

Results and discussion

The percentage of clones detecting polymorphism between CO 39 and each of four donor varieties varied significantly (Table 2). Compared with the 2 japonica parental varieties, polymorphic clones were approximately 60%. However, this value was cut in half when the parents were indicas. The lower level of polymorphism observed for the latter case is probably attributable to the fact that CO 39 is also an indica and is genetically less well differentiated from other indicas than from japonicas. This result is consistent with findings from a larger study of RFLP variation in cultivated rice (Wang and Tanksley 1989). It also suggests that resistance genes transferred from one subspecific group to another have a higher probability of being tagged in NILs than those transferred within a subspecific group, due to the greater probability of detecting introgressed segments. Since new markers are continually being added to the rice RFLP map, it should be possible to survey additional mapped clones, detecting polymorphism throughout the rice genome.

Table 2. Polymorphism detected between indica cultivar CO 39 (from India) and corresponding donor parents.

Donor	Race	Origin	Polymorphic clones	% genome searched[a]
5173	Indica	Colombia	41/142 (29%)	32
LAC23	Japonica	Liberia	91/142 (64%)	61
Pai-kan-tao	Japonica	China	92/142 (65%)	58
Tetep	Indica	Vietnam	46/142 (32%)	40

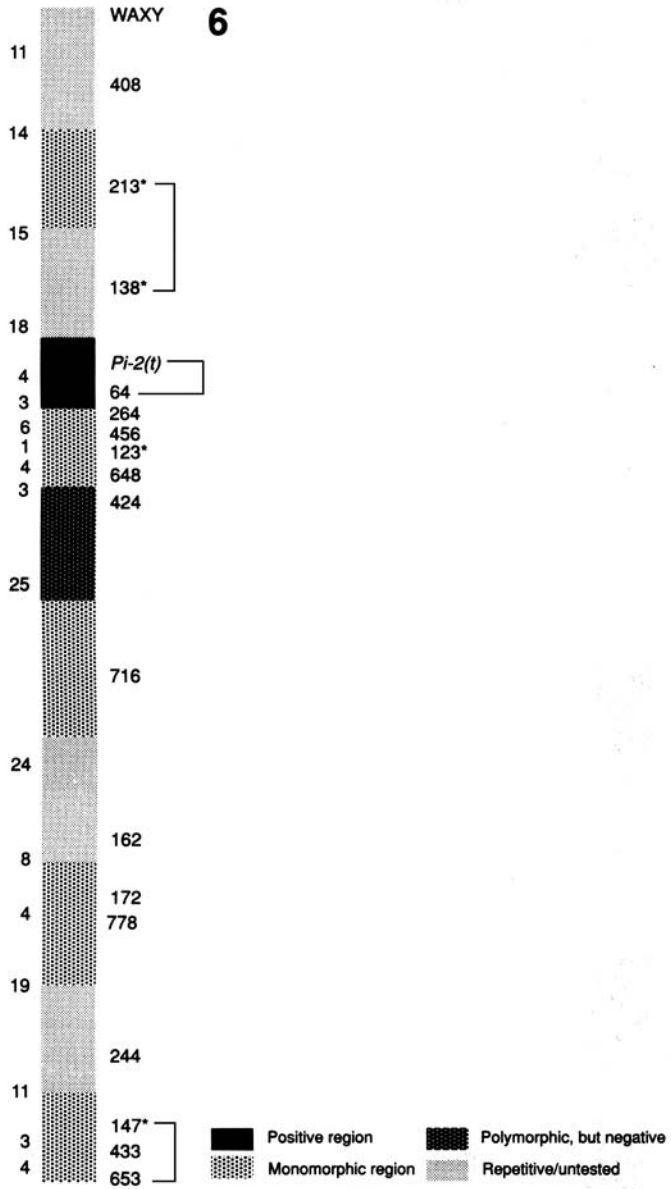
[a] The percentage of genome searched means total polymorphic regions over the entire genome in terms of centimorgans (Hanson 1959). The data were estimated from a survey of 108 random genomic clones.

The majority of polymorphic clones, tested by hybridization to NIL survey filters, produced identical restriction fragment patterns with DNA from CO 39 (i.e., same molecular weight hybridization bands for both NIL and CO 39). Thus, resistance genes are not likely to be near these markers. However, a few clones (2-6 for each NIL, about 6%) exhibited patterns different from those of CO 39, but the same as the donor's. Such clones were considered as putative positive clones potentially associated with Bl resistance genes. Some of these clones exhibited positive patterns on more than one NIL. This could be due either to clustering of resistance genes or the existence of a single locus conferring resistance to several races.

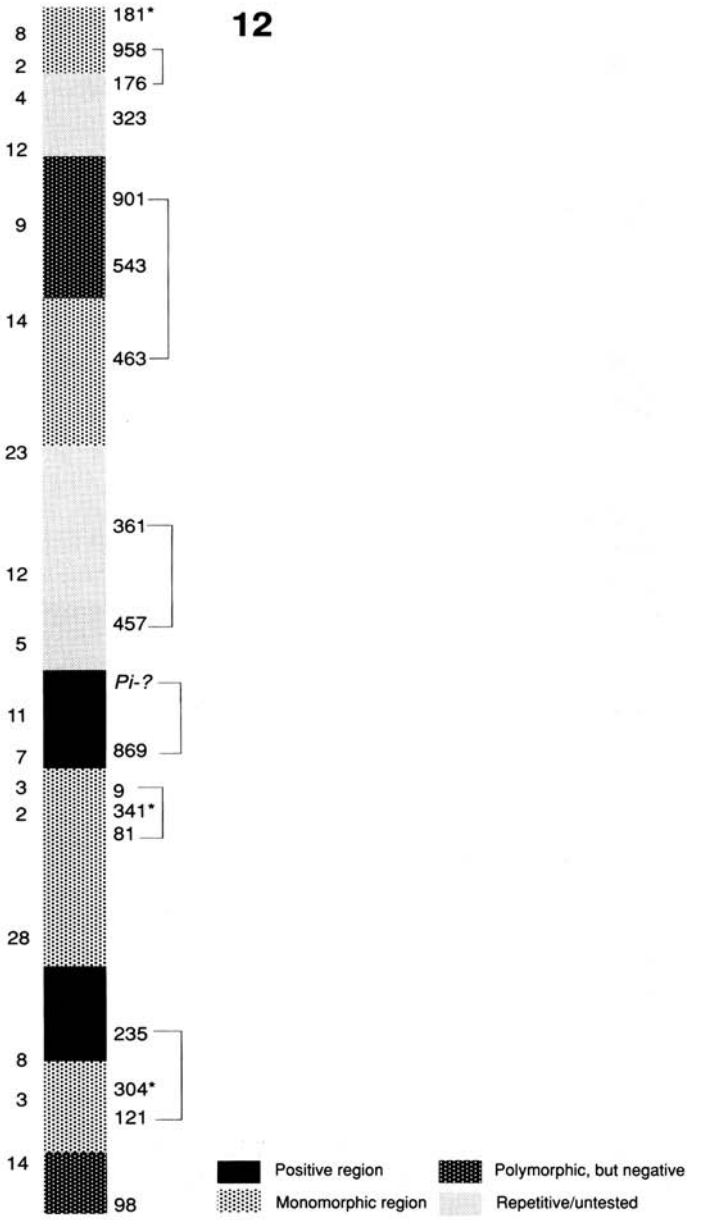
Once the putative positive clones are identified, they must be verified on a segregating population whose individual members are screened for disease resistance. In this study, we used F_3 families, derived from single F_2 populations. BC_6F_3 families were scored at IRRI for Bl reaction as either resistant (R), segregating, or susceptible. Remnant seed from the same F_3s was grown at Cornell for fresh-tissue harvest. Only homozygous resistant and susceptible members of an F_3 population were included in the verification filters. After hybridization with putative positive clones, a segregation of the donor-derived and CO 39-derived restriction fragments was monitored. If the donor-derived fragment cosegregated with the resistant phenotype, and the CO 39-derived fragment with the susceptible, the linkage was established. The map distance between the RFLP markers and resistance genes was calculated from the number of crossovers in the F_2.

Most of the putative clones turned out to be false positives, because the NILs were at an early stage of backcrossing (BC_6), and a few pieces of chromosomal segments not related to the resistance genes "contaminated" the NILs. However, two positive clones were found, each for linkage to different Bl resistance genes. RFLP analysis confirmed that one Bl resistance gene, *Pi-2(t),* derived from indica cultivar 5173, is closely linked to a single-copy DNA clone RG64 on chromosome 6. Four crossovers were found in the F_2 among 36 F_3 lines (26R:10S) from a cross between CO 39 and NIL C101A51, and only 2 crossovers among 35 F_3 lines (21R:14S) from a cross between CO 39 and NIL C104A51. Since C101A51 and C104A51 are in the same gene group (Table l), they may potentially have the same Bl resistance gene. Thus, the combined data were used to estimate the map distance. The distance between *Pi-2(t)* and RG64 is about 4.2+1.7 (S.E.) cM (Fig. 1). Another Bl resistance gene, *Pi-?,* derived from indica cultivar Tetep, is significantly linked to single-copy DNA clone RG869 on chromosome 12. The distance between *Pi-?* and RG869 is about 10.6+3.8 (S.E.) cM (Fig. 2). We are focusing on these two chromosomal regions to define the precise locations of *Pi-2(t)* and *Pi-?.* The orientations of the Bl resistance genes to the DNA can be clarified, and closer linkages can be achieved when the rice RFLP map is saturated with additional markers.

Currently, we are clarifying the orientations of *Pi-2(t)* to RG64, and of *Pi-?* to RG869. We are screening additional markers in these two chromosomal regions to identify other closely linked markers. The distance between a Bl resistance gene and a linked RFLP marker may be reduced in the future. Tightly linked DNA markers may



1. Map of rice chromosome 3. Note that gene *Pi-Z(t)* is linked to RG64 with a distance of 4 cM. The direction needs to be clarified. Kosambi cM are on the left of the chromosome line; marker designations are on the right. Bracket (]) shows orders where confidence level is below 99%.



2. Map of main linkage group of rice chromosome 6. Note that *Pi-*? gene is linked to RG869 with a distance of 11 cM. The direction needs to be clarified. Kosambi cM are on the left of the chromosome line; marker designations are on the right. Bracket (]) shows orders where confidence level is below 99%.

be useful in breeding programs. Homozygous resistant individuals can be identified in the F_2 of a cross, without F_3 confirmation. They may also be employed as powerful tools to map new resistance genes to BI isolates in both tropical and temperate countries. Comparisons among BI resistance genes identified in different countries have been very difficult. One reason is that the appropriate BI fungus races are not easily imported because of quarantine regulations. With tightly linked RFLP markers, gene comparisons become possible without using the pathogen. For example, *Pi-z,* derived from American cultivar Zenith, is also in the middle region of chromosome 6, based on a classical genetic study in Japan (Kinoshita 1986). The relationship of *Pi-2(t)* and *Pi-z* should soon be clarified. Gene *Pi-?* in C105TTP-1 is nonallelic to *Pi-2(t),* but its relationship to *Pi-l(t), Pi-3(t),* and *Pi-4(t)* is clear. Cl05TTP-1 is in the same group as C101PKT, which has *Pi-4a(t).* But *Pi-4h(t)* from the NIL C105TTP-4 is allelic to *Pi-4a(t),* and we would not expect to have two alleles at the same locus in one donor (Tetep). C105TTP-1 may therefore have a new gene that could be designated *Pi-5(t).* There is also the possibility that polymorphism exists within Tetep for this locus. This will remain unresolved until crosses are made between C105TTP-1 and the other NILs, or the probe RG869 is used on crosses with Cl01PKT to determine if it is linked to *Pi-4a(t).* These tests are in progress.

Blast resistance genes may ultimately be cloned via chromosome walking, although their gene products are not currently known. Cloning avirulence genes from *P. oryzae* Cav. is in progress (Valent et al 1991). In the future, the mechanism of host-pathogen interactions (gene-for-gene) may be investigated at the molecular level.

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Toward the integration of a restriction fragment length polymorphism map and conventional genetic map of rice *Oryza sativa* L.

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We composed a detailed genetic map for the nuclear genome of rice *Oryza sativa* L., using restriction fragment length polymorphism (RFLP) DNA markers and classically mapped isozyme, morphological, and physiological markers. This integrated map is based on the meiotic segregation of 209 RFLP DNA markers, 3 isozyme markers, 5 morphological markers, and 5 physiological markers detected in 144 F_2 plants from crosses between indica and japonica strains. Total map size is 1575 cM, which exceeds the conventional genetic map by 24%. By virtue of 3 independent methods—gene dosage analysis using hybrid primary trisomics, trisomic segregation analysis in F_2 populations, and linkage analysis of the RFLP markers and chromosome-specific marker genes—the RFLP linkage groups were assigned to the 12 rice chromosomes and the conventional genetic linkage groups. The orientations of the five RFLP linkage groups were aligned to the relevant ones in the conventional map. This integrated map may allow the mapping of genes of unknown location, including those for important agronomic traits and quantitative trait loci.

Recent advances in molecular biology allow detection of a new type of genetic marker that exhibits restriction fragment length polymorphism (RFLP). RFLP reveals differences in the nucleotide sequence of allelic DNA regions. Compared with conventional morphological markers, this type of molecular marker has several advantages for linkage mapping: 1) RFLP markers are expressed in a codominant mode of inheritance, 2) RFLP markers are not influenced by environmental or developmental effects, and 3) the number of RFLP markers is virtually unlimited. Linkage maps based on RFLP markers have been constructed for maize, tomato, rice, and some other crops (Helentjaris et al 1986, Landry et al 1987, McCouch et al 1988).

Linkage maps and genetic markers have potential application in selection systems for plant breeding (Tanksley et al 1989). For rice, a linkage map of conventional morphological markers and isozyme loci, including important agronomic trait loci such as disease and pest resistance, has been compiled (Kinoshita 1987). More

recently, a linkage map of RFLP markers has also been constructed for rice (McCouch et al 1988). However, the relationship between these two types of linkage maps remains to be clarified.

To utilize the linkage map and molecular markers as a device in the selection system for rice breeding, we first embarked on the construction of a linkage map of RFLP markers (Kishimoto et al 1989). The present study was undertaken to assign the RFLP linkage groups to the 12 chromosomes and to integrate the RFLP linkage map and the conventional genetic map.

Materials and methods

Plant materials, detection of RFLP, and linkage analysis are discussed here.

Plant materials

Four F_2 populations of crosses between strains of indica and japonica types with several genetic marker genes were used. Crosses and segregating marker genes of the F_2 populations are listed in Table 1. The chromosomal and map locations of the marker genes that were segregated in these F_2 populations have already been identified (Table 1). We examined 144 plants of an F_2 from Kasalath/FL 134. and 70 plants from the 3 other crosses. Phenotypes of morphological markers and genotypes of isozyme markers in F_2 individuals were evaluated on the basis of plant morphology and electrophoresis patterns. To assign RFLP markers to their respective chromosomes, we used the japonica/indica hybrid primary trisomics (some plants provided by T. Ogawa) and F_2 populations from the hybrid trisomics.

Detection of restriction fragment length polymorphism

Total DNA was isolated from the leaves according to the cetyltrimethylammonium bromide extraction method (Murray and Thompson 1980). Total DNA of parents and F_2 individuals was digested with a restriction enzyme, either *Bam*HI, *Bgl*II, *Eco*RV, or *Hin*dIII. Southern blotting and hybridization were performed according to the method of Southern (1975). Asize-selected (0.5-1.5 kb) *Pst*I genomic library of rice DNA from

Table 1. Cross combinations and segregating genetic marker genes in 4 F_2 populations.

Cross	Marker genes segregating in F_2[a]
Kasalath/FL 134	*d-30*(8), *chl-1*(5), *lg*(11), Ph(11), *Sdh-1*(4), *Est-2*(6), *alk*(6), *sp*(9), *Rc*(10), *Est-3*(?), *Lap*(?)
Surjumkhi/FL 209	*Bp*(1), *fgl*(7)
Kasalath/FL 193	*lax*(3), *z-1*(9)
FL 159/Dakanalo	*spl-7*(4), *nl-1*(2)

[a]Numbers in parentheses are numerical designations of the chromosomes (Nishimura 1961) on which marker genes are located.

a japonica variety, Nipponbare, was used as a probe source for RFLP detection. These probes were labeled with ^{32}P-dCTP by the random priming system. The filters after hybridization were washed to stringency detecting 50% homology. X-ray films were exposed to these filters for 1-4 d at -70 °C with intensifying screens.

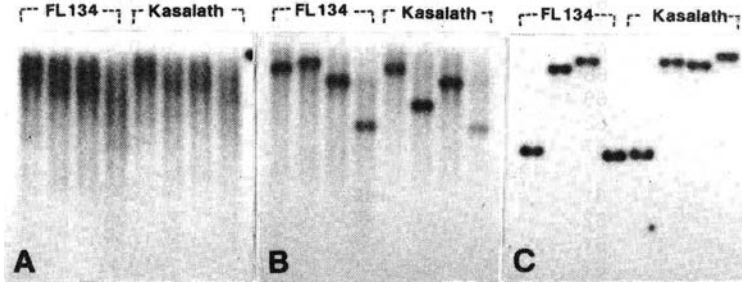
Linkage analysis

Linkage analysis was performed based on F_2 segregation patterns of each pair of markers. Recombination values were estimated by the maximum likelihood method, and the values with 1% significance in the chi-square test of independence were utilized for mapping. The three-point linkage test was used to determine the linear order of each marker. The centiMorgan distances for each pair of adjacent markers were estimated from recombination values with the Kosambi function (Kosambi 1944).

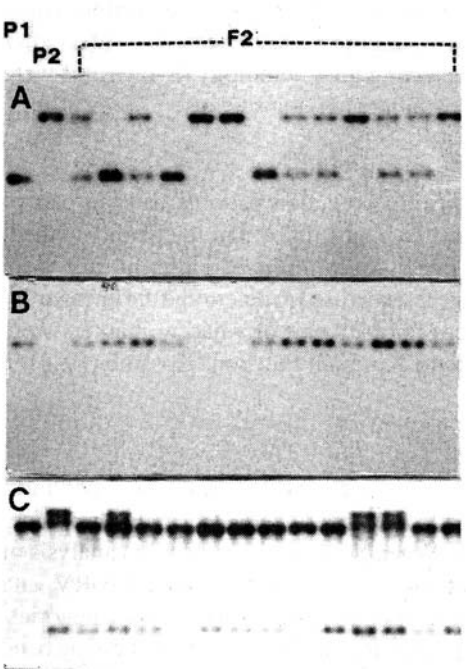
Results

To obtain RFLP probes, randomly cloned DNA fragments that revealed a polymorphism between Kasalath (indica) and FL 134 (japonica) were screened from a size-selected *Pst*I genomic library of rice DNA. When Southern hybridization analysis of 787 clones was performed using 4 restriction enzymes (*Bam*HI, *Bgl* II, *Eco*RV, and *Hin*dIII), the hybridization patterns of the clones were classified into 3 categories: smear (A), band in smear (B), and clear band (C) (Fig. 1). Of 472 clones showing band patterns, 262 showed a polymorphic pattern between Kasalath and FL 134. By using restriction enzymes *Eco*RV and *Bgl*II, RFLP was detected at a slightly higher frequency than by *Bam*HI and *Hin*dIII. Most of the 262 clones were classified as single copies based on the signal intensity of the band.

To construct a linkage map, we first evaluated the segregation of each RFLP, morphological, physiological, and isozyme marker in 144 F_2 individuals from the cross Kasalath/FL 134. Most clones showed a single locus with a codominant or dominant mode (Fig. 2A,B). However, some clones showed two or three loci (Fig. 2C). The segregation ratio of most RFLP and conventional genetic markers fitted the expected



1. Hybridization patterns of 3 types of randomly selected genomic clones by autoradiography. Total DNA of Kasalath and FL 134 was digested with 4 restriction enzymes, *Bam*HI, *Bgl*II, *Eco*RV, and *Hin*dIII (left to right lanes). Patterns A, B, and C were classified as smear, band in smear, and clear band, respectively.



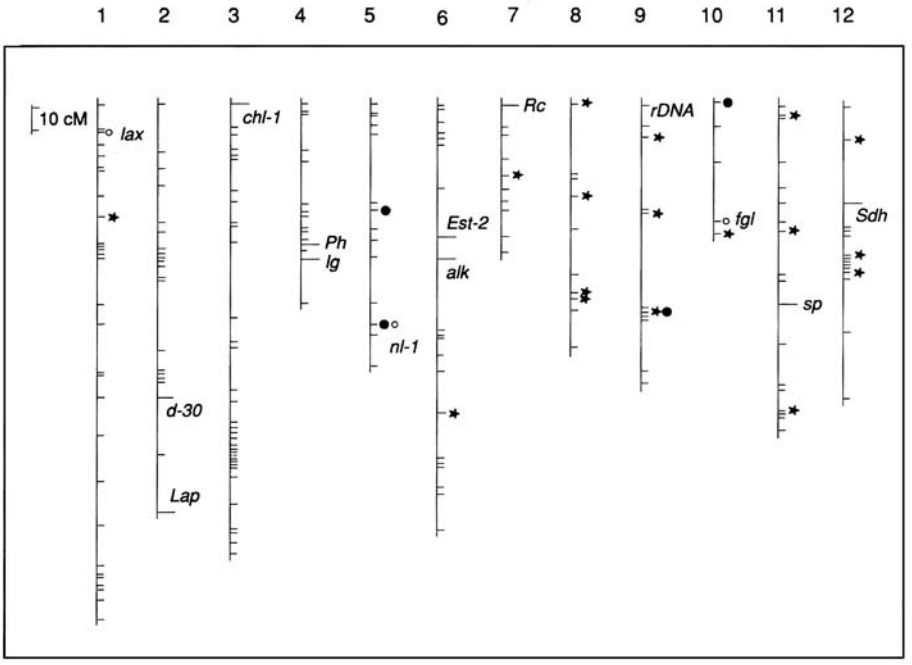
2. Segregation of some RFLP markers in F_2 population of Kasalath (P_1)/FL 134 (P_2). A = segregation of single locus with codominant inheritance mode; B = segregation of single locus with dominant inheritance mode: C = segregation of 2 loci with codominant or dominant inheritance mode.

Table 2. Segregation deviations of some RFLP markers in F_2 population of Kasalath/FL 134.

RFLP marker (numerical designation)	Segregation in F_2 population			Frequency of homozygote of Kasalath allele
	Homozygotes with Kasalath alleles	Heterozygotes	Homozygotes with FL 134 alleles	
PN 144	62	63	19	0.43
PN 51	67	59	18	0.47
PN 23	72	58	14	0.50
PN 15	67	43	14	0.54
PN 62	68	56	12	0.50
PN 74	68	63	13	0.47
PN 238	62	67	15	0.43
PN 65	57	--77--		0.40
PN 224	--129--		14	-[a]
PN 236	61	--82--		0.43
PN 28	62	--82--		0.43
PN 131	61	--77--		0.40
PN 129	61	65	18	0.42
PN 55	55	71	18	0.38

[a] Frequency could not be calculated because the homozygote of Kasalath allele and the heterozygote could not be distinguished.

ratio of codominance (1:2:1) or dominance (3:1). However, some RFLP markers showed significantly deviated segregation from the expected ratio (Table 2). The recombination values of possible pairs of markers were calculated based on segregation data by the maximum likelihood method, and three-point linkage analysis was performed to determine the linear order of markers. The linkage map thus constructed had 12 linkage groups containing 209 RFLP markers and 3 morphological, 4 physiological, and 3 isozyme markers (Fig. 3). By using some of the RFLP markers mapped on linkage groups, we examined the linkage relationship between RFLP markers and conventional genetic markers segregated in the F_2 populations of the other three crosses. The morphological markers *lax* and *nl-1,* and the physiological marker *fgl* were linked to RFLP markers PN 113, PN 25, and PN 133, respectively. Therefore, on the basis of conventional genetic markers of known chromosomal location, nine RFLP linkage groups were assigned to respective chromosomes. Moreover, the 17 and 4 RFLP markers were also assigned to respective chromosomes by using gene dosage analysis in hybrid primary trisomics and trisomic segregation analysis in the F_2 population, respectively. Finally, the 12 RFLP linkage groups were assigned exactly



3. Rice RFLP linkage map. Stars (★) show RFLP markers that were assigned to their respective chromosomes through gene dosage analysis in hybrid primary trisomics. Dots (●) show RFLP markers that were assigned to their respective chromosomes through trisomic segregation analysis. Two morphological markers, *lax* and *nl-1,* and one physiological marker, *fgl,* are shown near their respective linked RFLP markers (o). 1 to 12 = chromosome numbers following designation of Nishimura (1961).

to the 12 chromosomes (Fig. 3). For chromosomes 2, 3, 5, 6, and 11, alignment of RFLP linkage groups to conventional linkage groups was partially determined on the basis of conventional genetic markers shared by both linkage groups.

Discussion

Generally, F_1 plants of indica/japonica crosses show remarkable hybrid sterility. However, F_1 plants of Kasalath/FL 134 showed high seed fertility (86%), suggesting that distorted segregation in the F_2 was not caused by F_1 sterility. We calculated the recombination values based on a possible classification of segregating genotypes in pairs of loci (9, 6, and 4 classes). Therefore, we consider the map distances calculated in this study to be accurate. Thus we could partially integrate the RFLP linkage map and the conventional one on the basis of the 13 conventional genetic markers shared by both linkage groups. The total size of the linkage map is 1575 cM, which exceeds the conventional genetic map by 24%.

Some RFLP markers showed a deviated segregation in the F_2 population of Kasalath/FL 134. At these loci, the frequency of the Kasalath alleles in the F_2 progeny was higher than that of FL 134 alleles. These loci were mapped in a cluster on the linkage group corresponding to chromosome 3. Nakagahra (1972) and Nakagahra et al (1972) reported that gametophyte genes control the fertilization activity of pollen located on chromosome 3. Therefore, we conjecture that the distorted segregations observed in this study were caused by the existence of gametophyte factor(s) in this chromosomal region.

RFLP markers are potentially useful in many aspects of plant genetics and breeding (Lander and Botstein 1989, Tanksley et al 1989, Young and Tanksley 1989). In tomato, the loci underlying several important agronomic traits were identified using RFLP linkage analysis (Martin et al 1989, Paterson et al 1988, Sarfatt et al 1989, Young et al 1988). In rice, the conventional genetic map has been constructed with about 140 markers, including some important agronomic traits such as disease and pest resistance (Kinoshita 1987). However, most of the map locations of the agronomic trait loci remain to be analyzed. In the rice RFLP map constructed in this study, the RFLP markers were scattered throughout the 12 chromosomes. Therefore, the markers make it possible to clarify the map locations of the loci controlling the important agronomic traits. The markers and the map may become powerful tools in detecting or specifying the chromosomal region underlying quantitative loci.

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Intraspecific variation and genetic differentiation based on restriction fragment length polymorphism in Asian cultivated rice, *Oryza sativa* L.

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Restriction fragment length polymorphism of Asian cultivated rice *Oryza sativa* L. was examined in 135 cultivars to study the intraspecific variation and genetic differentiation of the crop. Most of the cultivars were land races or old cultivars, but some improved varieties were included. For Southern hybridization, 56 clones from a rice genomic library and a maize complementary DNA clone were selected as probes. Various kinds of polymorphism were detected, and almost all the cultivars could be distinguished from each other. Principal component analysis and cluster analysis were used to classify the cultivars into two major groups showing differences in phenol color reaction, *Est-3* alleles, and response to low temperature during the seedling stage. The groups are thought to correspond to the conventional varietal groups: indica and japonica (including javanica). Several smaller groups were found within each major group. Most of the smaller groups are distributed in particular regions.

The genetic variation of Asian cultivated rice *Oryza sativa* L. has been studied by many researchers to classify land race groups, to investigate phylogenetic differentiation, and to provide basic information for breeding.

Kato et al (1928) studied rice intraspecific hybrid sterility and found two groups of cultivars, which he designated as subspecies *indica* and *japonica.* Terao and Midzusima (1939), however, reported continuous variation in hybrid sterility among cultivars and suggested another group distributed on Java Island and vicinity. Oka (1953a,b) systematically surveyed variations in several physiological, biochemical, and morphological characters; performed genetic analyses of hybrid sterility; and classified rice varieties into continental and insular types, subdividing the latter into tropical insular and temperate insular. The three groups of rice are generally recognized as indica, javanica, and japonica. To make a precise classification, genetic variation at isozyme loci was examined (Glaszmann 1986, Nakagahra 1978).

Restriction fragment length polymorphism (RFLP) describes differences in the lengths of DNA fragments arising after treatment with a particular restriction endonuclease. Studies on RFLP in rice have been made mainly to construct linkage maps (Kishimoto et al 1989, McCouch et al 1988). Since variation is detected directly at the DNA level, RFLP can provide important information about genetic diversity and phylogenetic differentiation (Tanaka et al 1989).

Materials and methods

A total of 135 accessions of Asian cultivated rice *Oryza sativa* L. were used (Table 1); 123 were local cultivars collected from various areas of Asia, and 12 were improved varieties.

Germinating seeds were sown in small pots (5 cm diameter) filled with sterilized soil in a greenhouse. The total plant DNA of each cultivar was isolated from leaves of 4-wk-old seedlings using the cetyltrimethylammonium bromide method described by Murray and Thompson (1980).

For detection of RFLP, 57 clones were selected from those used as markers for linkage mapping of rice (Kishimoto et al 1989); all but one came from a genomic DNA

Table 1. Rice cultivars used.

Country or region	Cultivars (no.)	Code names
Japan	5	JP1-JP5
Northern China	2	NCl,NC2
Southern China	4	SC1-SC4
Yunnan Province, China	10	YN1-YN10
Philippines	5	PH1-PH5
Vietnam	9	VT1-VT9
Thailand	6	TA1-TA6
Myanmar	8	MY1-MY8
Laos	10	LA1-LA10
Malaysia	10	MA1-MA1 0
Indonesia	10	JN1-IN10
Nepal	10	NP1-NP10
Bhutan	3	BT1-BT3
Assam, India	2	AS1, AS2
Bangladesh	10	BA1-BA10
India	8	ID1-ID8
Northern Pakistan	5	PK1-PK5
Sri Lanka	5	SL1-SL5
Iran	2	IR1, IR2
Afghanistan	1	AN1
USSR	2	US1, US2
Italy	2	IT1, IT2
Africa	1	AF1
North America	3	NA1-NA3
South America	2	SA1-SA2
Total	135	

library from Nipponbare, a Japanese improved cultivar. The remaining one was maize waxy gene provided by R.B. Klogsen, Max-Planck-Institut für Züchutungsforschung, Germany. Insert DNA fragments from the clones were labeled with ^{32}P-dCTP, using the random primer method, and were used as probes for Southern hybridization (Southern 1975).

Plant DNA from each cultivar was digested with *Bam*HI, *Bgl*II, *Eco*RV, or *Hin*dIII. The resulting DNA fragments were resolved by agarose gel electrophoresis, denatured, transferred to nylon membrane filters, and immobilized (Southern 1975). The DNA attached to each filter was hybridized with a labeled probe by Southern hybridization. Then the filters were washed to the stringency of 50% sequence homology and exposed to X-ray films with intensifier screens at -80 °C for 2-4 d.

The variation between each pair of cultivars was expressed as the proportion of probes that showed different RFLP profiles. The method of cluster analysis of unweighted pair groups using arithmetic means, and principal component analysis (PCA) were used to investigate intraspecific variation and to classify the cultivars.

Results

RFLP led to classification of the cultivars.

RFLP variation

Various kinds of polymorphism were detected by Southern analysis of the DNA: dimorphism (detected in 18 of 57 clones), trimorphism (20), tetramorphism (9), pentamorphism (1), hexamorphism (7), heptamorphism (1), and tetradecamorphism (1). Almost all the 135 accessions used in the study could be distinguished from each other by RFLP except for 2 accessions from Yunnan Province, China (code names YN2 and YN5).

Twenty-five cultivars showed intravarietal heterogeneity for at least one RFLP clone. In such a case, the most frequent genotype estimated was used to represent the accession for PCA and cluster analysis.

Land race groups classified

The PCA scatter diagram revealed two major groups of land races (Fig. 1). The first and second principal components contributed 44.6 and 5.5% of the total variance, respectively. The two groups were distinguishable mainly by the first principal component, and only a few cultivars fell between them. They clearly correspond to two large clusters, tentatively designated as clusters I and II, in the dendrogram drawn by cluster analysis (Fig. 2). The cultivars in cluster I include those from Malaysia, Vietnam, Bangladesh, India, Yunnan Province of China, Sri Lanka, Myanmar, southern China, Thailand, and Nepal. Cluster II includes cultivars from Indonesia, Laos, Japan, Nepal, northern Pakistan, Yunnan Province of China, and Philippines.

Cluster I comprises smaller clusters designated Ia, Ib, Ic, Id, and Ie. The land races in clusters Ia, Ib, Ic, and Id overlap in the PCA scatter diagram, while those in cluster

Ie are clearly apart (Fig. 1). Cluster II contains smaller clusters, designated IIa, IIb, and IIc, the land races of which are distributed separately in the scatter diagram.

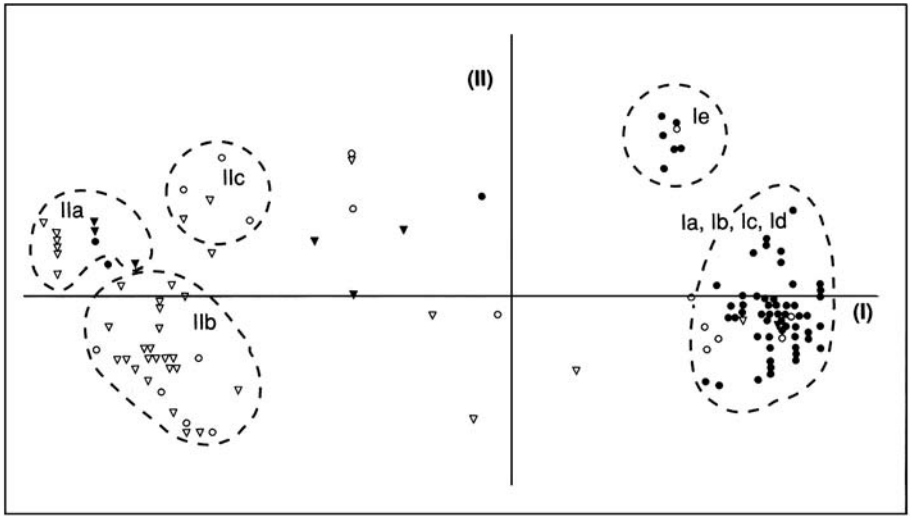
Discussion

Genetic variation was evaluated based on RFLP. Multivariant analysis of RFLP data clearly classified the cultivars into two large groups.

RFLP detected in rice cultivars

Almost all accessions used could be clearly distinguished from each other by RFLP, indicating that each pair of accessions showed a difference for at least one clone. Two Chinese cultivars (YN2 and YN5), which showed an identical profile for all 57 probes, exhibited similarity in plant morphology and esterase isozyme genotype, but differed in phenol color reaction.

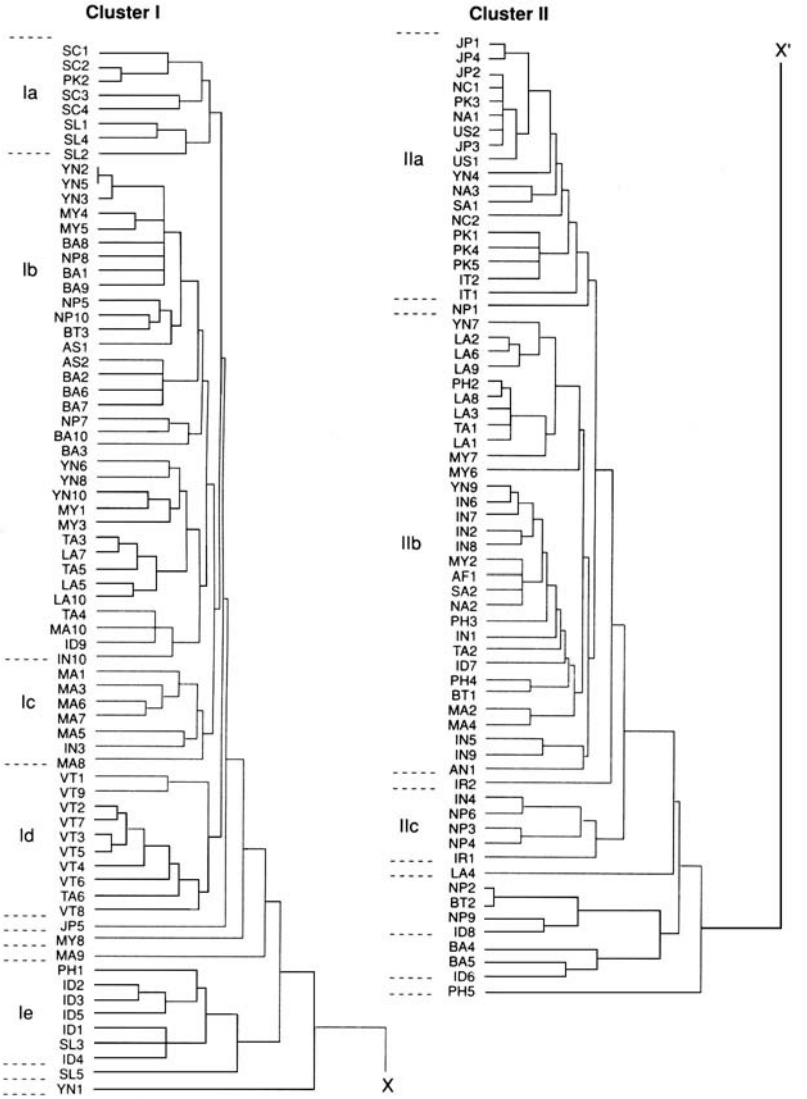
Genetic heterogeneity was noted in 25 cultivars. The genetic variation within a cultivar should have been reduced, because any rice material collected was subdivided into different accessions when different morphological forms were found mixed, and each accession was cultivated more than once for maintenance and multiplication. This heterogeneity implies considerable genetic variation within a land race population. It also suggests that RFLP analysis can be a powerful tool for genetic characterization and phylogenetic differentiation in cultivated crops, as contrasted with morphological characters, which can serve as a direct means for artificial selection.



1. Scatter diagram obtained from principal component analysis of RFLP of 123 land races of rice. Horizontal and vertical axes show first and second principal components, respectively. ● = *Est3*^{2} and *Ph*, o = *Est3*^{2} and *ph*, ▼ = *Est3*^{1} and *Ph*, ▽ = *Est3*^{1} and *ph*.

Intraspecific differentiation in rice

The cultivars in cluster I and those in cluster II were associated with genetic characters such as phenol color reaction and esterase isozymes (Fig. 1). The land races in cluster I usually showed a positive phenol color reaction (*Ph* allele) and had $Est3^2$ allele, while most of those in cluster II showed a negative reaction (*ph* allele) and had $Est3^1$. They



2. Dendrogram indicating relationships among 135 rice cultivars based on cluster analysis of RFLP. Clusters I and II are connected between X and X'.

also showed different responses to low-temperature stress at the seedling stage. The cultivars in cluster I were usually sensitive to low temperature, but those in cluster II were tolerant. The major land race groups classified as clusters I and II are thought to correspond to the conventional varietal groups indica and japonica (including javanica), respectively.

The two distinct groups of cultivars that have been genetically differentiated are supported by isolation mechanisms such as hybrid sterility and gametophyte genes (Nakagahra 1972). Those groups overlap geographically in Southeast Asia, where only a few land races were found to be intermediate between them.

Several smaller groups were classified within the major groups (Fig. 2). The cultivars in cluster Ie were distinct from the other accessions in cluster I. Most of the small groups were distributed in particular regions. For example, the cultivars in cluster Ia were collected mainly from southern China and Sri Lanka, Ic from Malaysia, Id from Vietnam, Ie from India, and IIc from Nepal. Cluster Ib consists of cultivars from Southeast Asia to the northern part of the Indian Subcontinent. Cluster IIa includes cultivars from Japan and the temperate zone of Eurasia. Most of the cultivars in cluster IIb were collected from Southeast Asia. Small indigenous land race groups are therefore thought to have developed in specific areas. Their geographical distributions sometimes overlap.

Further comparative analysis of RFLP; morphological, biochemical, and physiological characters; and other genetic characters, using a large number of land races and, if necessary, wild relatives, should be made to clarify the phylogenetic differentiation of Asian cultivated rice.

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Notes

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Cytoplasmic DNA markers, phylogeny, and systematics in Oryzeae

G. Second

Studies of cytoplasmic DNA restriction fragment length polymorphism (RFLP) were undertaken to investigate phylogenetic relationships among the Oryzeae (mostly genus *Oryza,* section Oryza, including not only cultivated rice but also the whole tribe Oryzeae). Comparisons with nuclear encoded markers such as isozymes and nuclear DNA RFLP markers were made. It appears that the diversity observed at the level of total chloroplast DNA generally supports the scenario of evolution proposed earlier on the basis of isozymes. Some discrepancies are easily resolved as cases of polyphyletic origin (introgression, allotetraploidization) of newly evolved species, assuming maternal inheritance of cytoplasm. The case of the *O. latifolia* allotetraploid complex from America, showing a unique chloroplast DNA although it was assumed to have evolved recently, could not be resolved by conventional interpretation, however. A hypothesis of recombinational origin, assuming occasional biparental inheritance of cytoplasm, was supported by additional RFLP observations. A new hypothesis for the origin of the D genome, unknown in nature at the diploid level, is thus proposed. Diversity at the mitochondrial DNA level, as seen through hybridization with 11 mitochondrial gene probes, paralleled that of the chloroplast DNA between genomes. It was low within species or even genomes. There was, however, more polymorphism in newly evolved species, including cultivated rice. Also, species with the CD genome showed particular relatedness with the E genome at the mitochondrial DNA level. The importance of the Oryzeae in evolutionary studies that bear on plant breeding is stressed. Phylogenetic analysis provides a basis for a revised systematics of genus *Oryza,* section Oryza, in which ancestral species are distinguished from "species" or forms that have evolved since the disturbance of the paleodistribution of *Oryza* by man, including cultivated and weedy forms as well as wild "species."

A sound taxonomy and the evaluation of germplasm diversity must reflect phylogenetic relationships, particularly when the treatment of genetic resources is concerned.

Isozymes, which are primary products of genes, provided a new insight into genetic diversification of the section Oryza (=Eu-Oryza) of genus *Oryza,* including cultivated

rice (summarized in Second 1985b). The observed variation clearly corroborated the earlier definition of genomes by cytogeneticists, and it was possible to adopt their nomenclature, from the A genome to the E genome. Much variation within genomes was also observed.

Reasoning from the geographic structure of the genetic variation seen at the isozyme level, in the framework of paleogeography and general knowledge of the botany and genetics of rice, I propose this scenario for the evolution of *Oryza* that summarizes the inferred phylogenesis.

Section Oryza is composed of two natural groups of species with an origin in the early differentiation of *Oryza* in Eurasia during the Tertiary Era: 1) the "Sativa" group, including all cultivated, weedy, and wild forms or species sharing the A genome; and 2) the "Latifolia" group of species with the diploid genomes B, C, and E and the tetraploid genomes BC and CD, all wild or weedy. Because rice seeds do not migrate naturally over long distances (a postulate validated by the consistency of the evolutionary model summarized in this paper), pan-tropical distribution of both groups resulted from the combined processes of 1) natural migration by land from Eurasia to Africa and Australasia along favorable routes (which occurred temporarily until the Pleistocene) and 2) the intervention of man, who carried seeds of wild species mixed with cultivated varieties in his migrations. (Most wild species considered here are sometimes observed in cultivated fields. Besides, wild species have received attention from man for medicinal or mythical purposes.) By transporting seeds across natural barriers to migration and by perturbing the natural habitats, man unconsciously promoted the evolution of new wild species and weedy forms of rice.

If this is correct, the surprisingly close genetic relationship between all *Oryza* species found in America (A and CD genomes) and some of the Old World species allows the conclusion that *Oryza* has been introduced to America by man. The origin of the D genome of the CD allotetraploid American species, however, remains unclear.

Additional genetic molecular markers are now available in an infinite number since the development of restriction fragment length polymorphism (RFLP) techniques for both nuclear and cytoplasmic encoded DNA. A nuclear DNA RFLP survey of the Oryza section of *Oryza* was recently made (unpubl. data). Not only does it provide additional information on some accessions, but new accessions were made available and studied for the first time. Additional information mostly confirmed the picture of genetic variation obtained at the isozyme level. This means that the genetic structure revealed is very strong and can be evidenced by using only a few loci. The only conflicting relationships were found in forms whose polyphyletic origin is consistent with the above-mentioned scenario: depending on the genetic loci considered, the affinity is then generally with one or the other parental species. In such cases, a limited number of loci are unable to provide an accurate picture. Polyphyletic origin was shown in particular for *O. rufipogon* from America (=*O. glumaepatula*) and from western India as well as for some weedy forms of *O. barthii* (=*O. breviligulata).* Information on newly collected accessions concerned mostly *O. officinalis* from China and the perennial form of *O. rufipogon* in Australia. *O. officinalis* from China was

shown to be different at numerous loci from *O. officinalis* from India. This variation, however, was unable to account for the origin of the D genome, which appeared to be a highly polymorphic genome not closely related to any known diploid form (the possibility of its future discovery at the diploid level appears to be very unlikely in America: Second 1990). Note, however, that neither *O. eichingeri* from Sri Lanka (Vaughan 1990) nor *O. officinalis* from Papua New Guinea has been studied. Comparing the CD genomes with diploid genomes, the E genome appeared the closest, after the C genome, confirming a trend also observed at the isozyme level. The perennial form of *O. rufipogon* from Australia appeared to be close to Asian *O. rufipogon,* contrary to the annual form of *O. rufipogon* from Australia (=*O. meridionalis*), which was confirmed to be highly divergent.

The potential of RFLP studies in phylogenetic investigations of the Oryzeae has not yet been fully utilized. Probes better characterized in terms of the evolutionary conservatism of the DNA fragments they hybridize to, mapped with confidence and used in large numbers, should give a complete picture of the genetic relationships among the Oryzeae tribe, both across wide genetic distances as well as between closely related forms. Introgressed forms in particular should be characterized in terms of which chromosome fragment(s) was introgressed.

We will restrict ourselves here to the information obtained from RFLP studies of the DNA encoded in the chloroplast and the mitochondria. Because the cytoplasm is generally maternally inherited in grasses, as in most plants, these markers should trace the phylogeny of the cytoplasm. In the case of genetic divergence in complete isolation during a long evolutionary time, both the nuclear and the cytoplasmic encoded markers are expected to diverge simultaneously and thus to show the same relatedness with other genomes. However, when independent evolutionary lines merge in allotetraploidization, hybridization, or introgression, nuclear and cytoplasmic encoded markers may be expected at times to show contrasting relationships. Moreover, we should bear in mind that nuclear and cytoplasmic DNA are coadapted (not all possible combinations are equally fit); that some DNA sequences occur ubiquitously in nuclear, chloroplast, and mitochondrial DNA; and that cytoplasm can occasionally be inherited biparentally, as is suggested in particular for rice (Second et al 1990).

Chloroplast DNA RFLP and phylogeny in the Oryzeae

Chloroplast DNA (ctDNA) is the most conserved during evolution, compared with mitochondrial and nuclear DNA. For this reason, and also because it has never been shown to recombine in nature in higher plants, it is thought to be an ideal marker of phylogenetic relationships, particularly above the species level.

Section Oryza

An innovative nonaqueous technique (Dally and Second 1989) was used to purify ctDNA from 320 single plants representing the breadth of the available variation within section Oryza. Complex restriction patterns obtained with the enzymes *Eco*RI and

*Ava*I were studied in all plants, and 32 plastotypes (chloroplast genotypes) were distinguished. These 32 plastotypes were further characterized for their restriction patterns with *Bam*HI, *Pst*I, *Hin*dIII, *Bst*EII, and *Sma*I enzymes. The restriction patterns were observed directly under fluorescence in ethydium bromide, but at least one specimen of each DNA pattern was saved through Southern blot transfer for later hybridization with selected probes. The molecular size of restriction fragments was carefully determined by comparison with a DNA "ladder" of known molecular size. The data permitted determination of the type of mutations, either additions/deletions or site changes, that explained the observed variation between patterns. Because reproduction of the ctDNA molecule is clonal (at least generally), a cladistic analysis (by successive branching, according to shared mutations) was preferred to the multivariate analysis adopted for nuclear encoded markers for which the importance of recombination in reproduction is paramount (Dally 1988, Dally and Second 1990).

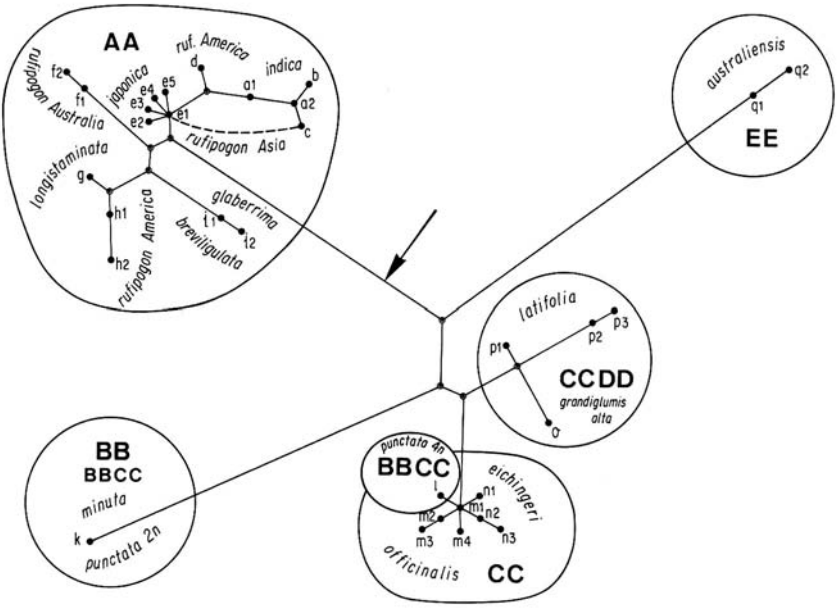
As the complete sequence of ctDNA from a japonica cultivar is now available (Hiratsuka et al 1989), it is possible to compare the observed patterns for the most common japonica-related plastotype with the patterns deduced from the sequence. The comparison shows that the estimation of molecular sizes of restricted fragments by Dally (1988) was accurate (generally less than 5% error). Major discrepancies occurred only in some fragments of *Ava*I and *Sma*I restriction patterns. Hybridization with probes showed they were due to errors of the sequence in the corresponding CG-base-rich restriction sites.

Major additions/deletions, which are detected with several enzymes, and many of the presumed restriction sites can be readily located on the physical map of rice ctDNA deduced from its sequence. Systematic hybridization of selected probes (covering the whole ctDNA molecule) with the DNA patterns saved on filters allowed resolution of most remaining ambiguities. Revision of the type of mutation presumed was necessary only in some cases where there were a large number of mutations to be explained between two patterns. It did not substantially modify the deduced cladogram. In short, the results obtained from direct observation of the restriction patterns are well substantiated by more refined verifications. They are also consistent with most observations made by other workers on rice ctDNA diversity (Ichikawa et al 1986; Ishii et al 1986, 1988).

Figure 1 shows the cladogram summarizing variation in the observed genetic structure at the ctDNA level in section Oryza. As the position of the "root" of the cladogram shows, two main groups of plastotypes are found. They correspond respectively to the Sativa and the Latifolia groups of species, confirming the validity of their recognition as natural groups at the phylogenetic level.

Comparing the genetic structure of section Oryza at the isozyme and ctDNA levels reveals many similarities, but there are also striking differences that we will now highlight separately for the Sativa and the Latifolia groups.

At the ctDNA level, the Sativa group is composed of two clusters: 1) *O. sativa* (with generally distinct plastotypes for indica and japonica varieties) along with its direct



1. Cladogram showing relatedness among 32 plastotypes distinguished in Oryza section (Dally 1988). Capital letters stand for nuclear genomes and small letters for plastotypes. Length of a branch is approximately proportional to number of mutations specific to that branch. Arrow indicates "root." Dashed line indicates a suggested possible origin in recombination of the *c* plastotype. As discussed in text, CCDD branches should also notably be considered as recombinant between BB and CC genomes plastotypes.

ancestor *O. rufipogon* from Asia (and some *O. rufipogon* from America); 2) a group of three branches corresponding respectively to *O. longistaminata* (unexpectedly, some *O. rufipogon* from America had two plastotypes very similar to the only plastotype found in *O. longistaminata*), *O. breviligulata* (with its cultivated form *O. glaberrima*), and *O. rufipogon* from Australia (interestingly, both the annual and the perennial forms shared the same two plastotypes in spite of their differences at nuclear encoded markers). The high degree of divergence found at the isozyme level for, respectively, *O. longistaminata* and the annual form of *O. rufipogon* from Australia, relative to other species in the Sativa group, was not observed at the plastotype level. This may be explained by nucleo-cytoplasmic substitution between forms early established in Africa and Australasia and more recent migrants from Asia to Africa and Australia. In Australia, this event would be recent (glaciation age?), consistent with the observation of apparent hybrid populations between the two forms (Second 1987). In Africa, this corresponds to an event presumed to have taken place much longer ago (2 million yr?) when the evolution of climate last allowed the migration of an annual form, the ancestor of *O. barthii*, and the reinforcement of the reproductive barrier with *O.*

longistaminata occurred (Second 1985a). In both cases, the chloroplast genome of the most recent immigrant from Asia was retained.

The fact that all forms of *O. rufipogon* from Australia (and also the "Oceanian" form of *O. rufipogon* from New Guinea [Ishii 1991) share the same two unique plastotypes supports their recognition as a distinct species, *O. meridionalis* Ng, with three subspecies: annual Australian, perennial Australian, and New Guinean. The perennial subspecies is, however, similar to Asian *O. rufipogon* at the morphological level as well as at most nuclear isozyme and RFLP loci. It may thus be interpreted as a result of hybridization between Asian *O. rufipogon* and *O. meridionalis.*

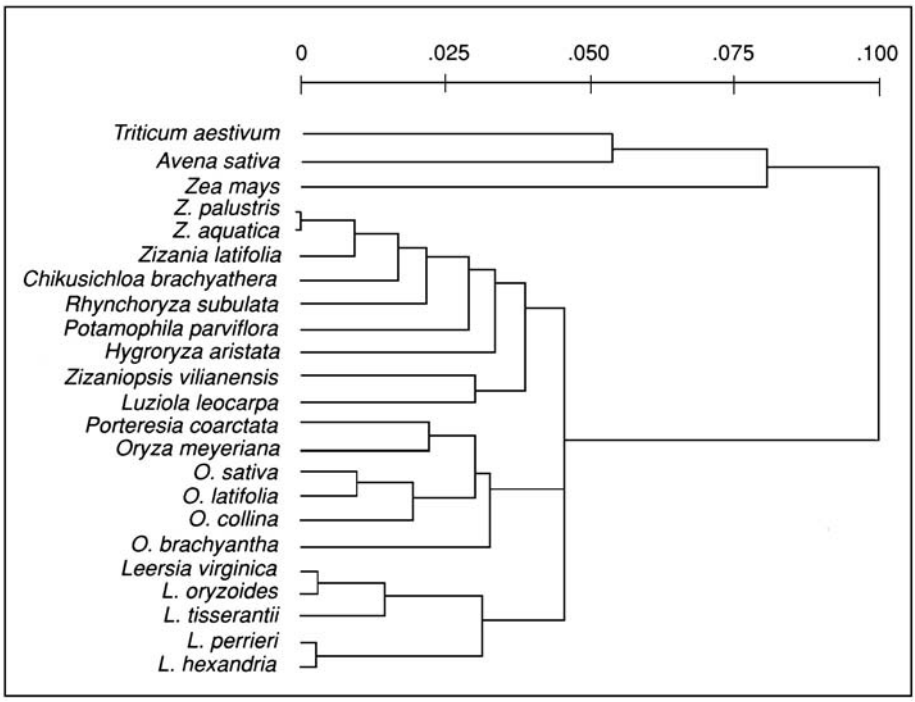
The high heterogeneity of the plastotypes of *O. rufipogon* from America and their close relation to Old World plastotypes again point to its polyphyletic and recent origin.

The Latifolia group of species appears composed of four clusters of plastotypes. Three of them correspond to the diploid genomes B, C, and E. The allotetraploids BC share a plastotype identical or close to either the B or C genome plastotypes. At least two events of allotetraploidization are then suggested for the origin of the BC genome. The fourth cluster corresponds to the CD genome, which appears particularly polymorphic.

The high degree of divergence of the B and E genome plastotypes is in line with their presumed long period of evolution in isolation in Africa and Australasia, respectively. Application of the molecular clock concept, based on published estimations of mutation rates for ctDNA, point, for both of them, to a time of divergence, around 15 million yr ago, in agreement with the proposed evolutionary scenario (Dally 1988). From the same perspective, however, the divergence of CD genome plastotypes is in clear contradiction with the presumed recent emergence of that genome in America. Conventional interpretation would point to an ancient differentiation of the CD genome or of one of its (missing) progenitors. Because this violates the postulate that seeds of rice do not spontaneously cross large stretches of ocean, of which we are confident because it appears to be validated by other observations, an alternative explanation was sought. Evidence for an exceptional case of rapid differentiation of that plastotype was found in its similarity, not only to C genome plastotypes, but also to the B genome plastotype (Dally and Second 1990). Mapping of the respective mutations and further characterization with additional restriction enzymes did point to an origin of the CD plastotypes in a combination (recombination or an unknown mechanism?) of derived mutations characteristics of both the C and B plastotypes (unpubl. data), but more data are necessary to support a positive conclusion.

Tribe Oryzeae

A preliminary analysis of phylogenetic relationships among tribe Oryzeae (as understood in Clayton and Renvoize 1986) at the ctDNA RFLP level was conducted by Zhang and Second (1990). Figure 2 illustrates the main results, which agree fairly well with the modern treatment of the genera *Leersia* and *Oryza.* A striking feature is, however, that two morphologically similar *Leersia* species, *L. tisserantii* from Africa



2. Average linkage dendrogram based on genetic distances for ctDNA RFLP among individual representatives of various species and genera of tribe Oryzeae and 3 other genera of grasses (Zhang and Second 1990).

and *L. perrieri* from Madagascar, appear to be widely divergent at the ctDNA level. as much so as the most divergent species in the genus *Oryza.* Both of them used to be placed in the genus *Oryza,* indicating their morphological relatedness to that genus. A tempting hypothesis is thus that these two *Leersia* species are relics of a common ancestor of both *Leersia* and *Oryza.* The common ancestor was born on the African plate and diverged early because of the separation of Madagascar from it. A similar hypothesis was proposed by Second (1985c) on the basis of the biogeography of the Oryzeae. A prediction made from that model was that species such as *L. tisserantii* and *L. perrieri* could be diploids, while all the *Leersia* species so far observed for chromosome number were reported to be polyploids (Pyrah 1969). A comparison of the number of bands in nuclear DNA RFLP patterns (per plant, among species) fulfills this prediction. Another prediction of the model was that the introduction of *Oryza* in Madagascar was recent, through the agency of man. This also appears to be likely.

Another striking result apparent in Figure 2 is the relatedness of the observed polyploid *Leersia* species with the presumed diploids. *Leersia hexandra* (distributed worldwide) appears closely related to *L. perrieri* (as confirmed also at the nuclear DNA level), while *L. virginica* (North American) and *L. oryzoides* (North temperate regions) are relatively closely related to *L. tisserantii.*

Mitochondrial DNA RFLP in section Oryza

Mitochondrial genes used as probes were hybridized on filters prepared from *Eco*RI-restricted total DNA and exhausted for their potential to hybridize with single-copy probes to a detectable level (unpubl. data). Checks for cross hybridization with ctDNA were made on filters prepared from purified ctDNA. Preliminary results are presented for 7 probes showing polymorphism out of 11 used.

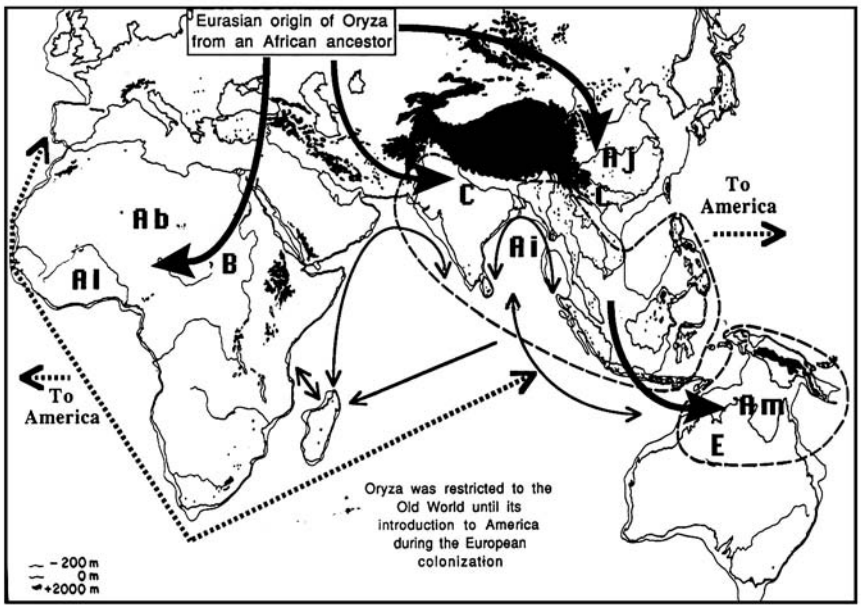
A low degree of polymorphism of the mitochondrial genome (chondriome) was detected within species (although several cases of polymorphism were found in cultivated varieties), but a high degree of polymorphism was observed between the main nuclear genomes. Table 1 omits within-species variation. Most of the probes distinguish the basic genomes A, B, C, and E. BC allotetraploids have an affinity with either the B or C mitochondrial type according to their relationships at the ctDNA level. However, there is some variation, compared with the parental genomes, in the allotetraploid species, and also in those C genome species considered to have recently introgressed some DNA from the B or E genome (*O. eichingeri, O. rhizomatis*). A similar trend of species considered to be recently derived to show more polymorphism was observed at the ctDNA level (Dally and Second 1990) but is more developed at the mitochondrial level. The CD species chondriome is similar to the E species chondriome in three cases out of seven probes showing polymorphism, reinforcing the tendency that its relationship to other species cannot be explained in a conventional way. Further investigation is under way.

Table 1. RFLP in mitochondrial DNA as revealed in section Oryza with 7 probes hybridized with total DNA digested by *Eco*RI (and 1 additional enzyme for *Cox*III, *Atp6,* and COB). Polymorphism within the A genome and within species for other genomes was neglected. Probes are symbolized according to usual nomenclature. (The complete reference of the probes will be acknowledged elsewhere.) Species analyzed were A = Sativa group, B = *O. punctata* (2n), C = *O. officinalis,* Cei = *O. eichingeri,* Crh = *O. rhizomatis,* BCpu = *O. punctata* (4n), BCmi = *O. minuta,* BCma = *O. malampuzhaensis,* CD = *O. latifolia* complex. Observed patterns are symbolized according to the genome or subgenome they characterize.

Probe	Primitive genomes				Derived species or genomes					
	A	B	C	E	Cei	rh	BCpu	BCmi	BCma	CD
*Cox*I	A	B	C	E	C	C	C	B	B	C
*Cox*III	A	B	C	E	C	C	C	B	B	CD
ATPA	A	B	C	A	C	A	C	B	B	A
ATP6	A	B	C	E	C	Co	C	BCmi	BCmi	CD
ATP9	A	B	C	E	Cpu	C	Cpu	C	C	C
COB	A	B	C	E	C	C+E	C	B	B	E
18+5s	A	B	C	E	c	C	C	B	B	E

Conclusion

The pattern of variation observed at the cytoplasmic DNA level fits nicely within the framework of the phylogenetic relationships worked out from the nuclear encoded markers. It reveals additional details, particularly where polyphyletic origins are concerned. The conservation of ctDNA allows one to obtain information across the whole tribe Oryzeae and provides evidence of surprising relationships. A summary of the evolutionary scenario for section Oryza is given in Figure 3. Differentiation of genomes in the Latifolia group occurred in allopatry on different continents: genome B in Africa, C in Asia, and E in Australasia. The absence of genome differentiation (as seen at the chromosome pairing level) among the Sativa group is attributed to its



3. Deduced paleodistribution of genus *Oryza* before man promoted its migration across natural barriers (modified from Second 1985c). Map shows main physical constraints that, along with favorable or unfavorable climate, regulate possibilities of natural migration: thin line along coast is -200 m isochore and approximates coastline when sea level was lowest, during glaciation age; blackened areas show zones above 2000 m. Himalaya appears to be the only mountain range that might have been a barrier to migration, approximately 2 million yr ago. Thick arrows indicate temporary routes of natural migration from Eurasian ancestor of African origin (common ancestor in Tertiary era → *Leersia* in Africa, *Oryza* in Eurasia). Possibilities of migration lasted longer for Sativa group (estimated between 15 and 2 million yr to Africa and between 15 million yr and the last glaciation age to Australasia, across amoving topography of islands) than for Latifolia group (estimated around 15 million yr to both Africa and Australasia). Basic subgenomes for Sativa group and genomes for Latifolia group are indicated: Ai = Asian *O. rufipogon,* indica type; Aj =Asian *O. rufipogon,* japonica type; Am = *O. meridionalis;* Ab = *O. barthii;* Al = *O. longistaminata;* B = *O. punctata;* C = *O. officinalis;* E = *O. australiensis.* Thin plain arrows indicate main maritime routes prior to 15th century, which likely promoted migration of weedy rice between continents. Dashed arrows indicate maritime routes developed since 15th century.

***Oryza*, section Oryza**

SATIVA GROUP (Genome A)

Ancestral species	Cultivated forms	Derived forms or "species"	
		Weedy forms	Newly evolved wild forms
		Lists by no means exhaustive*	
O. rufipogon Griff. (a "complex species")	*O. sativa***:	*O. spontanea:*	*O. nivara* (in West Indies =*O. rufipogon/O. breviligulata)*
	indica ssp	"red rice" (USA)	
	japonica ssp:	"akai-mai" (Japan)	
	tropical	"crodo" (France)	
	temperate	"pururе" (Zanzibar)	
	(+intermediates	etc...	American *O. rufipogon:*
O. barthii A. Chev.	and others)		(=*O. rufipogon/O. longistaminata/*
=*O. breviligulata*			*O. breviligulata,* and others...)
A. Chev & Roehr.	*O. glaberrima*	*O. stapfii*	
O. longistaminata A. Chev. & Roehr.		*O. madagascariensis* "Obake" forms = *O. longistaminata/O. sativa*	
O. meridionalis Ng			
annual ssp		Australian red rice	
perennial ssp			
New Guinean ssp		?	

LATIFOLIA GROUP (Genomes B to E)

Ancestral species	**Newly evolved "species"** Some names of imprecise applicatlon*	
	Genome C introgressed from B or E(?)	**Allotetraploid complex:**
O. officinalis Wall ex Watt (a "complex species", genome C)	Weedy *O. officinalis*	genome BC / genome CD
Chinese ssp	*O. eichingeri* complex	*O. punctata* 4n / genome D= modifled B+E?
South Asian ssp	*O. rhizomatis*	*(=O. schweinfurthiana?)* *O. latifolia*
O. punctata Kotschy (genome B)		*O. malampuzhaensis* *O. alta*
O. australiensis Domin (genome E)		*O. minuta* *O. grandiglumis*

*See Vaughan (1989 and 1990) for synonyms.

** Of hybrid origin between geographic forms of *O. rufipogon.*

4. The systematics of *Oryza*, section Oryza. Two largely independent groups of species are recognized: Sativa and Latifolia. Taxa are arranged by lines and columns. Columns are arranged according to an ''evolutionary concept'' of species: ancestral forms (in boxes), cultivated, weedy, and newly evolved wild forms (following the disturbance of distribution and habitats by man). Lines are arranged according to a ''biological species concept'' considering mostly the main genetic reproductive barriers. In Sativa group, for example, a single species would represent *O. rufipogon* and *O. sativa.* including all weedy forms. It should be called *O. sativa.* However, such possible revision of the nomenclature would not bear on a single universally recognizable species concept. Moreover, it does not appear to be practical. The present treatment is thus preferred.

On herbarium specimens. many taxa cannot be determined with accuracy morphologically, although knowing the geographical origin and habitat helps to discriminate them (see Vaughan 1989). On live accessions, there is clear congruence of morphology, cytogenetics, molecular markers for nucleus, molecular markers for cytoplasm, geographical origin, ecology, and the knowledge of reproductive barriers to characterize ancestral species. However, both Asian species *O. rufipogon* and *O. officinalis* appear to be ''complex species.'' As ancestral species, they include subspecies differentiated on both sides of the Himalaya. Moreover, they include a complex intermingling of ancestral and derived forms.

ecological adaptation, including temporary pools in arid savannah; this allowed it to migrate between continents of the Old World later than the forms of the Latifolia group, which are adapted primarily to more humid climatic areas (at least for its representatives from Asia). The A genome is, however, the most diverse of all genomes distinguished by cytogeneticists. Subgenome differentiation of the A genome clearly parallels in its geographic distribution the genome differentiation of the Latifolia group.

Confidence in the proposed evolutionary scenario led to preliminary evidence that a combination of two widely different plastotypes might explain the rapid differentiation of the CD genome plastotype from America. From an historical point of view, it makes sense that, after the emergence of BC allotetraploids, with both B and C plastotypes, in the Old World, and possible intervention of the E genome, the two of them were introduced to America (possibly as weeds), and their hybridization allowed the evolution of the CD plastotypes and nuclear genomes. The origin of the D genome, so far mysterious, could be in its rapid differentiation at the tetraploid level. A combination of the B and E genomes appears likely to be involved. This hypothesis for the origin of the D genome, presented here for the first time, is suggestive of the profound genome modifications that could be possible in breeding work. Adaptation of forms with new characteristics to new environments, such as those of the American continent, seems to have systematically involved recombination between African and Asian lineages. Transposed to plant breeding, this is the concept of transgressive variation through recombination.

The exact processes of recombination in the ctDNA molecule need to be worked out more precisely by sequencing. If proven, the fact that we were led to the likely existence of that phenomenon would exemplify that the Oryzeae provide an outstanding model for the study of plant evolution under domestication or human disturbance. The Oryzeae model, with a whole gradation of divergence time (from millions of years to a few centuries, as calibrated by events in the paleoenvironment and in history) and a wide variety of life history types and ecological situations, is a powerful tool for addressing evolutionary questions that have bearing in the breeding of rice at the molecular as well as organismic levels.

Phylogenetic information provides a basis for a revised systematics of *Oryza*, section Oryza, shown in Figure 4, in which ancestral species are distinguished from derived forms or "species," including cultivated and weedy forms, and also newly evolved wild species. These have escaped from the intermixing of different ancestral species through disturbance by man of natural habitats and distribution.

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Notes

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Biology and genetics of *Pyricularia oryzae* and *P. grisea* populations: current situation and development of RFLP markers

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Pyricularia oryzae and *P. grisea,* fungal pathogens of monocots, have different host ranges. Isolates pathogenic to rice (*P. oryzae*) are restricted to this host plant but are closely related to isolates pathogenic to other cereals or grasses (*P. grisea*). *P. oryzae* and *P. grisea* are heterothallic hermaphroditic ascomycetes, and their sexual state has been regrouped under the name *Magnaporthe grisea.* Crosses between isolates from almost all host range groups are possible in the laboratory, indicating the potential for gene flow in the field, as most of these forms coexist. The gene flow within rice pathogens is believed to be low, because most rice isolates are female sterile and can exchange genes only by outcrossing with hermaphroditic isolates pathogenic on other hosts. Thus, populations of rice pathogens might be clonal and relatively isolated from *P. grisea* populations. This hypothesis could be tested by studying the genetic structure of these populations with sufficiently polymorphic genetic markers such as restriction fragment length polymorphism (RFLP) markers. For that purpose, we cloned the ribosomal RNA gene cluster and sequenced two domains of the 26 S RNA gene. No polymorphic restriction sites were found within rice pathogens, but some unit length polymorphism was detected. We found no differences in the sequences of the 26 S RNA gene analyzed, either between rice isolates or between *Eleusine coracana* and rice isolates. Random single-copy probes from a rice pathogen (Guy11) lambda phage library were used to detect RFLP among rice, maize, wheat, and *E. coracana* isolates. To date, we have detected only a little RFLP within rice isolates, but much between isolates differing in host range. Thus, rice isolates and those from other hosts must have evolved independently for sufficient time to accumulate considerable specific RFLP.

Our knowledge of the population biology of the fungal plant pathogens *Pyricularia oryzae* and *P. grisea* (teleomorph, *Magnaporthe grisea*) has expanded in the past 10 yr with the accumulation of data on host range, sexual reproduction, and inheritance of pathogenicity. While these aspects are actively under study, others are underdeveloped, particularly the genetic structure of *P. oryzae* populations, because of the lack of genetic markers. We present an update of our knowledge of the population biology and

genetics of *P. oryzae* and *P. grisea,* together with the current state of development of restriction fragment length polymorphism (RFLP) markers necessary to study these populations at the genetic level.

Host specificity of *P. oryzae* and *P. grisea* isolates

Blast caused by the fungus *Pyricularia oryzae* is one of the major diseases of rice (Ou 1985). *P. grisea,* which is morphologically similar to *P. oryzae,* causes diseases on different cereals and grasses (Asuyama 1963, Mackill et al 1986, Ou 1985). Finger millet (*Eleusine coracana*) blast, which occurs in almost all millet-growing areas, is caused by *P. grisea* isolates pathogenic to *E. coracana* but not to rice (Kato et al 1977, 1980; Ramakrishnan 1963). Isolates pathogenic to wheat (but not to rice) have been recorded in the past 4 yr in Brazil, where they cause severe damage (Goulart et a1 1989). Other crop diseases due to *P. grisea* occur on ryegrass (Trevathan 1982), St. Augustine grass (Atilno 1983), *Pennisetum typhoides* (Ramakrishnan 1963), and *Panicum miliaceum* (Ramakrishnan 1963). *P. grisea* is also pathogenic to grasses frequently encountered around ricefields such as *Digitaria* sp. and *Eleusine indica* (Asuyama 1963, Kato and Yamaguchi 1980, Mackill et al 1986). It has been strongly suggested that all these isolates belong to the same species (Asuyama 1963, Kato 1978, Rossman et al 1990, Yaegashi and Udagawa 1978b), which should be named *P. grisea* (Rossman et al 1990), and that isolates with different host specificities could be grouped into *formae speciales* (Asuyama 1963). Results of some studies are contradictory or unclear; however, *P. oryzae* and *P. grisea* isolates can be classified according to their pathogenicity to differential hosts such as *E. coracana/E. indica, Eragrostis curvula* (weeping lovegrass), *Digitaria sanguinalis* (crabgrass), *Panicum repens, Setariae italica,* and rice (Asuyama 1963, Kato 1981, Kato et al 1980, Mackill et al 1986, Ramakrishnan 1963). Some other plant species such as *Lolium multiflorum* (ryegrass) and *Festuca elatior* are common hosts in the sense that they are attacked by isolates with different host ranges, including rice isolates (Kato et al 1980, Trevathan 1982). For example, isolates from *Eleusine coracana* are pathogenic to *E. coracana/E. indica* and a few other Gramineae such as *L. multiflorum, F. elatior,* barley, and maize (Kato et al 1977, 1980; Ramakrishnan 1963). However, they are not pathogenic to rice, *D. sanguinalis,* or *Eragrostis curvula.* Contradictory reports might be due to some variability in host range of a small number of isolates within a population. For example, among the populations of rice pathogens, only a few isolates are also pathogenic to *D. sanguinalis* (Ou 1985), *S. italica* (Kato et al 1980), or *E. curvula* (Valent and Chumley 1990), but all the other isolates are pathogenic only to rice and common hosts such as *L. multiflorum* and *F. elatior.* The existence of such isolates stresses the problem of genetic exchanges between *P. oryzae* and *P. grisea* isolates.

Sexual reproduction in *P. oryzae and P. grisea*

Sexual reproduction was first achieved in crosses between *P. grisea* isolates pathogenic to *D. sanguinalis,* showing that *P. grisea* is a heterothallic hermaphroditic ascomycete

with two alleles of one mating-type gene (Hebert 1971). The morphologies of the perithecia, ascus, and ascospores were used to classify the perfect state of *P. grisea* into *M. grisea* (Barr 1977, Yaegashi and Udagawa 1978). Perithecia were also obtained in crosses between *Eleusine coracana* isolates of opposite mating type (Yaegashi et al 1976, Yaegashi and Udagawa 1978), which are the most fertile among *P. grisea* isolates (Yaegashi 1977). But first attempts to cross rice isolates were unsuccessful (Yaegashi 1977, Yaegashi and Nishihara 1976). Nevertheless, crosses between isolates with different host specificities were possible (Yaegashi and Nishihara 1976), particularly between rice isolates and *E. coracana* isolates (Kato et al 1976, Valent et al 1986, Yaegashi and Asaga 1981, Yaegashi and Nishihara 1976, Yaegashi and Udagawa 1978), providing additional evidence for the grouping of all these isolates in the same species. These results led to an extensive search for rice isolates that could be crossed. Unfortunately, only very few hermaphroditic (female and male fertile) rice isolates were found among a large number screened (Kato and Yamaguchi 1982; Leung et al 1988; J.-L. Notteghem, unpubl. data; Ruy et al 1988; Valent et al 1986). The large majority of the rice isolates are female sterile (Itoi et al 1983; Kolmer and Ellingboe 1988; Leung and Williams 1985; J. -L. Notteghem, unpubl. data; Valent et a1 1986) and can behave as males with hermaphroditic isolates from other hosts or from rice. Frequently only one of the two mating types is present in the same area (Kato and Yamaguchi 1982; J. -L. Notteghem, unpubl. data; Yaegashi and Yamada 1986). These characteristics make the occurrence of sexuality in ricefields improbable, and, although perithecia were obtained experimentally from infected rice plants (Silue and Notteghem 1990), they were never observed in the field. Thus, there should be low or no sexual recombination between rice isolates. This might not be the case for *E. coracana* and *D. sanguinalis* isolates, which can undergo sexual reproduction between themselves and outcross with isolates having other host specificities. Thus, although rice isolates are likely to be clonal populations, some genetic exchanges could arise through outcrossing with isolates nonpathogenic to rice. The occurrence of this gene flow also depends on the number of genes necessary to confer pathogenicity on each host, which has great influence on the ease of recovering progeny with parental or wider host ranges.

Genetics of host specificity and avirulence

Crosses between isolates with different host ranges have shown that host specificity is controlled by either a few genes or multiple genes. When isolates pathogenic to E. *coracana/E.* indica were crossed with isolates pathogenic to *Eragrostis curvula,* each host specificity was shown to be controlled by a single gene (Valent et al 1986; Valent and Chumley 1987, 1990; Yaegashi 1978). Isolates pathogenic to both hosts were obtained in the progeny, showing that the host range could easily be extended through recombination; this might be one explanation for the existence of isolates with additional host specificities. The situation seems to be more complex in the case of isolates pathogenic to rice. In crosses between isolates from rice and those from other hosts (*E. curvula:* Kolmer et al 1988, Valent and Chumley 1990; *Eleusine coracana:*

Valent et al 1986, Yaegashi and Asaga 1981, Yaegashi and Yamada 1984), the viability of ascospores of the first generation is generally low, and only a few progeny (0-25%) are pathogenic to rice. Furthermore, progeny pathogenic to rice have low aggressiveness, frequently corresponding to a reduction in lesion size (Valent and Chumley 1990, Yaegashi and Yamada 1984). In a cross between an *Eragrostis curvula* isolate and a rice isolate, successive backcrosses of the progeny to the parental rice isolate were necessary to improve their aggressiveness to rice (Valent and Chumley 1990), indicating that the *E. curvula* isolate lacks several of the genes necessary to be fully pathogenic to rice. On the other hand, the *E. curvula* isolate carries avirulence genes that determine its nonpathogenicity toward specific rice cultivars (Valent and Chumley 1990). The same results were obtained with crosses between *Eleusine coracana* and rice isolates (Yaegashi and Asaga 1981, Yaegashi and Yamada 1984). These experiments show that isolates from hosts other than rice must accumulate many genes to be pathogenic to rice. Thus, it seems unlikely that new rice pathogens could easily result from the progeny of crosses between rice and *E. coracana* or *Eragrostis curvula* isolates. This suggests that the gene flow between rice and other host isolates is low, hence the genetic isolation of rice pathogens.

Isolates pathogenic to rice can be differentiated into races according to the resistant cultivars they can attack (Ou 1980, 1985). In many cases, resistance of the cultivar is due to one or a few dominant resistance genes (Ou 1980). Crosses between isolates pathogenic to rice were successful only recently, allowing the study of the heredity of avirulence. In most cases, avirulence to a particular resistant variety is controlled by a single gene (Leung et al 1988; Notteghem, pers. comm.; Valent and Chumley 1990).

Genetic distances between P. oryzae and P. grisea isolates differing in host range

The genetic distance between isolates was evaluated with isozymes in two independent studies. First, Matsuyama et al (1977) studied the polymorphism of isozymes (esterases and peroxidases) in the protein extracts of a collection of Japanese isolates. They showed that the rice isolates were more closely related to the *Eleusine coracana* and *E. indica* isolates than to the *D. sanguinalis, Eragrostis curvula,* or *S. italica* isolates. Ginger isolates were very different from all others. In a more extensive survey with 12 isozymes and isolates from all over the world, Leung and Williams (1986) found a close relationship between *Eleusine coracana, E. indica,* and rice isolates. *D. sanguinalis* isolates were very distant from all others. These last results were also confirmed by analysis of mitochondrial DNA RFLP (Leung and Taga 1989). Although these studies have not covered all the available variability of *P. grisea,* they indicate that the genetic distances might be very different between host-range groups. Some are clearly related, such as those from rice and *E. coracana,* but not the others, even if they can be intercrossed like the *D. sanguinalis* isolates. Recently, repeated dispersed DNA (MGR) sequences were found in the genome of *P. oryzae.* These MGR sequences hybridize only slightly to the DNA digests of isolates from other hosts, including those

from *E. coracana* (Hamer et al 1989), showing that they have accumulated numerous modifications to account for this low cross-hybridization. Thus, rice isolates must have diverged from others for sufficient time to accumulate such differences.

Polymorphism of *P. oryzae* populations

P. oryzae populations have been studied mainly through surveys of races in a large number of countries. In general, many races exist in the same geographical area (Ou 1980, 1985). Attempts to classify rice isolates by their virulence spectra on resistant cultivars have led to the hypothesis of two major groups of isolates, one specialized on resistant indica cultivars, the other on resistant japonica cultivars (Morishima 1969). This classification is not widely accepted, mainly because many isolates pathogenic to resistant indicas are also pathogenic to some resistant japonicas, the reverse being less true. Nevertheless, it is tempting to think that this specialization does exist, because indicas do not have the same resistance genes as do japonicas (Kiyosawa 1971). Thus, isolates pathogenic to each type of cultivar might have specific combinations of virulence genes.

Other data come from isozyme studies. Low polymorphism between rice isolates was detected by Leung and Williams (1986) using 12 isozymes and by Matsuyama and Kozaka (1971) using esterases. Both groups identified only three haplotypes among a worldwide set of isolates. Studies with other isozymes, but including esterases, revealed more polymorphism among rice isolates (Park et al 1986, Vales et al 1987), but only two major groups were identified by Vales et al (1987) independently of geographic origin. The two major haplotypes identified by Leung and Williams (1986) might be representative of the specialization of *P. oryzae* on indica or japonica cultivars, as there was a good correlation between the type of variety from which the isolates were obtained and their isoenzymatic haplotype. Although not yet conclusive, these studies stress the existence of genetic differentiation between rice isolates stemming from their probable coevolution with either indica or japonica rices.

Development of RFLP markers to study *P. oryzae* populations

DNA markers have not yet been widely used to study the structure and dynamics of *P. oryzae* populations, except that repeated dispersed DNA sequences have revealed RFLP among rice isolates from the same country (Hamer et al 1989). RFLP markers should provide a better view of the genetic structure of the *P. oryzae/P. grisea* populations as well as their relationships. These markers should show whether or not the populations on rice are clonal and separate from those pathogenic to other hosts. They will also allow the evaluation of genetic diversity within a particular geographic area or within a race group. These markers could also be used to test the hypothesis of coevolution between rice and *P. oryzae* during the domestication of rice, particularly in view of the differentiation of the indica and japonica forms. RFLP markers have proved very useful in population biology studies of many plant pathogens (bacteria

[Gabriel 1989] and fungi [Leong and Holden 1989, McDonald et al 1989, Michelmore and Hulbert 1987]). These studies have pointed out that isolates with different host ranges are in general very distinct genetically from each other, because they have accumulated considerable specific RFLP. These results support the idea that host range is a primary determinant of the diversification of these pathogens. Depending on the pathogen studied, this was less true when comparing isolates with different virulence spectra, because they might have part of their polymorphism in common with those of other races. This can be explained either by gene exchanges between races or by independent acquisition of the same virulence. These studies are encouraging in the sense that RFLP markers can be good indicators of the genetic relationships and exchanges between isolates of a particular pathogen.

Sequence and RFLP analysis of the ribosomal RNA gene cluster

We have sequenced two domains (D1 and D2) of the 26 S RNA gene that are good phylogenetic indicators, as already shown by the comparison of species in genus *Fusarium* (Guadet et al 1989). The Dl domain, 250 bp long, is quite conserved. Usually there are no differences within one species, and the D1 might be used to compare genera and species (Guadet et al 1989). The D2 domain, 230 bp long, is more polymorphic, since several differences can be found among isolates of the same species (Guadet et al 1989). We have sequenced the D1 and D2 domains of one rice isolate (Guy11 [Leung et al 1988]) and of two isolates from *E. coracana* from different geographic origins (from H. Kato, University of Kobe, Japan). The sequences of the two domains are the same in these three isolates, which is further evidence for their classification as a single species. Their comparison with sequences of other ascomycetes showed that *Pyricularia* spp. are related, as expected, to other Pyrenomycetes such as *Neurospora crassa, Podospora anserina,* and *Gibberella fujikuroi.*

The ribosomal DNA (rDNA) gene cluster of *Pyricularia oryzae* was cloned from a lambda phage genomic library of the rice isolate Guy 11 by heterologous hybridization with a rDNA probe from *Podospora anserina.* When used as a probe with 12 enzymes, it did not detect differences in restriction site among the rice isolates, but did detect a few differences between rice and other host isolates (one *Dra*I site was shown to be absent in isolates from *E. coracana,* but present in rice isolates). On the other hand, *Eco*RI digests allowed the detection of length polymorphism among rice isolates, as shown by the variation in length of one of the three bands obtained (2.70-2.90 kb). These length variations are thought to originate in the nontranscribed spacer as deduced from a partial restriction map of the *Pyricularia oryzae* rDNA unit and the position of the ribosomal DNA genes through the alignment of the map with restriction sites conserved among ascomycetes (Garber et al 1988). This type of polymorphism in rDNA is frequent within a species in ascomycetes (Garber et al 1988) and is widespread among rice isolates, allowing their classification into three groups by length (Table 1) independently of their geographic origin or their ability to attack resistant indicas or japonicas.

Table 1. Length polymorphism of the rDNA unit among *Pyricularia oryzae* isolates.

Origin	Rice isolates[a]		
	Group 1 (8.2 kb)	Group 2 (8.1 kb)	Group 3 (8.0 kb)
Ivory Coast	Cd2[b]	Cd46[b] Cd101[c] Cd128[b]	Cd41[b] Cd112[b]
Brazil (Br), Colombia (Cl), and French Guyana (Guy)	Guy11[b]	Br26[b] C16[c]	Br11[b] Br19[c] Br25[c]
Malaysia (Ms) and Philippines (Ph)		Ph11[c] Ph14[c] Ph28[b]	Ph12[b] Ph24[b] Ms2[c]
Japan		P2B[b] Ina72[b]	Ina168[b] Jp10[b]

[a]Size of rRNA gene cluster, in kb, is in parentheses. [b]Isolated from and/or pathogenic to japonica cultivars. [c]Isolated from and/or pathogenic to indica cultivars.

RFLP detected by random single-copy genomic probes

Probes from a genomic gene bank of *P. oryzae* isolate Guy 11 cloned in a lambda phage vector were tested for RFLP detection. We selected nine rice isolates pathogenic to either indicas or japonicas of different geographic origins, and three isolates from other hosts *(E. coracana,* wheat, maize). Clones from the gene library were chosen at random and tested on genomic DNA of the 12 selected isolates digested by 6 restriction enzymes. With 5 probes and 6 restriction enzymes, little RFLP was detected among rice isolates, since only 8% of the bands were polymorphic (8 of 102 bands observed). Each of these eight polymorphic markers was detected in a different isolate. Therefore, it was not possible to classify the isolates. This shows that rice isolates are quite homogeneous genetically. On the contrary, all the probes detected RFLP between rice isolates and isolates from other hosts, since 37% of the bands were polymorphic. The polymorphism we observed between rice and *E. coracana* isolates was much higher than that reported by isozyme studies (Leung and Williams 1986). We could compute similarity coefficients between these groups (Table 2) and construct a phenogram using the Fitch-Margoliash method (Fig. 1). Wheat and *E. coracana* isolates were clearly related compared with maize or rice isolates. The maize isolate appeared to be different from the three others and might belong to a particular group of *P. grisea* isolates.

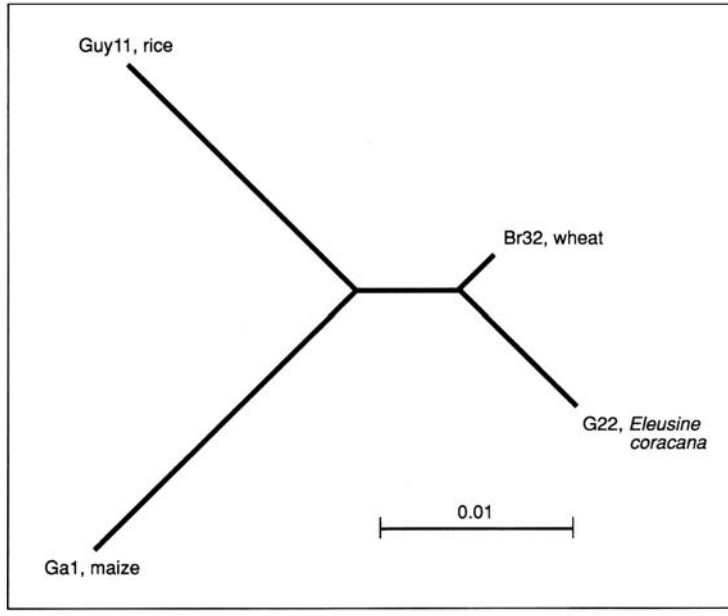
Conclusions

We used RFLP to study the relationships between *P. oryzae* and *P. grisea* isolates differing in host range and to evaluate the genetic diversity of isolates pathogenic to rice *(P. oryzae).* Our first results show that there is significant RFLP between isolates

Table 2. Similarities between *Pyricularia* isolates differing in host range, deduced from FLP analysis with random single-copy probes.[a]

Isolate	Similarity coefficient[a]			
	Rice	Maize	Wheat	*Eleusine coracana*
	Guy11	Ga1	Br32	G22
Guy11	1	0.718	0.794	0.764
Ga1		1	0.810	0.779
Br32			1	0.917
G22				1

[a]Guy11 from French Guyana, Gal from Gabon, Br32 from Brazil, G22 from Japan. Similarity coefficients were computed as percentage of restriction fragments shared by 2 isolates. 102 bands were used for this computation (5 probes x 6 enzymes).



1. Phylogenetic tree of *Pyricularia* isolates differing in host range, constructed by the Fitch program of the PHYLIP 3.0 package, using Table 2 data (similarity coefficients) transformed into nucleotide diversity by formulae (20) of Nei and Miller (1990).

differing in host range. The level of polymorphism among rice isolates is low, as we did not find restriction site polymorphism within the rDNA gene cluster, and only a little RFLP with random single-copy probes. On the other hand, we found some variations in rDNA unit length that were more frequent than those detected with single-copy probes. In plants, such rDNA length variations are supposed to arise from unequal

crossing-over within a family of repeated sequences located in the nontranscribed spacer (Rogers et al 1986). Therefore, if the same mechanism is involved in fungi, it could be more frequent than the occurrence of point mutations within a restriction site. This situation suggests that different probes might reveal polymorphism that could have different probabilities of appearing with time. This might also be the case when comparing repeated dispersed sequences that are thought to be a family of transposable elements (Hamer et al 1989), which might reveal many more differences than those due to spontaneous base-pair modifications. Thus, it seems important to compare independently the polymorphism due to different mechanisms of variation, especially when comparing closely related individuals. It is then necessary to accumulate enough information on single-copy probes to evaluate the genetic diversity of the rice pathogen populations.

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Discussion

Session 9: RFLP analysis of rice genomes

Q—*Brar:* What is your opinion on the use of oligonucleotide polymorphism in rice mapping and gene tagging? Do you expect it to be an important supplement to the restriction fragment length polymorphism (RFLP) technique?

A—*Tanksley:* Compared with standard RFLP analysis, the use of oligonucleotide primers with polymerase chain reaction (PCR) can significantly reduce the labor and time required to monitor a molecular marker linked to a gene of interest. However, a number of limitations remain with the use of PCR. Depending on the types of primers used, PCR will normally detect less polymorphism than regular RFLP markers or will behave in a dominant or recessive manner. Future research may allow us to overcome these problems.

Q—*Zhang Qifa:* You relate the genetic map to the physical map. How did you figure out how many nucleotides there are between restriction fragments?

A—*Tanksley:* Using pulsed fields gel electrophoresis and rare cutting restriction enzymes, it is possible to construct chromosomal restriction maps from which base pair distances can be estimated.

Q—*Brar:* Once the linkages between RFLP probes and agronomic traits become known, do you anticipate any difficulty in using such markers to detect polymorphism, particularly in elite lines having complex pedigrees?

A—*Tanksley:* The utility of RFLP linkage in breeding will be determined by two factors: 1) the level of polymorphism of the linked marker among rice cultivars, and 2) the genetic relatedness of the varieties corresponding to the crosses in which the marker is being selected. With regard to the first point, it will be valuable to have high density RFLP maps so that, in the event one linked marker is not polymorphic in a particular cross, a closely linked, polymorphic marker can be utilized.

Q—*Chaudhuri:* Please explain whether your RFLP map and Dr. Tanksley's RFLP map coincide at any point. It might help us to make a better physical map of the rice chromosomes.

A—*Yano:* Since the probe sources of the linkage maps are different, there is no way of saying. But it is possible to infer chromosomal correspondence between the RFLP linkage maps.

Q—*Chaudhary:* Your conclusion about genetic diversity in populations of *Pyricularia oryzae* is that it is very "narrow." The prior evidence of pathologists and breeders is to the contrary. Varietal resistances break down frequently. Would you comment on this?

A—*Notteghem:* Genetic variability is wider if we consider *Pyricularia* spp. isolated from various hosts than if we examine only *P. oryzae* isolated from rice. To

examine genetic diversity within *P. oryzae* isolates, we must screen more probes.

Q—*Chaudhuri:* In your analysis Nepal and India are in different groups, although it is believed that Nepali and Indian cultivars are close. What is the basis of your selection of cultivars?

A—*Kawase:* We checked the geographical origins and ecological backgrounds of the accessions as precisely as possible. Cultivars collected from Nepal had wide genetic variation. Some of them were classified into Cluster I and others in Cluster II. In Cluster I, a few cultivars from Nepal were closely related to those from Bhutan and Assam State of India. In Cluster II, three Nepali cultivars were grouped in Cluster IIc together with Indonesian cultivars and an Iranian cultivar. Two other Nepali cultivars showed close relationships to a cultivar from Bhutan and another from India. So, both Nepali and Indian cultivars have wide variation, and some of them showed close relationships. The number of the cultivars used was limited, and we are going to examine many more varieties.

Q—*Zhang Qifa:* You detected up to tetradecamorphism with a single probe. How do these morphs differ from each other in number of bands or in mobility? Since there are many phenotypes detected with each probe, how did you process the data in principal component analysis (PCA)?

A—*Kawase:* For each accession, three or four bands differing mainly in mobility were detected. We scored 1 or 0 depending on whether the cultivar showed each type or not, respectively. This kind of data matrix was put into the computer for PCA.

Q—*Shao Qiguan:* Could ecological principles be useful in selecting samples for RFLP mapping (analysis taking)?

A—*Kawase:* The ecological background of the accessions is very important. We selected our materials from various areas throughout Asia. We are going to examine many more accessions after checking their passport data.

C—*Glaszmann:* Based on isozymes, the pattern of diversity is much more complex in the Indian subcontinent, where types other than the classical indicas or japonicas seem to exist. Particular behaviors are also known for sterility in hybrid combinations. This can be related to what is observed in your work: in the indica cluster, the last varieties that cluster came from India; in the japonica cluster, the last varieties to cluster came from Nepal. Thus, the number of varieties from the Indian subcontinent in your sample could probably be profitably extended.

Q—*Chaudhuri:* Do you plan to study the delta T-base sequence change in different genomes as Dr. David Khone did with primate genomes, which showed a final correlation between nucleotide base sequence change and ecological time period?

A—*Second:* I am not planning to do any sequencing; I will leave it to the specialists. It is an interesting suggestion. However, note that in an allotetraploid, because

all sequences are initially duplicated, some of them might not be subjected to stabilizing selection and might therefore evolve more quickly.

Q—*Bennert:* What information has been obtained from molecular studies concerning the relationship between the weedy *Oryza spontanea* species and *O. sativa?* What definition of species was used to designate *O. spontanea* as a distinct species?

A—*Second:* To my knowledge, no molecular data support *O. spontanea* as a distinct species. In my table, the species concept really applies to the seven primitive species only. All others are derived forms. They are arranged in columns according to an evolutionary concept and in rows according to a biological concept, but many names are imprecise. Besides, the biological species merge in the derived forms so that the concept loses ground. From my point of view, weed forms have various names in many countries and they do not correspond to a species rank. However, if you recognize the weeds as part of *O. rufipogon,* you should also recognize *O. sativa* as part of *O. rufipogon,* which should then be called *O. sativa.* I do not think that this would improve rice systematics. The fundamental point is that molecular markers show that gene flow exists between cultivated and weed forms and it deserves further attention.

C—*Second:* The Chinese people might well have been the first to distinguish Keng and Hsien types in *O. sativa.* However, as a scientist, I follow the first scientific description in choosing names, unless they have become confusing. To my knowledge, the subspecies indica and japonica were scientifically described and named by Kato, and it seems to me that what we observe confirms what he described.

C—*Wang Xiangmin:* The subspecies names indica and japonica have been used by rice specialists for a long time. Dr. Ting Ying, the late President of the Chinese Academy of Agricultural Sciences, proposed using the traditional Chinese names Hsien and Keng, respectively. Dr. T.T. Chang proposed to use sinica instead of japonica. Although the name of japonica was to be used according to rules of nomenclature, the Chinese rices have a very long history, and the classification into Hsien and Keng types is of ancient origin. Perhaps we can use sino-japonica to name the subspecies.

SESSION 10

Molecular Genetics of Rice Proteins

Rice endosperm proteins and their accumulation signals

N. Mitsukawa and K. Tanaka

Characteristics of rice endosperm proteins are described, focusing on their distribution inside rice endosperm cells. The signal sequence of each protein is of special interest. Prolamin polypeptides that specifically accumulate into starchy endosperm protein body type I (PB-I) are described, and their families and amino acid structures are deduced from the information obtained through analyses of corresponding complementary DNAs. The distribution of 26-kDa a-globulin in the endosperm cell is also described. The signal sequences of the rice endosperm proteins so far analyzed are classified and correlated with their distribution in endosperm cells.

Endosperm proteins are synthesized during seed development. Many endosperm proteins may be essential for the organization of endosperm cells. These proteins are not distributed uniformly throughout the endosperm tissues. Usually, an individual protein is expressed in a specific cell group, and even inside an endosperm cell each protein collects in a specific area. Some of the endosperm proteins are storage proteins and serve as nitrogen reservoirs. The characteristics of proteins so far characterized are listed in Table 1.

To understand the mechanisms that distribute endosperm proteins to specific sites, we need structural analyses of individual proteins from the start of biosynthesis until translocation to the final deposition sites. Some mechanisms for prolamin and glutelin accumulation have been discerned (Krishnan et al 1986, Tanaka and Ogawa 1986). Synthesis of prolamins probably starts on the messenger RNAs (mRNAs) coding for prolamin precursor; then the nascent polypeptide with signal sequence attaches to the endoplasmic reticulum (ER) to form the so-called rough endoplasmic reticulum (RER) (Tanaka and Ogawa 1986). During passage through the ER, the signal sequence is removed cotranslationally, and a mature polypeptide is formed; it is then deposited inside the ER cisterna, forming a 1- to 2-µm spherical protein body, PB-1 (Tanaka and Ogawa 1986). This protein body usually displays a concentric ring structure when a thin section, poststained by uranyl acetate and lead citrate following glutaldehyde and

Table 1. Characteristics of rice endosperm proteins.

Protein	Molecular size (kDa) by			Signal sequence		Localization	Gene families (no.)	Intron	Copy number
	Precursor	SDS-PAGE	From cDNA (amino acids)	Length	Charged amino acid				
Glutelins	59 (preproglutelin)	37-39 Acidic subunit	31.8-32.2 (278-282)	24	Arg		>4	2	>20
	57 (proglutelin)	22-23 Basic subunit	21.5-22.2 (191-197)			PB-II			
(a-globulin	28	26	18.9 (146)	22	Lys	ER, PB-II	1	?	1-2
Allergen	18	16	14.8 (137)	27	Lys	?	?	?	?
Prolamins									
16 kDa	18	16	?	?	?	PB-I	?	?	?
13 kDa	15	13	14.8-15.8 (123-137)	19	Lys	PB-I	3	no	50
10 kDa	12	10	12.3 (110)	24	Lys	PB-I	1	no	1-2
Oryzacystatin	-	11.5	11.3(102)	-	-	Cytosol	1	?	2-3

osmium tetraoxide staining, is observed under the transmission electron microscope (Yamagata and Tanaka 1986).

On the other hand, glutelin biosynthesis starts on the mRNAs for preproglutelins with signal sequences as with prolamin synthesis (Furuta et al 1986). The nascent precursor polypeptide soon attaches to the RER-inserting signal sequence inside the membrane. The signal sequence is soon removed from the preproglutelin polypeptide. The proglutelin polypeptide thus formed is first kept in the ER cisterna, then transferred to the golgi machinery, and finally transported into a vacuole-type protein body (PB-II), which is 2-4 μm in diameter with no concentric ring structure under the electron microscope.

Waxy protein in the endosperm is well known to be organized in amyloplasts, associating strongly with starch granules, and this protein is responsible for amylose content in endosperm starch. The sorting of this enzyme protein is controlled by an NH_2-terminal structure of about 30 amino acids in length (Klösgen et al 1989). The NH_2-terminal structure is also responsible for distributing this protein into the chloroplasts. Although information about rice waxy protein is not as complete as that about other cereals such as maize, no doubt a similar mechanism works in rice endosperm.

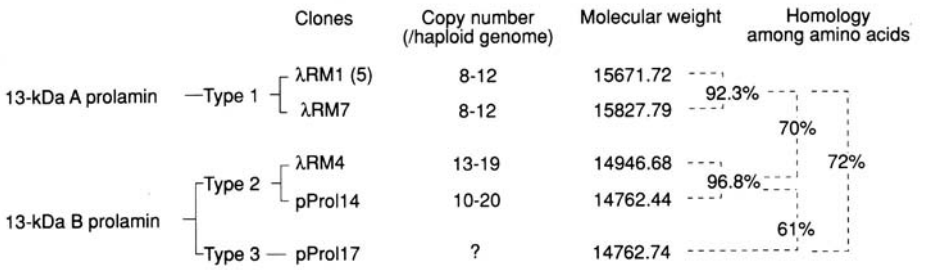
Rice allergen is also known to increase during maturation, and the polypeptide carries a signal sequence on the NH_2-terminal (Fujii et al 1990), but the accumulation site is not known.

Oryzacystatin is a well-characterized rice endosperm protein having no signal sequence; thus it is believed not to be accumulated inside specific organelles in the endosperm (Abe et al 1987).

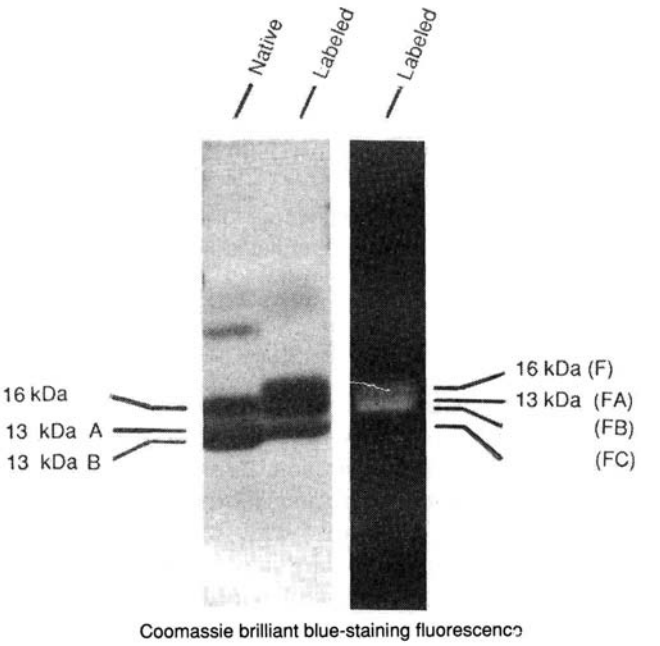
Rice prolamin families and their structure

Many cereal prolamins are composed of multigene families; thus they are groups of polypeptides having microheterogeneity inside their polypeptides (Kim and Okita 1988). All these polypeptides, however, accumulate at a specific site called a "protein body." Rice also has several groups of prolamins. One is 10-kDa prolamin, very rich in sulfur-containing amino acids (Hibino et al 1989, Masumura et al 1989). The other is 13-kDa prolamin, which is further divided into A (larger) and B (smaller) types (Kumamaru et al 1988). Prolamin A consists of more than seven and prolamin B more than five polypeptide groups. The total number of polypeptide groups making up 13-kDa prolamin is more than 12, distinguishable by electrophoresis (Kumamaru et al 1988); 16-kDa prolamin contains fairly high amounts of sulfur-containing amino acids, but we lack information on its structure.

Thus, rice prolamin is composed of many polypeptides, especially 13-kDa prolamin polypeptides consisting of groups of multigene families (Kumamaru et al 1988). They all accumulate specifically in PB-I (Ogawa et al 1987). So we can surmise the existence of a signal or signals in a rice prolamin polypeptide. To learn the signal that may be responsible for recognizing the PB-I membrane, we analyzed detailed structures of rice prolamin complementary DNAs (cDNAs). We already deduced seven 13-kDa



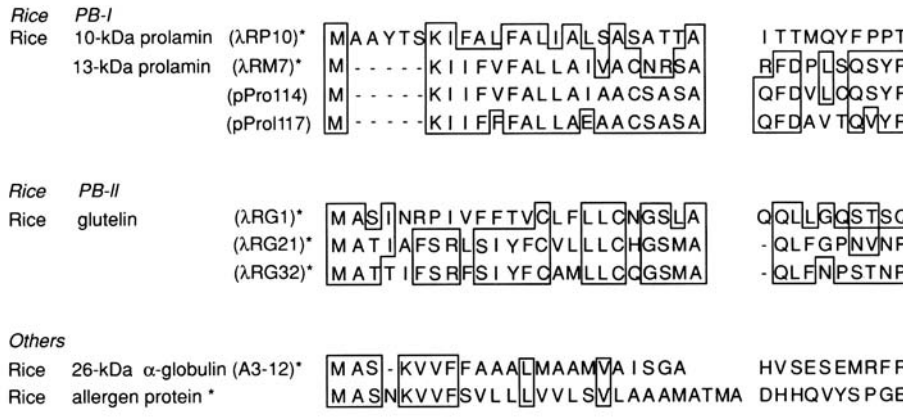
1. Homology among 13-kDa prolamins, indicated by broken lines.



2. Labeling of rice prolamin by 4-aminosulfonyl-7-fluoro-2, 1, 3 -benzoxadiazole, F=fluorescence; FA,FB, and FC = fluorescent-labeled 13-kDa prolamins.

prolamin polypeptides from cDNA sequences. They are classified into three groups by their sequence homology, as shown in Figure 1.

The 13-kDa prolamin was further separated by fluorescent labeling with 4-amino-sulfonyl-7-fluoro-2, 1, 3-benzoxadiazole (ABD-F) (Kirley 1989). As shown in Figure 2, 13-kDa prolamin A,B was further divided into 13-kDa FA, FB, and FC. FA and FB



3. Structures of signal sequences of rice endosperm proteins. Homologous amino acids are boxed. * = isolated and characterized in the authors' laboratory.

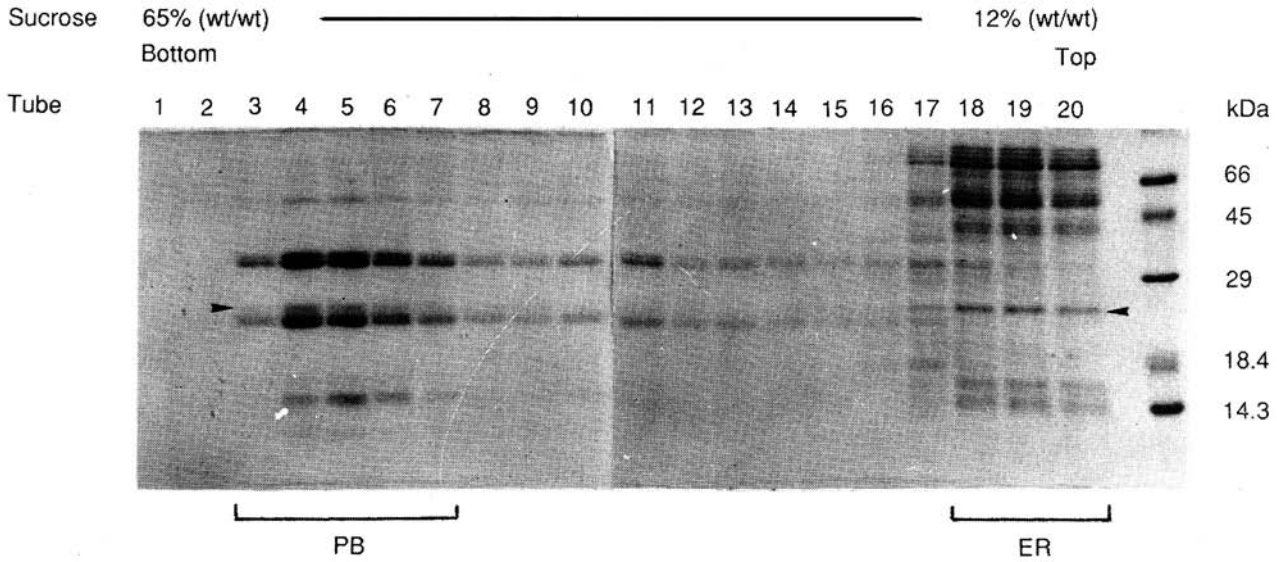
polypeptide groups showed increased molecular size with addition of dye molecule. However, FC showed no change in molecular size. From the nature of the labeling, the FA, FB polypeptide should contain cysteine residue(s) in each polypeptide, but FC should not.

Prolamin polypeptide pProl14 (Kim and Okita 1988) obtained through cDNA analysis contains no cysteine residue. The homology among 13-kDa polypeptides so far obtained is summarized in Figure 1. Thus, l ARM7 type (Masumura et al 1990) is the largest and is thought to correspond to one of the 13-kDa prolamin A polypeptides. The classification suggests that the 13-kDa prolamin B is formed from the A group, deleting an amino acid group in the octapeptide region. Beside this deletion in the case of pProll4, all cysteine residues in l RM7 were changed to other amino acid residues by point mutations. This process might produce a polypeptide group that cannot receive dye molecules, as shown in Figure 2.

Comparison of many 13-kDa prolamin polypeptides (Fig. 3) revealed that signal sequence regions are conserved throughout the mutation process of the 13-kDa prolamins. The specific sequence observed in the signal peptides of 13-kDa prolamin is also strictly conserved in 10-kDa prolamin. These results strongly suggest that the signal sequence is one of the important factors responsible for the specific accumulation of prolamin into PB-I.

Distribution of 26-kDa a -globulin

Rice starchy endosperm contains a -globulin polypeptide, which has a molecular size of 26-kDa when estimated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Tanaka et al 1980). This polypeptide is unique among major Gramineae storage proteins in that it is globular. Figure 4 shows its distribution in the



4. Fractionation of rice endosperm proteins by sucrose density gradient. Coomassie. brilliant blue stained. PB = protein bodies, ER = endoplasmic reticulum.

endosperm cells. This figure is the result of a SDS-PAGE of the proteins distributed into each fraction obtained after fractionation by sucrose density gradient of the maturing endosperm homogenate. PB-I distributes in tubes 4, 5, and 6, because 10–, 13-, and 16-kDa prolamins are strongly accumulated in these tubes. Similarly, PB-II distributes in tubes 3 and 4 as 22- to 23-kDa glutelin basic subunits and 37- to 39-kDa glutelin acidic subunits coexisting in these fractions.

In contrast, the band of 26-kDa a-globulin distributes widely from tube 20 (ER-region) to tube 4 (PB-region). The 26-kDa α--globulin was formerly described as having a specific accumulation site in PB-II (Tanaka et al 1980). However, this figure shows that the a-globulin is not restricted in PB-II but is dispersed throughout the ER membranes. Recently, 26-kDa α--globulin cDNA was obtained and analyzed (Shorrosh et al 1990). The signal sequence of the 26-kDa α--globulin shows no homology with the rice glutelin signal sequence (Fig. 3). As already well demonstrated, rice glutelin specifically accumulates in PB-II (Tanaka et al 1980). For this specific sorting in PB-II, signal sequences appearing in the preproglutelin (59-kDa) polypeptides might be indispensable (Fig. 3) (Masumura et al 1988).

Classification of signal sequence

Endosperm cells of rice seeds are highly organized, and the proteins accumulating at specific sites must be controlled through sophisticated mechanisms. We lack sufficient information on this topic. One of the important factors responsible for the specific sorting of endosperm proteins may be signal sequence, especially at the NH_2-terminal of the individual protein (Masumura et al 1990). Signal sequences so far obtained from rice endosperm are listed along with their NH_2-terminal sequences of mature polypeptides in Figure 3.

One conspicuous feature is that the signal sequence of each storage protein that accumulates in PB-I or PB-II is unique and is thought to be responsible for the specific sorting into PB-I or PB-II. The signal sequences appearing in the proteins that accumulate in PB-I form one group. This group has the following characteristics:

- The signal sequence is composed of 19 or 24 amino acids having a highly hydrophobic domain at - 12 to -8.
- The signal sequence of this group has one Lys as a charged amino acid close to the initiation Met.
- -KI—FALLA—A———A- is a consensus amino acid sequence.

Another group, preproglutelin, which comes to the PB-II-related membrane, has the following characteristics:

- It has 24 amino acids and is rich in hydrophobic amino acids.
- This type of signal sequence contains Arg as a charged amino acid instead of the Lys of the PB-I polypeptide precursor.
- The conserved amino acid sequence is MA ———— C—LLC-GS-A.

Although proteins that also accumulate in endosperm cells may have their own signal sequences for specific sorting, we do not have enough evidence to find out their

consensus sequences for sorting. Those signal sequences that were observed in the NH_2-terminals of the proteins appearing in endosperm cells are also listed in Figure 3.

Prospectives

The final goal of our research is to improve the quantity and quality of rice endosperm proteins. To reach this goal, there are several alternative approaches. The first important direction is to enhance the expression of nutritionally desired proteins using biotechnological methods. The second way is to introduce a foreign peptide that is nutritionally good and easy to digest. For the second approach, the foreign protein must bear the signal sequence that leads it to the target site.

We recently constructed genes that produce a chimeric bovine a_{S1} casein peptide bearing PB-I-type signals. In vitro trials to discover the sorting abilities of these chimeric polypeptides into PB-I were successful.

We are now close to realizing an artificially designed rice seed that accumulates foreign polypeptides in the endosperm cells.

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Genetic manipulation of storage proteins in rice

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The 3 seed storage proteins of rice (cultivar Basmati 370)–60kDa albumin, 8.3-kDa prolamin, and 28-kDa and 21-kDa subunits of glutelin—were purified and partially characterized with respect to amino acid analysis, NH_2-terminal amino acid sequence, and fluorescence analysis. In the case of rice prolamin, the increase in relative fluorescence at 50% ethanol concentration suggested that it underwent further folding before it unfolded at 40% ethanol concentration. The genomic library of rice was constructed in the Charon 40 vector after separating the stuffer region by restricting with *EcoRI* and *Nae*III. The library was then screened using a glutelin-specific, 33-base-long oligoprobe and a kafirin complementary DNA probe to identify clones containing glutelin and prolamin genes, respectively. To quantify rice storage proteins, three immunoassays were developed, of which the dextran conjugation system for rice albumin was found to be the most sensitive and time saving.

The immediate objectives of our work are to gain information concerning the structural features of the albumin, prolamin, and glutelin storage proteins and the genes encoding them, and to learn how the expression of these genes is regulated developmentally. Ultimately, we hope to improve the nutritional status of rice through specific modifications of its storage proteins. In this paper, we present data on partial structural characterization of albumin, prolamin, and glutelin; on cloning of genes encoding prolamin and glutelin; and on development of a novel method for quantifying albumin.

Partial biochemical analysis of rice seed storage proteins

To get an idea of the proportions of seed storage protein fractions in rice (cultivar Basmati 370), defatted seed meal was fractionated on the basis of differential solubility as determined by Osborne (1907), and the four sets of endosperm-localized proteins, namely albumin, globulin, prolamin, and glutelin, were isolated. Unlike most of the major cereal proteins, rice has glutelin as its main protein (Table 1).

Table 1. Composition of rice *(Oryza sativa,* cultivar Basmati 370) seed storage proteins.

Protein fraction	Solubility	Protein (%)	Biological value
Albumin	Water	0.5 - 7.0	74.5
Globulin	Salt	2.0 - 8.0	54.9
Prolamin	Alcohol	1.0 - 5.1	3.1
Glutelin	Urea and ß-mercaptoethanol	85.0 - 90.0	31.2
Total		100	42.0

Albumin, prolamin, and glutelin were used for further characterization. Rice albumin, purified on Sepharose CL-6B and Con-A Sepharose columns, was a monomer of 60 kDa with a pI value of 6.5. Prolamin, purified on diethyl amino ethyl (DEAE) cellulose A-50 and Octyl Sepharose CL-4B columns, showed the presence of a single subunit of molecular weight 8.3 kDa. Glutelin, purified on a DEAE-Sephacel column, showed 2 subunits of molecular weights 28 kDa and 21 kDa. All the rice proteins were glycoprotein in nature. Partial N-terminal sequencing of the three proteins up to the first six or seven amino acids from the NH_2-terminal end was determined using the dimethyl aminobenzene isothiocyanate-phenyl isothiocyanate double coupling method of Chang (1983), as follows:

Albumin: Asn-Asp-His-Gly-Val-Thr-Gly
Prolamin: Asn-Pro-Trp-Gly-Cys-Tyr-Glu
Glutelin
Subunit I: Gly-Leu-Pro-Glu-Gly-Met (21 kDa)
Subunit II: Gly-Gln-Asp-Glu-Thr-Phe (28 kDa)

The amino acid composition of all three proteins (Table 2) was elucidated by hydrolyzing them as described by Moore and Stein (1963) and then analyzing them on a Beckman automatic amino acid analyzer (Model 120 B) by the method of Spackman et a1 (1958).

Fluorescence studies of albumin, prolamin, and glutelin

The fluorescence spectra of rice proteins were determined under nondenaturing and denaturing conditions and were recorded on an AMINCO SPF-500 spectrophotometer.

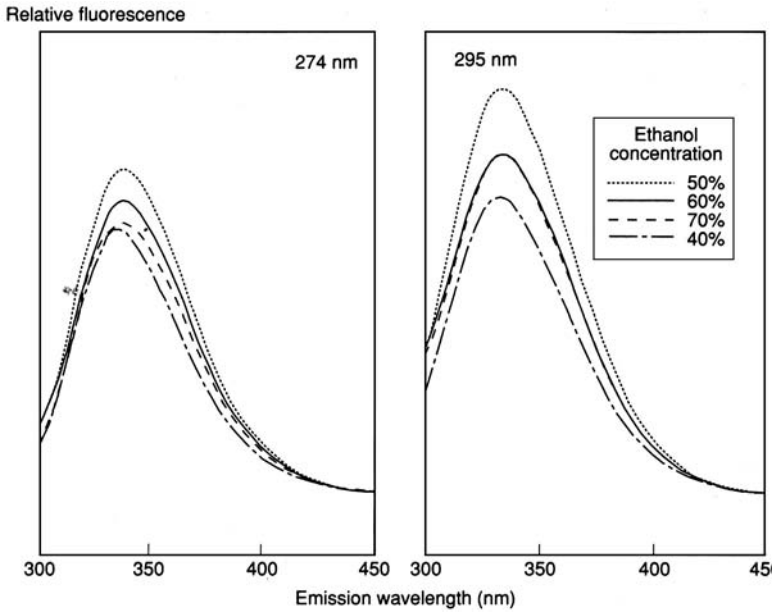
When native albumin and prolamin were excited at 274 and 295 nm excitation wavelength, the emission spectra were characterized by one emission peak in the range of 330-350 nm. These results are typical of proteins containing both tyrosine and tryptophan residues. During denaturation studies with guanidine hydrochloride (Gdn.HCl), the relative fluorescence intensity of albumin, prolamin, and glutelin

Table 2. Amino acid composition of rice albumin, prolamin, and glutelin.

Amino acid	Albumin	Prolamin	Glutelin		
			Subunit I	Subunit II	Total
Lysine	1.6	0.9	2.7	4.6	4.1
Histidine	1.4	1.1	2.1	1.9	2.0
Arginine	3.8	6.0	7.1	6.8	8.1
Aspartic acid	9.4	7.1	8.9	11.0	10.1
Threonine	2.9	2.6	4.0	4.8	4.5
Serine	5.9	5.0	7.1	6.3	6.6
Glutelin	11.8	18.1	19.7	12.1	14.6
Prolamin	5.6	4.9	6.1	6.3	6.1
Glycine	9.9	3.9	8.3	7.4	7.9
Alanine	11.1	6.4	5.8	7.2	6.4
Valine	9.9	7.1	5.6	6.7	6.2
Methionine	2.5	0.6	0.9	1.8	1.7
Isoleucine	3.1	4.6	3.4	4.8	4.2
Leucine	7.3	10.9	3.1	3.4	3.2
Tyrosine	2.8	9.1	3.6	3.2	3.5
Phenylalanine	2.0	6.7	5.2	5.1	4.9
Tryptophan	1.4	1.0	0.8	1.3	1.1
Cysteine	-	-	0.8	0.9	0.8
Cystine	2.6	0.5	-	-	-

decreased at 274 and 295 nm, with a shift in the emission peak, indicating unfolding of the protein molecule as expected (data not shown).

When rice prolamin was excited at 274 or 295 nm in 70% (vol/vol) ethanol, a single emission peak with maximum emission at 335 or 336 nm, respectively, was observed (Fig. 1). The tyrosine-to-tryptophan ratio is 9:l in rice prolamin. Because of the dominance of tryptophan fluorescence in native protein, the presence of even a low level of tryptophan has a significant impact on the observed fluorescence spectrum of prolamin. As seen in Figure 1, at 274 nm excitation, rice prolamin shows an increase in relative fluorescence with a decrease in ethanol concentration from 70 to 50%, and a drastic decrease in relative fluorescence with a decrease in ethanol concentration from 50% (vol/vol) to 40% (vol/vol). When rice prolamin is excited at 295 nm, no change is observed in relative fluorescence at 70% (vol/vol) or 60% (vol/vol) ethanol. With a decrease in ethanol concentration from 60% (vol/vol) to 50% (vol/vol), however, there is a sudden increase in relative fluorescence intensity. The latter undergoes a significant decrease at 40% (vol/vol) ethanol. A decrease in the relative fluorescence of a protein with a decrease in ethanol concentration (hydrophobicity) indicates the process of protein unfolding (denaturation). In the case of rice prolamin, the increase in relative fluorescence at 50% ethanol suggests that it undergoes further folding before it unfolds at 40% ethanol. This is an intriguing observation and reflects the occurrence of specific conformational changes in the molecule with a decrease in hydrophobicity in the surrounding environment. The change from predominantly μ-



1. Effect of ethanol concentration on fluorescence emission of rice prolamin excited at 274 and 295 nm.

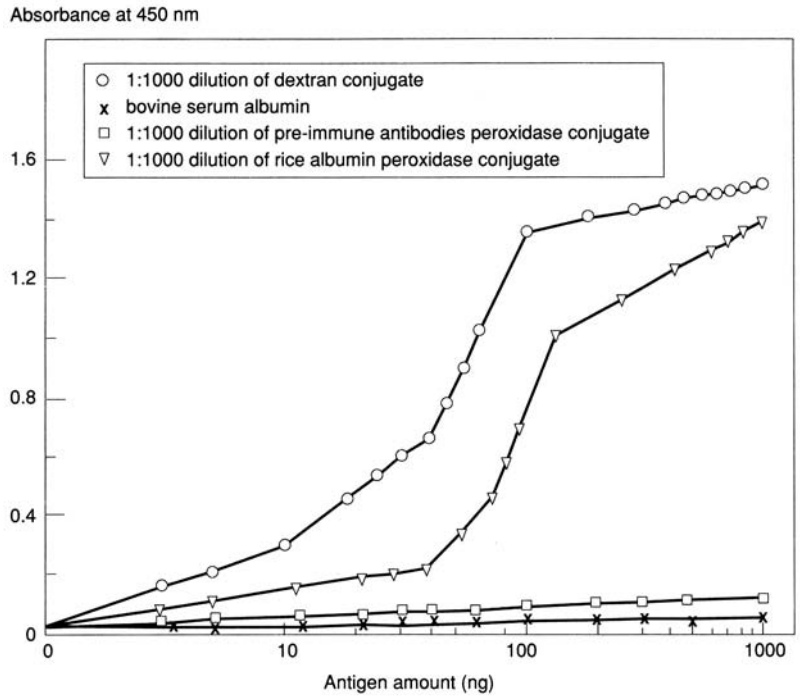
class to a+b class proteins, and a decrease in relative fluorescence intensity with decrease in hydrophobicity, can possibly be correlated with the cleavage of prolamins during seed germination. A seed is a dehydrated mass that absorbs water during germination, when there is a decrease in hydrophobicity. This helps prolamin to be susceptible to proteolytic cleavage and to undergo degradation during germination. Leszczynski and Rose (1986) stated that there is no evidence of random coils (loops) being ready targets for proteolysis. So it can be assumed that the a- and b-secondary structures are the only target sites for proteolysis.

Cloning of prolamin and glutelin genes

We decided to isolate glutelin and prolamin genes from rice to study their expression and structural features.

Construction of a rice genomic library

Production of truly representative libraries for crop plants is the collective effect of many tricky steps due to unique features of plant cells like the presence of a high proportion of repeat-DNA sequences, starch, etc. Pure, total rice DNA of molecular weight 50-60 kbp was digested with restriction enzyme *Eco*RI, and the digested DNA in the size range of 10-23 kbp was isolated on a sucrose density gradient (Maniatis et al 1982). The genomic library of rice DNA was then constructed in a recent-vector



2. ELISA of rice albumin. The antigen was adsorbed on microtiter plate cells, and rice albumin antibodies-dextran conjugate (1:1000) was allowed to bind to these antigens. The bound conjugate was quantified.

DNA from bacteriophage lambda (Charon 40) after separating the stuffer region by restricting with *Eco*RI and *Nae*III (Dum and Blattner 1987). This library represented eight times the rice genome.

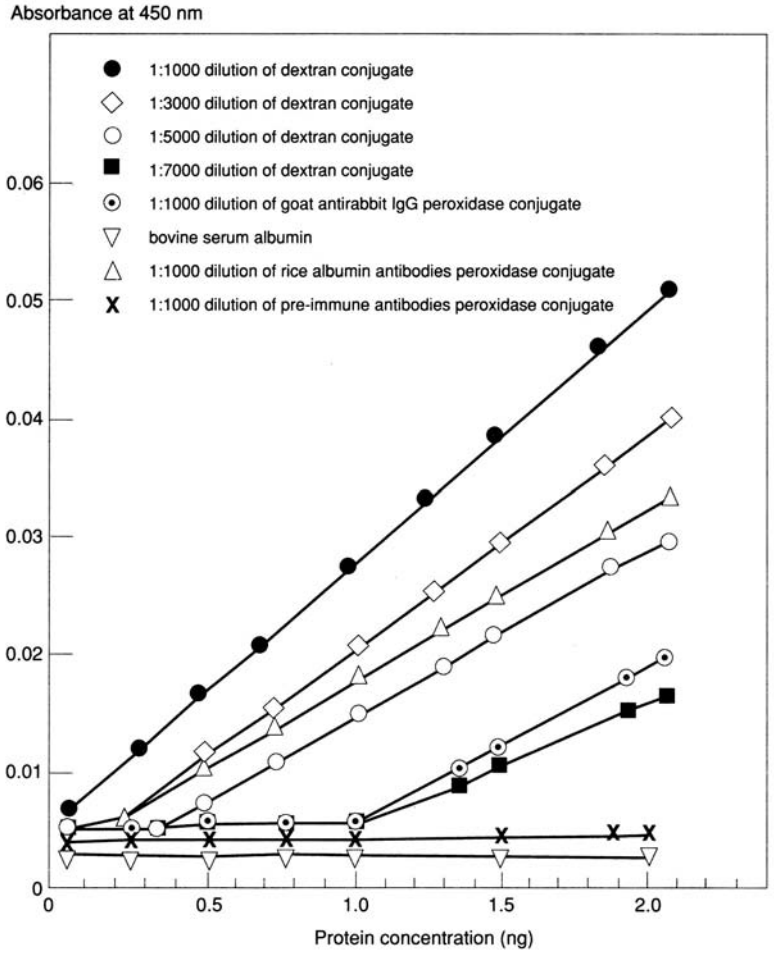
Identification of rice prolamin and glutelin genes

For the identification of the glutelin gene, the library was screened using a single-stranded oligo probe of 33 bases that was picked up from the known complementary DNA (cDNA) sequence of the glutelin gene of japonica rice (Takaiwa et al 1987). Seven glutelin clones were identified using plaque hybridization techniques. The insert size of these clones was 12-13 kbp. Kafirin, a prolamin gene of *Sorghum bicolor* (DeRose et al 1989), was used as a probe to identify prolamin genes from our rice library, and a few clones were identified. The insert size of these clones was 15-16 kbp.

Further work on subcloning these genes is in progress.

Construction of complementary DNA library

To prepare the cDNA library, total RNA was isolated from panicles harvested between 6 and 8 d after anthesis. The quality of the RNA was checked by spectrophotometric scanning and by electrophoresis on agarose-formaldehyde gel. Poly A^+ RNA was

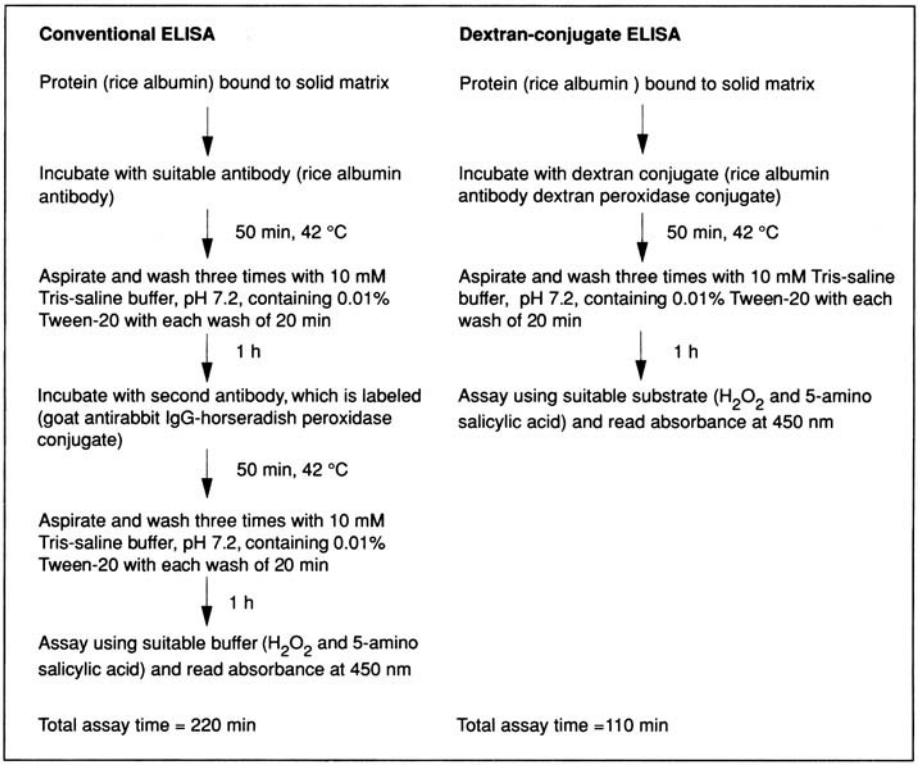


3. ELISA of rice albumin using various dilutions of rice albumin antibodies-dextran-peroxidase conjugate. The antigen was adsorbed on the microtiter plate well and then ELISA was performed.

isolated from this RNA by oligo dT cellulose chromatography. The biological activity of this fraction will be checked by in vitro translation, and it will then be used for cDNA synthesis.

Quantification of albumin, prolamin, and glutelin

During the immunological characterization of rice storage proteins, a few novel modifications of the radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) techniques were carried out that were more sensitive and time saving than routine RIA and ELISA.



4. Schematic representation for comparing conventional ELISA and ELISA using rice albumin antibody-dextran-peroxidase conjugate.

An improved ELISA method for rice albumin included conjugation of rice albumin antibodies and the enzyme peroxidase together with dextran. Using this conjugate, albumin as low as 0.1 ng was detected. The dextran conjugate had tenfold greater efficiency in quantifying albumin than the commercial goat antirabbit immunoglobulin G (IgG) conjugate and was threefold more efficient than the rice albumin peroxidase conjugate (Fig. 2,3). To further confirm the efficiency of our method, we compared the time required to complete one assay (Fig. 4). It is clear from Figure 4 that, while the conventional ELISA using goat antirabbit IgG conjugate takes about 220 min to complete, ELISA using the dextran conjugate is completed in just 110 min, thereby saving half the time. In the case of rice glutelin, the solid support used for binding glutelin was an Immulon I microtiter plate, and it was then quantified with commercial goat antirabbit IgG conjugate by conventional ELISA. This could detect glutelin as low as 5 ng. Prolamin, being a highly hydrophobic protein, could not be dissolved in phosphate-buffered saline as in conventional RIA. The iodination of prolamin was thus carried out in 70% ethanol. The addition of bovine serum albumin resulted in the precipitation of labeled prolamin, which later redissolved by itself during dialysis

against 70% ethanol. Using those immunoassay systems, the maximum synthesis of rice albumin, glutelin, and prolamin was found to take place within 18-20,6-8, and 10-14 d after anthesis, respectively.

Perspectives

This study has provided insights into the structural details of albumin, prolamin, and glutelin. We propose to carry out further experiments to learn the secondary structure of these proteins. This information will be useful in the genetic manipulation of seed storage proteins in rice by site-directed mutagenesis to improve the crop's nutritional value without affecting the structure and localization of these proteins.

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Glutelin genes in wild rices

Hsin-Kan Wu and Tien-Chin Chen

The genomic DNA of 14 rice species was extracted, *Eco*RI digested, blotted, and hybridized to one of our glutelin-coding sequence probes. The number of glutelin genes varied among the species. When the same DNA blotted filter was probed with the 5' region of glutelin gene type II, only some of the glutelin genes belonged to type Il. To isolate more glutelin genes from wild species, the polymerase chain reaction technique was applied. One glutelin gene with a new 5' region was located in the genomic DNAs of *Oryza officinalis* and *O. eichingeri,* both having genome CC.

Glutelin complementary DNAs (cDNAs) were first isolated from cultivated rice in 1985 by Tanaka and Ogawa (1986). The probe they used was reverse-transcribed from poly(A)RNA extracted from the rough endoplasmic reticulum of rice caryopses. The isolated cDNAs were sequenced and classified into two types, I and II (Takaiwa et al 1987a). The sequence of glutelin gene type II was finally determined (Takaiwa et al 1987b). Five glutelin genes from cultivated rice have so far been reported (Takaiwa el a1 1988). In our laboratory, restriction enzyme mapping and partial sequence analysis of our own glutelin cDNA clones allowed us to classify them into seven groups (Wu and Fu 1988). More than 20 wild species of rice have been assigned to 7 genomes, viz., AA, BB, BBCC, CC, CCDD, EE, and FF. We have been interested in analyzing glutelin genes in these wild species. This paper reports detection of glutelin genes with 5' regions of various lengths in wild rice species.

Materials and methods

Fourteen *Oryza* species including the two cultivated ones were used. Table 1 shows their genomes, ploidy, and accession numbers. The second and third leaves picked from healthy plants were quickly frozen in liquid N_2, lyophilized, and kept at -70 °C until use.

For genomic DNA isolation, 1 g freeze-dried leaves were ground in a mortar and pestle in liquid N_2. The leaf powder was ground further in 20 ml Kochert extraction

Table 1. Rice species used.

Species (cultivar)	Acc. no.[a]	Genome
Oryza sativa (Nipponbare)		AA
O. sativa (Caloro)		AA
O. sativa (Taipei 190)		AA
O. sativa (Labelle)		AA
O. sativa (Sando earliness)		AA
O. sativa (Taichung Native 1)		AA
O. sativa (Bulu)	Acc 17827	AA
O. sativa (Bulu)	Acc 17851	AA
O. sativa (Taichung glutinous 70)		AA
O. glaberrirna	W 0025	A^gA^g
O. nivara	Acc 103821	AA
O. rufipogon	Acc 103823	AA
O. perennis	Acc 103849	AA
O. perennis	W 0107	AA
O. breviligulata	W 0049	A^gA^g
O. punctata	W 1577	BB
O. punctata	W 1564	BBCC
O. minuta	W 0045	BBCC
O. officinalis	W 1275	CC
O. eichingeri	W 1519	CC
O. latifolia	W 0019	CCDD
O. grandiglumis	W 1194	CCDD
O. australiensis	W 0008	EE
O. brachyantha	W 1057	FF
O. australiensis	Acc 101467	EE

[a] Acc = from the International Rice Research Institute, Philippines; W = from the National Institute of Genetics, Mishima, Japan.

solution (10 mM Tris-HCl, 50 mM NaCl, 10 mM ethylene diamine tetraacetic acid (EDTA), 1% phenol, and 2% Sarkosyl). Still further grinding was done with addition of 2 ml 10% sodium dodecylsulfate (SDS) and then 20 ml chloroform-isoamkyl alcohol (24: 1). Extraction was done in a 60 °C water bath for 60 min, then 5 min at room temperature. The supernatant was extracted for 30 min with an equal volume of phenol, and nucleic acid was precipitated with 2 M NH_4OAC and 1.5 volumes of isopropanol. RNA was removed by RNase digestion. Finally, the DNA was precipitated with 0.1 volume of 3 M NaOAC and 2 volumes of cold ethyl alcohol.

For Southern blot analysis, *Eco*RI was purchased from Boehringer Mannheim. Digested genomic DNA samples were fractionated by electrophoresis in 0.8% agarose gel. DNA fragments were transferred to a nylon membrane (from Amersham), which was hybridized to the ^{32}P-labeled probes at 42 °C and autoradiographed. The hybridization solution was 50% formamide, 5× Denhardt's solution, 5× sodium chloride-sodium citrate, 0.4% SDS, 2.5 mM EDTA, 50 mM sodium phosphate, 5% dextran sulfate, and 100 µg denatured salmon sperm DNA/ml.

One probe was our own isolated cDNA 47D46, of the glutelin gene coding sequence, 1.5 kb in length. The two other probes, Spa and Spb—1.2 and 0.9 kb,

respectively—were prepared by polymerase chain reaction (PCR) from the 5' region of a genomic clone that is positive to one of the glutelin cDNAs of gene type II.

APCR apparatus from Coy Laboratory Products was used. The temperature settings for melting, annealing, and polymerization were 94, 42, and 72 °C, respectively.

Results and discussion

The kinds and distribution of glutelin genes in rice species were determined. Rice glutelin is likely under the control of a gene family.

Several glutelin genes in a species

After probing the genomic DNAs with a cDNA probe of glutelin coding sequence, 47D46, the positive bands appeared different, both in number and molecular weight, from one species to another (Table 2). Most entities of the AA genome had bands of 2.8, 4.4, 6.5, and >22 kb in common, among which 4.4 and >22 kb were major bands. *O. perennis* was an exception; it had 2 more bands of 5.4 and 9.0 kb, the latter being shared with *O. rufipogon.* Among the cultivated rices, Nipponbare and Sando earliness each had an additional band of 1.7 kb. The 2 entities of the BBCC genome had major bands of 18, 12, 5.4, and 5.0 kb and minor bands of 6.3 and 5.6 kb. The BB genome had 3 bands of 18, 6.3, and 5.4 kb, all shared with BBCC. CCDD entities had major bands of 13, 7.8, 6.3, and 5.0 kb and minor bands of 1.3, 2.3, 3.3, 3.4, and 6.5 kb. Entities of the CC genome and the CCDD genome had bands of 3.4, 5.0, 6.3, and 7.8 kb in common. In the EE genome, 2 entities had 1 major band either of 9.0 or 6.3 kb and 2 minor bands of 1.2 and 1.3 kb. The FF genome had one major band of 5.4 kb and shared a minor 9.0-kb band with the EE genome.

The distribution of the glutelin-positive bands in the BB, BBCC, CC, and CCDD genomes was distinct from that in the AA, EE, and FF genomes. It may be inferred that genome BB contributes the most bands to genome BBCC and that genome CC contributes the 5.0-kb band to BBCC, but 3.4, 5.0, 6.3, and 7.8 kb to CCDD. This is not surprising, because these are related diploids and tetraploids. Among the unrelated species, EE and FF had only one band (9.0 kb) in common. AA shared a 5.4-kb band with FF, while EE shared 2 bands of 6.3 and 9.0 kb with BB and BBCC. These results reflect the appropriateness of the traditional designation of genomes based on classical genetic studies.

Because, within the composing sequence of glutelin gene type II, no *Eco*RI cutting site had been used to cut the genomic DNA of the species, each band that reacts positively to the coding sequence of glutelin gene type II in a species may well represent a DNA segment in which a glutelin gene is embodied. Many glutelin genes may be clustered in the major bands. If this is true, the fewest kinds of glutelin gene in rice species varies from 1 to 7 (Table 2).

Not all glutelin genes in rice species belong to type II. When the two probes of glutelin 5' region sequences were used to hybridize the *Eco*RI-cut genomic DNA of the species, most of the major bands that show positive reaction to the glutelin coding

Table 2. Rice DNA bands hybridized to a glutelin cDNA.[a]

Genotype	Entities (no.)	Major band (kb)											Minor band (kb)						Total
		>22	13	18	12	9.0	7.8	6.1-6.5	5.2-5.6	5.0	4.4	2.8	2.7	12	9.0	6.1-6.5	3.4	2.3	1.7	
AA	5	✓				✓2[b]			✓1		✓		✓3			✓3				6
AA[b]	10	✓3									✓		✓			✓			✓3	5
AgAg	1	✓									✓	✓				✓				4
AgAgb	1	✓									✓	✓				✓				4
BB	1			✓					✓							✓				3
BBCC	1			✓	✓				✓							✓				4
	1			✓	✓				✓	✓						✓				5
CC	1						✓			✓						✓	✓			4
	1		✓							✓					✓	✓				4
CCDD	1	✓	✓					✓		✓						✓	✓	✓		7
	1		✓				✓	✓									✓			4
EE	1					✓														1
	1							✓												1
FF	1								✓					✓	✓					3

[a]An arabic figure next to a check mark denotes the number of entities. Otherwise, the check mark denotes all entities in a row.[b]Cultivated rice.

sequence reacted positively to either 0.9 kb, 1.2 kb, or both. In contrast, some bands such as 9.0, 6.3, 5.6, and <3.4 or >22 kb in the various species showed no positive reaction to the 2 probes, This probably means that the glutelin genes embodied in these bands do not have a 5' region of glutelin gene type II.

In cultivated rice (AA genome), some positive bands shifted from 6.5 to 3.4 in one case, and from 1.7 to 2.8 kb in another case. Such shifting may indicate that the two glutelin genes have a mutation such that an *Eco*RI cutting site had been formed somewhere between the coding sequence and the 5' region. These two genes may be of the same gene type II even though some of their bases had mutated.

Distribution of glutelin gene in rice species

To find any new 5' regions of the glutelin gene, PCR was applied using genomic DNA of the species as templates and the two 25 mer oligo ends of type II 5' regions as primers. In each species, PCR bands varying from 1.9 to <0.5 kb were obtained, among which bands of 0.9 and 1.2 kb were the major ones. However, the FF genome lacked 0.9-, 1.2-, and 1.9-kb bands; it instead had 0.5- and 0.8-kb minor bands. The EE genome lacked a 1.2-kb major band. The CC genome did have an additional major band of 0.5 kb, but not of 0.9 kb. Hybridization experiments showed that bands 0.9, 1.2, and 0.5 kb (of the CC genome) but not others had high homology. The results show that glutelin genes in the CC, EE, and FF genomes may have a different 5' region than those of other species. Whether or not all the remaining PCR bands belong to the 5' region of the glutelin gene is not known, but this can be further tested.

Using the *Eco*RI-cut band DNAs positive to the glutelin coding sequence probe as templates, and hybridizing the PCR bands obtained to the probe prepared from the 5' region of glutelin gene type II showed with which *Eco*RI-cut band the glutelin gene with a specific 5' region was associated. In *O.perennis* (AA genome), both 0.9- and 1.2-kb 5' regions were present in the 9.0-kb bands, only 0.9 kb in the 4.4-kb bands, and none in the >22-kb band. *O. punctata* (BB genome) had both 0.9- and 1.2-kb 5' regions in the 18-kb band, but only 0.9 kb in the 6.3- and 5.4-kb bands. For *O. brachyantha* (FF genome), neither the 0.9-kb nor the 1.2-kb 5' region was present in the 12-, 9.0-, or 5.4-kb band. Thus, a glutelin gene with its specific 5' region may be associated with a defined *Eco*RI-cut band. Sometimes, glutelin genes with different 5' regions were clustered in the same band.

More PCR bands prepared from the 5' region of glutelin genes are being cloned and used to probe the EcoRI-cut bands positive to the glutelin coding sequence to discover the distribution of glutelin genes in rice species.

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Notes

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Discussion

Session 10: Molecular genetics of rice proteins

C— *Juliano:* The term indigestible protein given to PB-I or spherical protein bodies in rice endosperm is misleading. All protein bodies in raw milled rice are 100% digestible by growing rats. The digestibility of protein bodies in cooked rice is 85-90% in both rats and humans. Thus, the indigestible nature of the core fraction of PB-I is not due to primary protein structure but to changes in secondary/tertiary structure due to cooking.

Q— *Tukeda:* You have shown the strategy for genetic improvement of storage protein, but you have not given the timetable. When will practical breeders accept your results?

A— *Tunaka:* It depends on the establishment of transformation systems. However, recent developments in the systems promise to provide such new transformants in the quite near future.

Q— *Potrykus:* How do you plan to approach modification of the storage protein? In which direction do you plan to go?

A— *Mitsukawu:* To improve the digestibility and nutritive quality of rice storage proteins, we are trying to introduce bovine casein polypeptide (soluble in water) into PB-I using the function of rice prolamin signal polypeptide. We have already finished constructing a hybrid gene that is expected to produce in the developing endosperm cell a chimeric protein with the signal sequence for PB-I.

Q— *Bennett:* Is there a functional assay for the signal sequence that directs rice endosperm proteins into protein bodies? For example, is rice glutelin or prolamin transported into protein bodies in transgenic dicots?

A— *Mitsukawa:* Only rice prolamin precursor polypeptides were targeted at maize protein bodies (PB-I type); glutelin precursor polypeptides could not be. A chimeric bovine casein polypeptide carrying the rice 10-kDa signal sequence was incorporated into a maize protein body in an in vitro system.

Q— *Yong Soo Hwang:* Did you sequence wild type glutelin? What percentage of homology did you find between wild-type and cultivar glutelin?

A— *Wu:* We have not yet sequenced the glutelin genes located in the genomic DNA of wild species. However, the two types (I and 11) of DNA have been partly sequenced. The homology of the coding region between the two types varies from 90 to 95%.

Q— *Wang Xiangmin:* Did you use *O. granulata* in your comparison of wild species? Has the genome formula of this species been identified? In the list it seems to be classified as CCDD.

A— *Wu:* No, we did not use *O. granulata.* We used *O. grandiglumis.*

SESSION 11

Molecular Genetics of Disease Resistance

Defense gene regulation

C.J. Lamb, Qun Zhu, R.T. Yamamoto, A. Beeche, A.J. Nelson, and M.A. Lawton

To investigate the mechanism of disease resistance in a monocot system, we have isolated genes from rice encoding two key defense enzymes: phenylalanine ammonia lyase (PAL) and chitinase. These sequences were identified by hybridization with dicot sequences or by polymerase chain reaction-mediated amplification using oligonucleotide primers to conserved regions of the gene in dicot systems. Rice contains small gene families for both PAL and chitinase. Treatment of rice cell suspension cultures with fungal elicitors causes a rapid increase in PAL and chitinase enzyme activities. Northern blot analysis, using rice PAL and chitinase sequences as homologous probes, shows that the elicitor causes a very rapid but transient induction of PAL and chitinase transcripts. Constructs were made containing a rice chitinase promoter fused to the *GUS* reporter gene, and expression of *GUS* was examined in transgenic tobacco. Both organ-specific expression and tissue-specific expression were observed. The rice chitinase promoter was induced in tobacco both by wounding and by treatment with a fungal elicitor. Work has also begun on the isolation and characterization of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, the enzyme that catalyzes the first step of the isoprenoid biosynthesis pathway involved in the production of rice phytoalexins. These studies of defense genes will provide the basis for dissection of disease resistance mechanisms in rice, and will allow manipulation of the level and pattern of defense gene expression in transgenic plants.

Microbial diseases are a major factor reducing rice productivity, but little is known about the molecular mechanisms underlying natural resistance to disease in rice compared with well-characterized dicots such as soybean and tobacco. Dicots respond to environmental stresses by rapid and specific changes in RNA and protein synthesis (Chappell and Hahlbrock 1984, Cramer et al 1985, Ebel 1986, Lawton and Lamb 1987, Somssich et al 1986, Templeton and Lamb 1988). The response of the plant to microbial attack involves *de novo* synthesis of an array of proteins designed to restrict the growth of the pathogen. These proteins include 1) proteinase inhibitors and certain hydrolytic enzymes such as chitinase and glucananse, 2) hydroxyproline-rich glycopro-

teins and enzymes contributing to reinforcement of cell walls, and 3) enzymes for synthesis of phytoalexins.

Chitinase catalyzes the hydrolysis of the b-1,4 linkages of the *N*-acetyl-D-glucosamine polymer, chitin, a compound present in the cell walls of many fungi. In dicots chitinase activity is markedly increased by wounding or fungal attack and by treatment with microbial elicitors (Boller 1987; Boller et al 1983; Dixon and Lamb 1990; Lamb et al 1989, 1990). Purified plant chitinase attacks and partially digests isolated cell walls of potentially pathogenic fungi, and is responsible for inhibition of fungal growth (Schlumbaum et al 1986).

The first reaction in the pathway for biosynthesis of polyphenolic compounds including lignin, cinnamic acid esters, and flavonoids is catalyzed by phenylalanine ammonia lyase (PAL) (Jones 1984). PAL activity increases in response to infection and wounding (Lawton et al 1983, Liang et al 1989).

The enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) catalyzes the conversion of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) into mevalonate (MVA) in the first committed step of the pathway for isoprenoid synthesis. Higher plants produce a vast array of isoprenoid compounds, including phytoalexins for defense against microbial infection (Gray 1987). Induction of HMGR activity precedes accumulation of terpenoid phytoalexins in a number of dicot systems (Oba et al 1985, Suzuki et al 1975). In rice the phytoalexins momilactone A and B are induced along with HMGR by wounding and fungal infection (Cartwright and Langcake 1980, Cartwright et al 1977).

The present research examined the expression of these genes in rice plants during infection with microbial pathogens and further characterized the structure and expression of these genes.

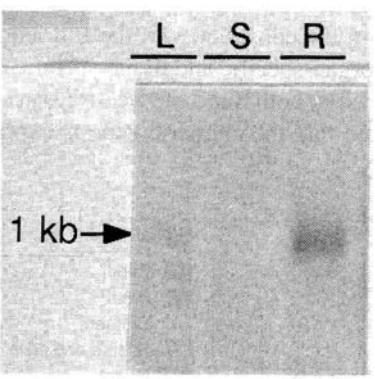
Chitinase

A rice genomic library (from N.-H. Chua) was screened using a fragment from the conserved region of the bean chitinase gene as a probe. From the 12 positive clones obtained, 3 were characterized by restriction mapping and Southern blot hybridization, and 1 was completely sequenced (pRch10). This gene proved to be a basic chitinase, with a hevein domain of approximately 49 amino acids. Based on the deduced amino acid sequence, the catalytic domain of the gene is highly homologous to dicot chitinase genes, and the hevein domain is homologous to other genes such as WIN1, WIN2, wheat agglutinin isolectin, and rubber hevein. The isolated chitinase gene fragment is 3035 bp in length and does not contain an intron. Primer extension analysis has revealed the transcriptional start site to be the “A” at nucleotide 1885. A hydropathy index plot has revealed a hydroprobic 5‘ terminus of the gene, as expected for a signal peptide. At the 5’ end of the gene, putative TATA and CAAT boxes are located 44 and 75 bp upstream, respectively, from the transcription start site. Between these two boxes, the DNA sequence is very rich in GC. Two inverted GC boxes are present at positions -55 to -65, and a sequence similar to an inverted elicitor-inducible motif occurs at position

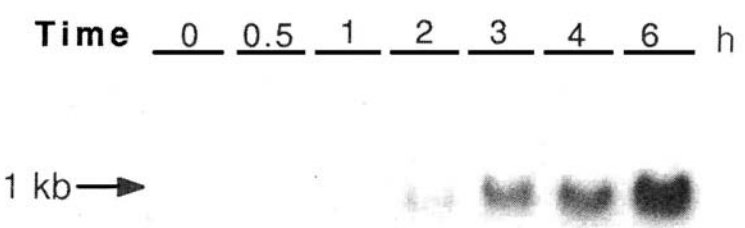
-107. A duplicated TGTCCAGGT motif, which is a constitutive footprint within the parsley PAL promoter, is located at position -752. In the 3' region, two putative polyadenylation signals have been located at positions +1054 and +1093. Their sequences fit the proposed consensus polyadenylation sequence for plants (NGAATAA) (Heidecker and Messing 1986).

Southern blot analysis of rice genomic DNA, using a conserved fragment of the pRch10 clone as probe, revealed eight *Eco*RI bands, six *Cla*I bands, six *Hin*dIII bands, and six *Pvu*II bands. This suggests that there are at least six chitinase genes in the rice genome.

Northern blot analysis of RNA from different rice organs, using the same probe as for Southern blot, indicated that organ-specific expression of chitinase occurs (Fig. 1). Chitinase was strongly expressed in roots, whereas it was only weakly expressed in stems and leaves. Expression of chitinase was induced by fungal elicitor (Fig. 2). This was also observed when a gene-specific probe for the pRchl0 clone was used.



1. Organ-specific transcription of chitinase gene. Total RNA was isolated from leaf (L), stem (S), and root (R) tissues of sterile-grown rice seedlings. Northern blot was probed with 422-bp fragment from rice chitinase gene. Transcript size is approximately 1 kb.



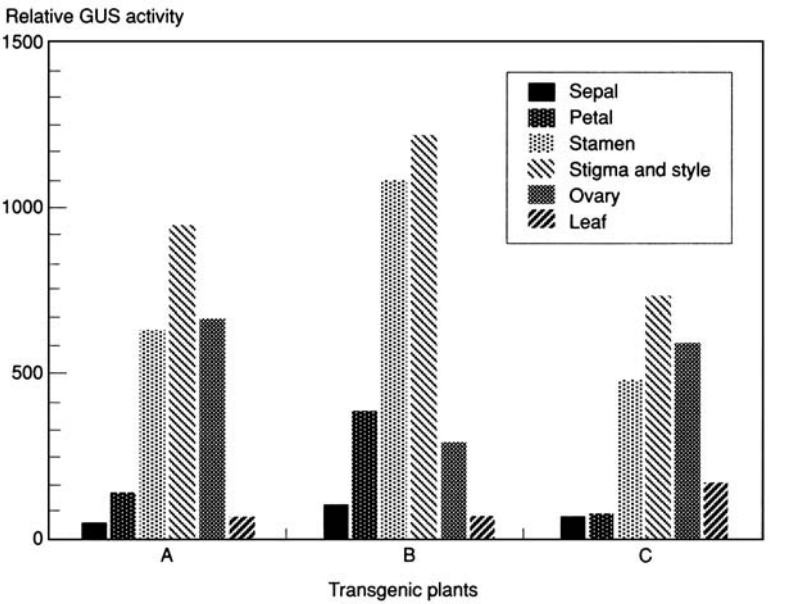
2. Induction of chitinase by fungal elicitor. Rice cell suspensions were treated with elicitor from *Phytophthora megasperma* var. *glycinea* (PMG). Total RNA was isolated from cells at various times following elicitor treatment. Northern blots were probed as in Figure 1.

Chitinase gene transcripts began to accumulate about 3 h after elicitor treatment and reached a maximum about 6 h after treatment.

To gain information about the protein product encoded by the pRchl0 gene, a fragment from the coding region (+85 to +1026) was fused to the TrpE promoter and transformed into *Escherichia coli.* Three constructs were made. The construct pBZ7-1 was fused in the reading frame; the constructs pBZ7-2 and pBZ7-3 were shifted 1 and 2 bases, respectively, from the pBZ7-1 fusion. A 37.5-kDa protein band was observed in extracts from cells transformed with pBZ7-1, whereas no band was detected in extracts from cells transformed with either pBZ7-2 or pBZ7-3. The protein band reacted positively with antibodies to bean chitinase, indicating that it was indeed a chitinase protein.

To further characterize the expression of the rice chitinase gene, constructs were made containing the chitinase promoter fused to the *GUS* reporter gene. Several constructs were made containing serial deletions of the rice chitinase promoter and 5' endpoints at -1512, -336, and -160. The constructs were transformed into tobacco via *Agrobacterium*-mediated transformation.

Transgenic tobacco plants containing the construct with the full length (-1512) chitinase promoter were examined for organ-specific and tissue-specific expression. ß-glucuronidase (GUS) activity, measured by both fluorometric assays in cell extracts and histological assays in situ, indicated that the chimeric gene is expressed most

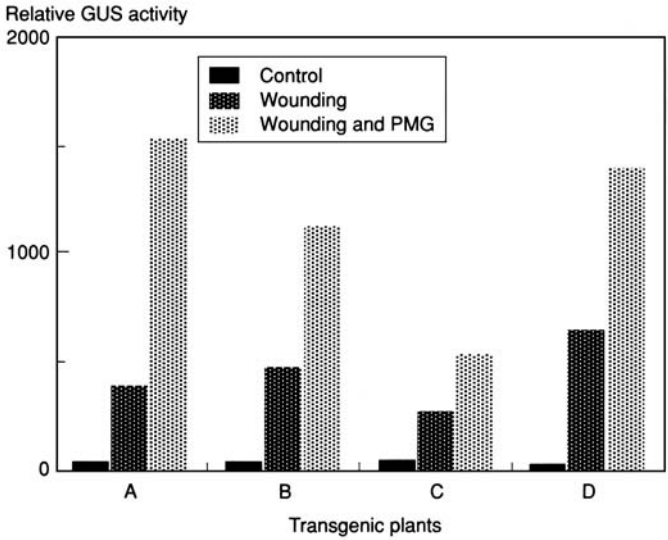


3. Localization of GUS activity in flowers of transgenic tobacco containing rice chitinase promoter. Promoter region (-1512 to +76) was inserted into pBI101.2, and recombinant plasmid containing chitinase-*GUS* gene fusion was transformed into tobacco leaf discs using *A. tumefaciens* LBA4404. GUS activity in floral parts of 3 transgenic plants was determined by fluorometric assay in cell extracts.

highly in roots and at only low levels in leaves and stems. This organ-specific expression pattern appears to be developmentally regulated: higher levels of expression of *GUS* were observed in young leaves than in old leaves. In tobacco roots, tissue-specific expression of *GUS* was observed. A high level of expression was observed in the tip (containing the root meristem), the vascular tissue (both xylem and phloem), and the epidermis. Organ-specific expression of *GUS* was also observed in tobacco flowers. Expression was highest in the stigma and style regions of the carpels, moderate in the stamen and in the ovaries of the carpels, and low in the petals and sepals (comparable to levels observed in leaves) (Fig. 3).

Expression of the chitinase construct in tobacco was examined following wounding and fungal elicitor treatment. Approximately 10-fold induction of *GUS* expression occurred after wounding, and this induction was enhanced another 2- to 3-fold following elicitor treatment (Fig. 4). The same pattern of induction was observed for all chitinase-*GUS* gene fusions, including the construct containing the smallest promoter, deleted to -160. This indicates that some wound-inducible and/or elicitor-inducible element is present in the first 160 nucleotides upstream of the transcription start side. Results from a gel retardation assay of rice nuclear extracts, using the -160 promoter fragment as a probe, indicated that indeed some protein factor may bind to this region of the promoter.

Constructs were also made containing the cauliflower mosaic virus 35S promoter fused to the chitinase coding region and the nopaline synthase terminator. Expression of these constructs in transgenic tobacco will be studied to attempt to identify a



4. Induction of GUS activity by wounding and fungal elicitor treatment in transgenic tobacco containing rice chitinase promoter-*GUS* gene fusion. Constructs were prepared and transformed into tobacco as in Figure 3. GUS activity was assayed in 4 transgenic plants at various times following wounding with or without elicitor treatment.

phenotype for the chitinase gene. All constructs will be expressed in rice as soon as a reliable rice transformation system is established in our laboratary.

Phenylalanine ammonia lyase

Several degenerate oligonucleotides corresponding to amino acid sequences in conserved regions of the dicot *PAL* genes were synthesized and used as primers for polymerase chain reaction (PCR). Three distinct products were obtained with nucleotide sequences clearly homologous to the dicot *PAL* sequences. One of these PCR product clones (R-3c2, 470 bp) was used to probe a rice genomic DNA library (from N.-H. Chua), and 10 positive clones were purified. Restriction fragment length polymorphism analysis indicated that these clones fell into at least two major classes. Representatives of the two classes, Rp2-23 (2.3 kb) and PZB 17-50 (4.4 kb), were subcloned, in addition to another hybridizing fragment, PZB8-36 (3.6 kb). Partial nucleotide sequences were determined for Rp2-23 and PZB8-36 to confirm that they contained rice *PAL* gene sequences.

Rp2-23 was truncated, containing the second exon and much of the intron of a *PAL* gene, but not the 5' portion of the intron, the first exon, and the 5' promoter regions. Sequencing of PZB8-36 showed that the 3' region has about 60% identity with the rice *PAL* gene sequence reported by Minami et a1 (1989). Sequence comparison of the 3' region with Rp2-23 showed these 2 genes to have 67% similarity. These data suggest that there are at least three *PAL* genes. A genomic Southern blot, using the 470 bp PCR product as a probe, indicated that there are probably 4 *PAL* genes in the rice genome.

By overlap subcloning, a 3-kb *Xho*I fragment (PZB8-3), believed to contain a region of the gene upstream from the 3.6-kb fragment (PZB8-36), was subcloned and sequenced. The 3-kb fragment presumably contains the intron as well as the first exon and part of the promoter region. It was shortened by *Sph*I digestion at the 3' end to exclude the intron region. Further dissection of this fragment will allow characterization of the first exon and the promoter region of this rice *PAL* gene. Work has also started on the PZB17-50 clone. A physical map has been constructed from restriction digests, and several small fragments are being sequenced.

Northern blot analysis, using the rice *PAL*470-bp fragment as a probe, indicated that the *PAL* transcripts are induced by treatment with fungal elicitor (Fig. 5). Induction begins at 3 h and continues for at least 10 h. The induction is more rapid than for the chitinase gene.

3-hydroxy-3-methylglutaryl coenzyme A reductase

A heterologous *Arahidopsis* gene fragment (450 bp) from a conserved region of the *HMG-CoA* reductase gene was used to probe a rice genomic library. Approximately 20 clones were purified, and restriction digests indicate that these clones fall into at least 3 classes. A 4-kb *Sac*I fragment from several of the clones was subcloned, further digested, and hybridized to yield a fragment small enough for investigation of sequence identity with other *HMGR* genes.

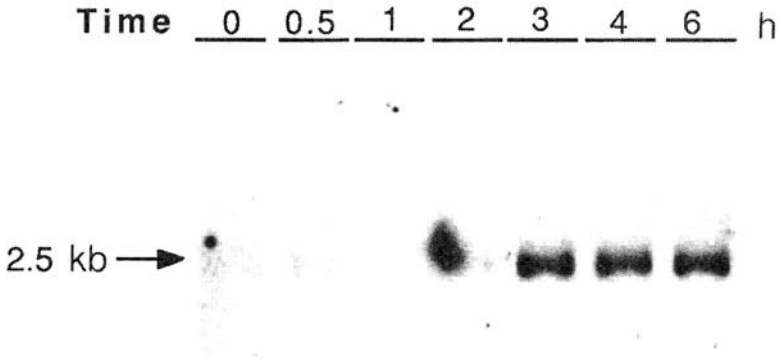
Discussion

We have successfully isolated a chitinase gene, a full-length *PAL* gene, and a truncated 3' region of a *PAL* gene. In addition, we have begun isolation of HMGR genes from rice. Our results show that the chitinase gene is a basic chitinase, containing a hevein domain. The entire gene has been sequenced, including the promoter region, and several potential regulatory sites have been identified. Transcription of the chitinase gene has been shown to be induced by both wounding and fungal elicitor treatment.

Constructs containing the rice chitinase promoter fused to *GUS* have been transformed into tobacco, and resultant *GUS* expression patterns indicate that the rice chitinase promoter is under organ-specific and tissue-specific control. Interestingly, *GUS* expression under the rice chitinase promoter is highest in tobacco roots, particularly in vascular and epidermal tissues. This would be expected for effective defense against fungal infection. In addition, *GUS* is highly expressed in tobacco flowers, particularly in the stigma and style regions. The role that chitinase may play in flowers is unknown. These results for rice chitinase parallel those in dicot systems and suggest that a similar mechanism of stress induction may operate in dicots and monocots.

Physical maps of one full and one partial *PAL* sequence have been completed. Both sequences differ from the published rice *PAL* sequence (Minami et al 1989). Sequencing information from the full-length *PAL* clone indicates that it contains two exons separated by an intron. The partial clone contains only the second exon. In addition, work has begun on a third *PAL* clone, which presumably differs from the other two.

Our work may lead to a better understanding of defense responses in rice, and will allow comparison of these responses in monocot and dicot systems. Once the defense genes have been isolated and characterized, studies of the regulation of these genes in



5. Induction of *PAL* transcripts by fungal elicitor. Total RNA was isolated from rice suspension cells at various times following treatment with fungal elicitor. Northern blots were probed with 470-bp PCR ¶product from rice *PAL* gene.

both a heterologous (tobacco) and in a homologous (rice) system will provide the necessary information to genetically engineer rice for effective resistance to microbial diseases.

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Molecular biology of rice tungro viruses: evidence for a new retroid virus

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Rice tungro spherical virus (RTSV) particles contain a single-stranded RNA genome with a major species of about 10 kb. RTSV particles have several species of coat protein. Two major proteins (35.5 and 25 kDa) were detected in protein extracts from infected leaves that were probed on western blots with a polyclonal antiserum raised against purified virus: a third protein (29 kDa) was found after Coomassie Brilliant Blue staining of gels. Two species of coat protein (36 and 32 kDa) were detected in rice tungro bacilliform virus preparations by western blotting. The particles contain double-stranded DNA of 8.3 kbp that has structural features (different conformations and singlestrand discontinuities) resembling those of caulimovirus DNA. Preliminary sequence data also revealed a primer binding site and a putative promoter sequence similar to those of cauliflower mosaic virus DNA.

Tungro, the most important virus disease of rice in Southeast Asia, is caused by a complex of two viruses. Rice tungro spherical virus (RTSV) is transmitted in a semipersistent manner by the rice green leafhopper *Nephotettix virescens,* but induces few symptoms. Rice tungro bacilliform virus (RTBV) causes the severe symptoms of the disease in most rice cultivars, but on its own is not transmitted by leafhoppers. Together, both viruses are leafhopper-transmitted and cause the disease (Cabauatan and Hibino 1985, Hibino 1983, Hibino et al 1978, Omura et al 1983, Saito 1976).

Continued attempts at breeding tungro resistance into rice have achieved mostly short-lived results. Dahal et al (1990) reported that most such resistance has been to the leafhopper vector, and that adaptation of the vector to new cultivars has led to the breakdown of resistance.

Among recent developments in plant virology is the concept of nonconventional resistance, in which the expression of virus-related genes or sequences leads to interference in some stage of the virus infection cycle (Sanford and Johnston 1985). In practice this has occurred when the coat protein gene of certain viruses is introduced

into and expressed from the host genome (Baulcombe et al 1987, Baulcombe 1989, Van Den Elzen et al 1989, Loesch-Fries 1989, Wilson 1989). The expression of satellite sequences has also been shown to reduce the replication and symptom expression of the parent virus (Harrison et al 1987).

The concept of nonconventional resistance opens up many other ways by which virus resistance may be induced in plants (Hull 1990). However, for any given virus in any host there are three major requirements before nonconventional resistance can be tested, namely knowledge of the genome organization, the mechanism of expression of the virus, and methods for transforming the host species.

Systems for regenerating both major types of rice from protoplasts have been developed (Abdullah et al 1986, Wang et al 1989), and transformed japonica rice plants have been produced (Raineri et al 1990, Shimamoto et al 1989, Toriyama et al 1988, Zhang et al 1988). However, despite the importance of tungro, relatively little is known about the molecular characteristics of the two viruses. We here describe some recent advances in the molecular biology of the tungro viruses. Besides providing information concerning nonconventional resistance, these results have revealed some features of RTBV that may have other uses in the genetic manipulation of rice.

Rice tungro spherical virus

RTSV has isometric particles 30 nm in diameter that most likely have icosahedral symmetry. Coomassie Brilliant Blue staining of gels in which sodium dodecyl sulfate-denatured coat protein has been electrophoresed reveals 3 bands with molecular weights of approximately 35,500, 29,000, and 25,000 (Jones et al 1991). When the proteins from such gels are transferred to nitrocellulose by western blotting and probed with an antiserum to RTSV, usually only the largest and smallest proteins are revealed. This would suggest that the 29-kDa protein is not antigenically active during antibody production.

The nucleic acid from RTSV is digested by RNAse under both high and low salt conditions, suggesting that it is single-stranded RNA. On gel electrophoresis there is a major band of about 10 kb (Jones et al 1991). RTSV RNA is selectable on oligo dT columns, and complementary copy DNA synthesis can be primed by oligo dT, which suggests that it is polyadenylated.

Plant viruses such as comoviruses, nepoviruses, and potyviruses are known to have polyadenylated single-stranded RNA and have (+)-strand genomes that express by the polyprotein mechanism. That the capsid of RTSV is formed of several protein species suggests that there may be proteolytic processing of a precursor protein involved, but this must be confirmed.

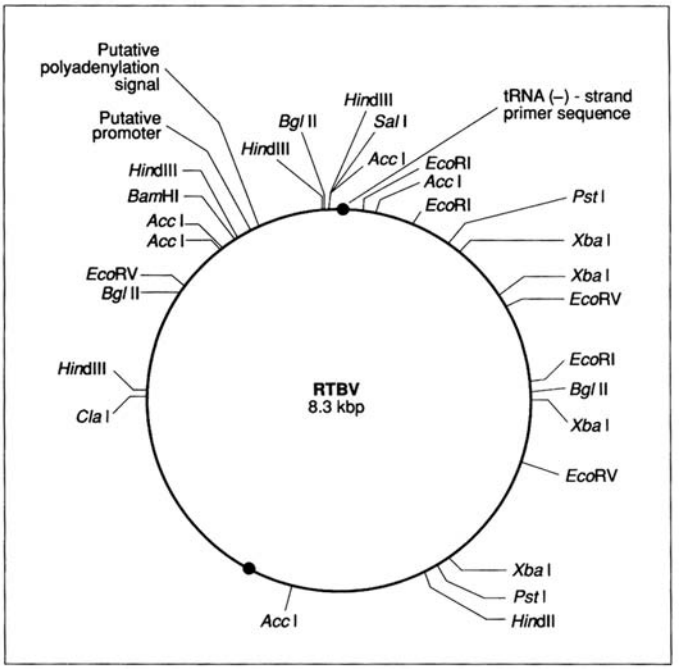
As RTSV has not yet been fully characterized, it cannot be assigned with confidence to any recognized plant virus group. However, similarities to maize chlorotic dwarf virus (MCDV), which is also transmitted by leafhoppers in a semipersistent manner, have been noted (Gingery 1988). Recent molecular characterization of MCDV RNA (Ge et al 1989) supports this relationship.

Rice tungro bacilliform virus

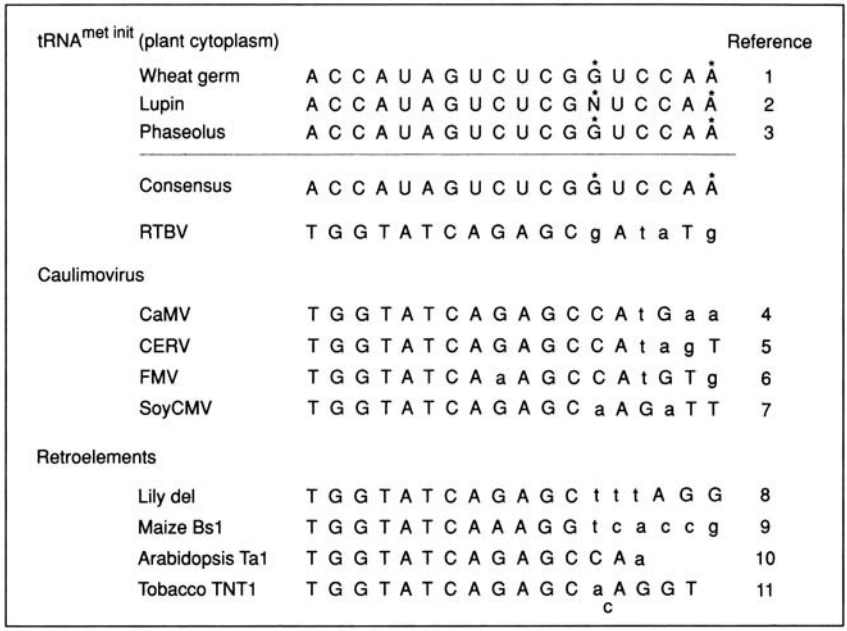
The particles of RTBV are about 30 nm in diameter and somewhat variable in length, with a modal length of 130 nm. RTBV coat protein usually migrates on gel electrophoresis as major bands of 36 and 32 kDa. The proportions in the two bands vary from preparation to preparation (Jones et al 1991).

Jones et a1 (1991) reported that the nucleic acid of RTBV is circular double-stranded DNA of about 8.3 kbp (Fig. 1). The DNA has two discontinuities at specific sites, one in each strand. In these features RTBV resembles several mealybug-transmitted viruses that have bacilliform particles (Lockhart 1990) and for which the name badnavirus (*ba*cilliform *dna virus)* is being proposed. It should be noted that RTBV DNA is larger than those of the mealybug-transmitted badnaviruses (7.5 kbp). The badnavirus genomes also resemble those of caulimoviruses, which are circular double-stranded DNAs of 7.8-8.0 kbp and have discontinuities at specific sites. However, caulimoviruses have isometric particles, and there is no cross hybridization between caulimovirus and badnavirus genomes.

Further similarities to caulimoviruses are revealed by our recent sequencing of regions of the RTBV genome. In the region of one of the discontinuities (Fig. l), a



1. Map of circular double-stranded DNA of RTBV showing restriction endonuclease cutting sites and sites of single-stranded discontinuities (•). Positions of tRNA complementary sequence and putative promoter and polyadenylation signal described in text are indicated.



2. (-)-strand primer sequences of RTBV (putative), various caulimoviruses, and plant retroelements compared with 3' sequences of plant cytoplasmic tRNA$^{\text{methionine initiator}}$. Mismatches in complementarity are shown in lowercase, * = methylated nucleotides in tRNA. References: 1 = Ghosh et al (l982), 2 = Sprinzl et al (1987), 3=Canaday et al (1980),4 = Franck et al (1980), 5 = Hull et al (1986), 6 = Richins et al (1987), 7 = Hasegawa et al (l989). 8 = Smyth et al (l989), 9 =Johns et al (1989). 10 = Voytas and Ausubel(1988). 11 = Grandbastien et al (1989).

sequence complementary to tRNA $^{\text{methionine initiator}}$ was found (Fig. 2). This resembles the (–)-strand DNA priming site of caulimoviruses and of various plant retrotransposons. In fact, all reverse transcribing elements so far described from higher plants have a tRNA$^{\text{methionine initiator}}$ priming site (Fig. 2). This is in contrast to retroviruses and reverse transcribing elements from animals (Table 1). There are at least two tRNA primers for mouse retroviruses, and a further two for mouse retroelements. The three Drosophila retroelements each have a different tRNA primer. The reason for the conservation of tRNA primer in plant retroelements in contrast to those of animals is unknown. Possible reasons include the relative availability of suitable tRNAs in the two systems, or that plant retroelements might have recently evolved rapidly from a common ancestor.

Southern blotting of total DNA from RTBV-infected rice probed with cloned RTBV DNA reveals a small virus-specific species of 500-600 bp. This resembles in size the strong-stop DNA of cauliflower mosaic virus (CaMV) (Covey et al 1983), which results from the premature termination of (–)-strand synthesis owing to failure to switch strands from the 5' to the 3' terminus of the RNA template. CaMV strong-stop DNA thus defines the length between the (–)-strand priming site and the 5' end of the template 35S RNA and hence the promoter site of the 35S RNA. The sequence of

Table 1. tRNAs that are (-)-strand primers in a selection of animal retroviruses and retroelements.[a]

Item	tRNA	Reference
Retroviruses		
Oncoviruses		
Rous sarcoma virus	TrP	1
Murine leukemia virus	Pro	2
Mouse mammary tumor virus	LYS	3
Lentiviruses		
Human immunodeficiency virus	Lys	4
Visna	Lys	5
Spumaviruses		
Human spumaretrovirus	Lys	6
Retroelements		
Mouse VL30	Gly	7
Mouse Intracisternl A particle	Phe	8
Drosophila Copia	Met initiator (internal)	9
Drosophila za7	Ser	10
Drosophila 412	Arg	11
Drosophila Gypsy	Lys	12
Yeast Ty	Met initiator	13

[a]References: 1 = Harada et al (1975), 2 = Harada et al (1979), 3 = Peters and Glover (1980), 4 = Ratner et al(1985), 5 = Sonigo et al (1985), 6 = Maurer et al (1988), 7 =Adam et al (1988), 8 = Mietz et al (1987), 9 = Kikuchi et al (1986), 10 = Inouye et al (1986), 11 = Yuki et al (1986), 12 = Marlor et al (1986), 13 = Clare and Farabough (1985).

Virus	*TATA box*	*Poly A signal*	*Gap 1*	*Total*	*Reference*
RTBV	- - - TATATAA - -	214 - - ATATTAT - -	389 - - TGGT	610	
CaMV	- - - TATATAA - -	188 - - AATAAAA - -	419 - - TGGT	614	1
CERV	- - - TATATAA - -	191 - - ATAATAA - -	677 - - TGGT	875	2
FMV	- - - TATATAA - -	170 - - TAATAAA - -	679 - - TGGT	856	3
SoyCMV	- - - TATAAAT - - - - - - -	2028 - - - - - - - -	TGGT	2028	4

3. Landmarks in sequences of RTBV and various caulimoviruses around major RNA promoter and polyadenylation signals and (-)-strand primer binding sites. References: 1 = Franck et al (1980), 2 = Hull et al (1986), 3 = Richins et al (1987), 4 = Hasegawa et a1 (1989).

RTBV in the region indicated by the small DNA species revealed a homology with the TATA box of the CaMV 35s promoter (Fig. 1,3). Downstream of that was a sequence resembling the consensus sequence for the polyadenylation signal. The topography of these sequence features of RTBV is compared with those of caulimoviruses in Figure 3. There is a remarkable similarity in the sequences and in their positions relative to each other with the exception of soybean chlorotic mottle virus (SoyCMV). It is not known why SoyCMV differs so markedly from the others.

The preceding sequence data are strongly suggestive of RTBV having a reverse transcription mode of replication that would involve a slightly more-than-full-length RNA. Preliminary northern blotting of total RNA from RTBV-infected tissue indicates that such a virus-specific RNA exists. The promoter of the CaMV 35S RNA is widely used as a strong constitutive promoter for plant transformations. However, the CaMV 35S promoter is not as efficient in monocots as in most dicots. Thus, a promoter from RTBV might prove to be an asset in genetic manipulation of monocots. RTBV is found mainly in phloem tissue (Saito et a1 1986), however, which raises the possibility that the promoter is phloem-specific. These sequences will obviously attract a great deal of attention in the near future.

Conclusions

There has been rapid progress in the understanding of the molecular biology of the two very different tungro viruses. Both viruses have features that are unusual among plant viruses, and one is a new retroid virus. However, it is too early to predict approaches that would have a reasonable chance of successfully conferring nonconventional resistance on rice.

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Recent advances in genetic analysis of rice-blast fungus interaction

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The fungus *Magnaporthe grisea* (*Pyricularia oryzae*) is a heterothallic ascomycete that causes rice blast (BI) disease. By genetic analysis, a number of loci conditioning virulence on and pathogenicity to rice have been identified. A genetic map of the fungus has been constructed using restriction fragment length polymorphism (RFLP) markers and chromosome-specific DNA probes. Combined RFLP and clamp-contoured homogeneous electric field electrophoresis analysis has established 14 linkage groups with 76 markers, including the mating type locus and 2 lactate dehydrogenase loci. Transformation with a cosmid encoding hygromycin B resistance has been achieved. This transformation system is generally applicable to field and laboratory strains, and together with the available genetic map, will allow molecular cloning of genes controlling pathogenesis. Concurrent with the analysis of the pathogen, host response to fungal infection is being studied in wild rice species *Oryza minuta* (2n = 48), which shows strong resistance to BI. Preliminary histological studies of infection showed a reduction in spore attachment, penetration, and growth of the infection hyphae in *O. minuta.* Backcross progeny with chromosomes introgressed from *O. minuta* to *O. sativa* have been produced and are being used to investigate the critical stages responsible for BI resistance.

Rice blast (Bl) caused by *Magnaporthe grisea* (anamorph *Pyricularia oryzae*) is the most widespread fungal disease of rice. The disease occurs in almost all rice-growing areas of the world and is most serious in temperate and tropical nonirrigated (upland) environments. In temperate regions, which include major rice-growing countries such as China, India, Korea, Japan, and the United States, the Bl problem is perpetuated by the high pathogenic variability of the fungus. In the upland environment, Bl exacerbated by drought is the major constraint to rice production. Upland rice is grown on over 18 million ha in Latin America, Africa, and Asia (IRRI 1989), and since upland rice is often associated with resource-poor areas, Bl has a severe impact on the welfare of poor farmers. As rainfed rice is becoming increasingly important in tropical Asia, there is growing concern that seasonal drought and increased use of fertilizers will cause the crop to become vulnerable to Bl (J.M. Bonman, IRRI, pers. comm.). Despite

the magnitude of the problem, satisfactory control of Bl has been achieved in tropical irrigated rice primarily through the use of resistant varieties (Bonman and Mackill 1988). This gives hope that control is possible in other environments.

Advances in biological sciences have offered new opportunities for solving recalcitrant production problems in agriculture. So far the most promising new approach in disease control has been the use of cross-protection in engineering virus resistance (Baulcombe 1989). No approach, however, has been developed for diseases caused by bacteria and fungi. As host-pathogen interactions in bacterial and fungal diseases are highly complex, an immediate solution to the problem through genetic engineering seems unlikely. A long-term strategy is to apply available techniques to understand both the host and the pathogen as fully as possible so that an integrated approach, which may include integrated pest management, genetic engineering of Bl resistance, conventional plant breeding, and biocontrol, can be used to manage the disease.

Research efforts on the Bl fungus and Bl resistance over the past several years have advanced our understanding of the pathogen and host resistance to it. Genetic crosses can now be made between laboratory strains of the fungus and, to a lesser extent, between field isolates; a number of factors involved in pathogenesis have been genetically defined; DNA-mediated transformation systems have been developed for the fungus; and a genetic map based on molecular markers has been constructed. Progress has also been made in the development of rice genotypes that will be useful for the analysis of host-pathogen interactions: near-isogenic lines (NILs) with single resistance genes in a common genetic background have been developed (Mackill et al 1988), and recombinant/translocation lines with resistance gene(s) from wild rice have been produced by interspecific hybridization (Amante-Bordeos et al 1991). The objective of this paper is to review the progress made in our understanding of the Bl fungus and its interaction with the rice plant. We will discuss experimental results as well as recent relevant literature in the hope of stimulating future research on this important disease.

The pathogen

Rapid progress has been made over the last 5 yr in our understanding of the genetics and genome organization of the Bl fungus. Also, molecular and genetic tools are now available for dissecting the processes involved in pathogenesis.

Genetic analysis

The sexual stage of *P. oryzae—M. grisea—was* discovered by Hebert (1971), who succeeded in crossing two isolates from crabgrass (*Digitaria sanguinalis*). Since then, much effort has been made to obtain the perfect stage in rice-infecting isolates of *P. oryzae,* but with limited success (Kolmer and Ellingboe 1988, Valent et al 1986, Yaegashi and Asaga 1981).

Much of the problem is due to the fact that almost all field isolates from rice are nonfertile or can function only as males in a cross. Only recently has the sexual stage

between two field isolates from rice been produced (Leung et al 1988). This was attributed to the discovery of a hermaphroditic rice isolate by J.L. Notteghem in French Guyana and to the continuous selection of fertile strains in the laboratory. The isolate Guy11 is highly pathogenic to specific rice varieties but not to most IR varieties, which are derived from indica rices (H. Leung and E.S. Borromeo, IRRI, unpubl. data).

The use of Guy11 in genetic crosses has advanced our understanding of the genetic control of pathogenesis. Using isolate Guy11, four genes controlling pathogenicity to rice have been identified (Leung et al 1988). Crosses between resistant rice lines Denorado and Norin 22 and susceptible Kinandang Patong and line 51583 showed a single-dominant resistance gene segregating in the F_2. F_3 families are being produced to conduct parallel genetic analysis in the host to determine whether genes conditioning resistance in rice correspond to pathogenicity loci in the fungus. Using isolate Guy11, Ellingboe et al (1990) reported at least seven avirulence genes segregating in a set of rice varieties from the United States. Valent (1990) reported that three avirulence genes derived from a weeping love grass *Eragrostis curvulus*-infecting isolate of *M. grisea* control specificity to three rice varieties. Furthermore, single genes controlling host specificity on weeping love grass, finger millet *Eleusine coracana,* and goose grass *E. indica* have been identified (Valent et a1 1986, Yaegashi 1978).

Although data seem to show a predominance of major genes in the control of host or cultivar specificity, minor genes modifying the interaction phenotypes are also present. Intermediate types with varying lesion size and number have been commonly observed but little studied, partly because the intermediate phenotypes are not adequately defined or described in most disease rating systems. Genetic analysis of intermediate interactions may be highly relevant to our understanding of host-pathogen interactions occurring in the field.

In addition to genes involved in host or cultivar specificities, other factors involved in pathogenesis have been identified. Tricyclazole protects rice plants by its inhibitive effect on melanin synthesis in the B1 fungus (Woloshuk and Sisler 1982). Melanin synthesis has further been shown to be required for successful formation of appressoria and for penetration of rice leaves and plastic surfaces (Howard and Ferrari 1989). Chumley and Valent (1990) genetically delineated the pathway leading to melanin synthesis in *M. grisea.* Three *loci—ALB, RSY,* and *BUF—*were shown to be epistatically related, with albino (ALB) epistatic to rosy (RSY) and rosy epistatic to buff (*BUF*). Addition of scytalone to buff mutants can restore pathogenicity. So far the genetic evidence suggests that the *BUF* locus encodes 3-hydroxynaphthalene reductase, converting 1,3,8-hydroxynaphthalene to vermelone.

In an effort to understand the early infection process, Hamer et al (1988) tested the ability of conidia to adhere to various surfaces under hydraulic shear forces. They identified a mucilage at the apex of the conidium that is essential for effective adhesion on glass and Teflon surfaces. To isolate mutants defective in the early infection process, Hamer et al (1989) screened for prototrophic survivors of ultraviolet mutagenesis that failed to form appressoria on Teflon films. A class of morphological mutants with abnormally shaped conidia (named Smo for spore morphology) were

recovered. Rice plants infected with Smo mutants produced fewer and smaller lesions, suggesting that the Smo mutation affects pathogenicity on rice. These studies clearly show that multiple-gene traits are involved in allowing the fungus to successfully infect the rice plant. Hence, a control strategy should consider the multiple pathways involved in this highly complex host-pathogen interaction.

Transformation

One approach to studying the genes involved in *M. grisea* pathogenesis is to directly isolate them by molecular cloning. With an efficient transformation system, DNA sequences from one strain can be transferred to a recipient strain; the transformants could then be selected for altered phenotype.

The first successful transformation in *M. grisea* was achieved by Parsons et a1 (1987), who used plasmid pMA2, which carries the *ArgB*$^+$ gene (encoding ornithine carbamoyltransferase) from *Aspergillus nidulans,* to complement an *arg*$^-$ auxotroph of *M. grisea.* About 35 transformants/µg of vector DNA were obtained. Incorporation of *M. grisea* genomic segments into the transforming vector did not significantly increase the transformation frequency; but integration by homologous recombination was observed when the vector containing a *M. grisea* sequence was linearized. Since then, two additional transformation systems using different selection techniques have been reported: Daboussi et al (1989) used the nitrate reductase gene from *Aspergillus* to transform a chlorate-resistant mutant of *M. grisea;* and Leung et al (1990) succeeded in transforming the fungus to hygromycin B resistance using the vectors pAN7-1 and pAN7-2, which contain the *Escherichia coli* hygromycin B phosphotransferase gene linked to A. *nidulans* regulatory sequences (Punt et a1 1987).

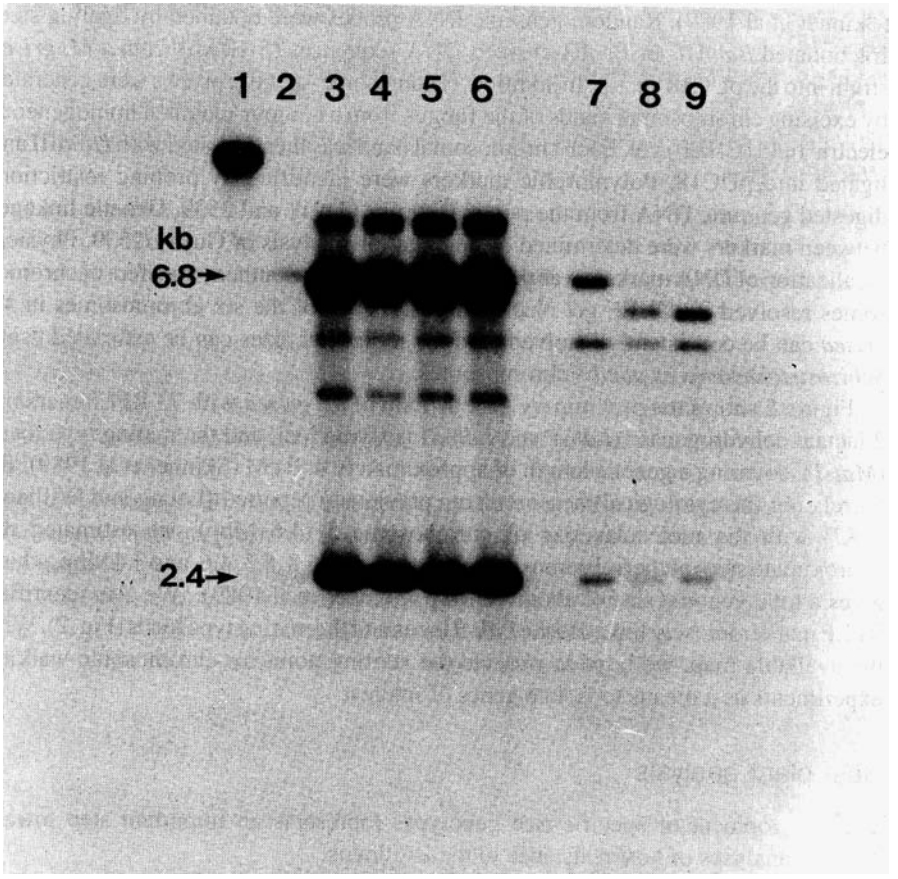
The frequency of transformation to hygromycin B resistance varied among recipient strains, ranging from 1 to 10 transformants/µg of vector DNA. Backcrossing of hygromycin B-resistant transformants with single-copy or low-copy vector integrations to the wild-type parents showed simple Mendelian inheritance of hygromycin B resistance in the progeny (Table 1). Pathogenicity tests showed that most transformants were as virulent as the untransformed wild-type strains from which they were derived.

Table 1. Segregation of hygromycin B resistance in crosses between transformants and wild-type strains of *Magnaporthe grisea.*

Cross	Cross type[a]	Ascospore progeny		c^{2b}
		Resistant	Susceptible	
GT05-1/2539	S/WT	26	37	1.92
GT03-1/2539	T/WT	64	54	0.85
GT06-1/2539	T/WT	41	29	2.06
2539T06-1/Guy11	T/WT	78	49	6.62*
GT10-1/2539	T/WT	12	12	0.0

[a]Integration pattern: S = single-copy, T =tandem, WT =wild-type strain with no vector integrated.
[b]Based on a 1 resistant : 1 susceptible monogenic model: * =significant deviation from 1:1 ratio at P = 0.05.

Most single-conidial isolates from lesions produced on rice or weeping love grass by transformants maintained their hygromycin B resistance, although some showed intermediate levels of resistance. Southern blot analysis revealed rearrangement of integrated DNA patterns among isolates of intermediate hygromycin B resistance (Fig. 1). Since hygromycin B selection is generally applicable to field and laboratory strains, it is likely to be the system of choice for future molecular analysis of the B1 fungus.



1. Southern hybridization analysis of transformant 2539T1-1 and single conidial cultures reisolated from weeping love grass *Eragrostis curvulus* following inoculation with 2539T1-1. DNA was digested with *Eco*RI and probed with ^{32}P-labeled pAN7-2. Lane 1 = lambda *Hin*dIII DNA (350 ng); lane 2 = *Eco*RI-digested pAN7-2 (0.1 ng); lane 3 = 2539T1-1; lanes 4-6 = 3 hygromycin B-resistant single conidial cultures isolated from single lesion on weeping love grass after inoculation with transformant 2539Tl-1; lanes 7-9 = 3 single-conidial cultures showing intermediate levels of hygromycin B resistance, isolated from single lesion on weeping love grass. Note reduction of copy number in lanes 7-9. Size markers indicate expected fragment sizes of pAN7-2 digested with *Eco*RI.

Genetic map

Through the work of several laboratories, the number of genetic markers in *M. grisea* is increasing rapidly, yet little is known about their linkage relationships. Nagakubo et al (1983) reported 5 linkage groups based on 11 fungicide resistance and auxotrophic markers, but these markers are too few and scattered in the genome to be useful for obtaining close linkages with genes of interest. To understand the genome organization of the fungus and to develop a tool for gene cloning, a genetic map based primarily on restriction fragment length polymorphism (RFLP) markers has been constructed (Skinner et al 1989). Random genomic DNA probes were obtained by ligating size-fractionated *Bam*HI- or *Eco*RI-cleaved DNA sequences (5-10 kb) from a *M. grisea* strain into the pUC18 vector. In addition, chromosome-specific probes were generated by excising chromosomal bands of the fungus from a contour-clamped homogeneous electric field (CHEF) gel. Each chromosomal band was then digested with *Hind*III and ligated into pUC18. Polymorphic markers were identified by probing restriction-digested genomic DNA from the parental strains Guy 11 and 2539. Genetic linkages between markers were determined by segregation analysis of Guy11/2539. Physical localization of DNA markers was performed by probing Southern transfers of chromosomes resolved by CHEF gel electrophoresis. Four of the six chromosomes in *M. grisea* can be consistently resolved, and the molecular sizes can be estimated using *Schizosaccharomyces pombe* chromosomes.

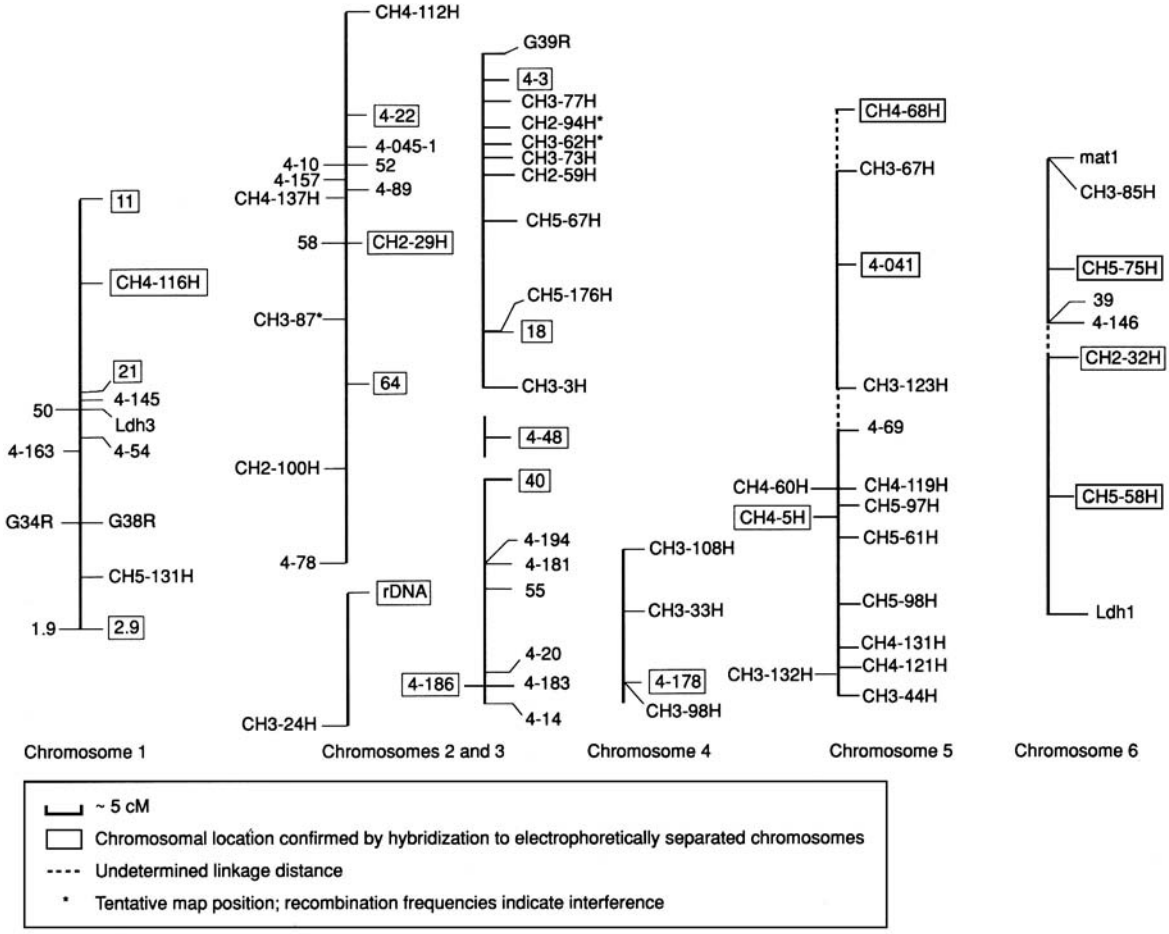
Figure 2 shows the preliminary genetic map of *M. grisea* with 73 RFLP markers, 2 lactate dehydrogenase *(Ldh-1* and *Ldh-3)* isozyme loci. and the mating type locus (Mat-I), covering a genetic length of approximately 400 cM (Skinner et al 1989). By correlating the cytological measurements previously reported (Leung and Williams 1987) with the molecular size of chromosome 5 (4.6 Mbp), we estimated the approximate sizes of the 6 chromosomes to be 9.5, 7.3, 5.8, 5.3, 4.6, and 3.4 Mbp, which gives a total genome size of about 36 Mbp (Skinner et al 1989). We also identified RFLP markers closely linked to the *Ldh-3* locus and the mating type locus (Fig. 2). With the available map, we hope to provide the starting point for chromosome walking experiments as a means to isolate genes of interest.

Host plant analysis

The development of specific rice genotypes represents an important step toward detailed analyses of host resistance to the Bl fungus.

Near-isogenic lines of indica rices

To study the genetics of Bl resistance, a series of NILs with single resistance genes from indica rices has been produced by repeated backcrossing of the resistant parents to a common susceptible parent CO 39 (Mackill et al 1988). These lines will be valuable in studying the effects of single or combined resistance genes on host-pathogen interaction. Use of the NILs for isolating Bl resistance genes is discussed by Yu et al (1991).



2. Genetic map of *Magnaporthe grisea* based on segregation analysis of progeny from Guy11/2539.

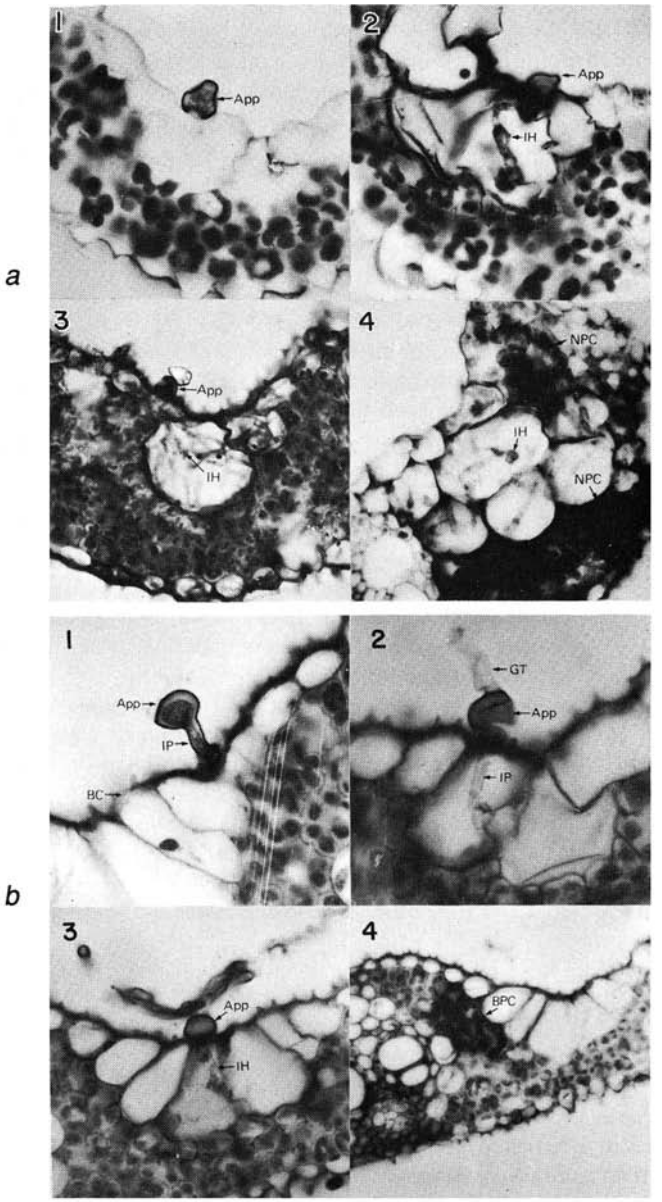
Production of recombinant and translocation lines with resistance genes from wild rice

At IRRI, we initiated a study of disease resistance genes derived from wild rices. *O. minuta,* a tetraploid wild rice species commonly found in the Philippines, was identified as a potential source of resistance to B1 and bacterial blight. Crosses between an *O. minuta* accession no. 101141 and rice breeding line IR31917 were made to transfer the resistance from *O. minuta* to cultivated rice and to examine the nature of the resistance present in the wild species. By embryo rescue and backcrossing, BC_2 plants with 24-37 chromosomes and varying degrees of fertility were recovered. Results suggest that B1 resistance was incorporated into the rice genome either by recombination or by translocation of a small *O. minuta* chromosome fragment. Details of the methodology and results of this breeding project are described by Amante-Bordeos et a1 (1991).

BC_2F_2 progeny from B1-resistant plant 75-1 were analyzed histopathologically. Preliminary observations on the infection process of the fungus in *O. minuta* showed that fewer spores were attached to the leaf surface of *O. minuta* than to a cultivated rice leaf. A lower percentage of bulliform cells (5.6%) of *O. minuta* than of *O. sativa* were penetrated, and no hyphae were established (Fig. 3) (Bemardo 1989). Since there are substantial differences between the leaf anatomies (number of papillae and trichomes and the size of silica-cork cell) of *O. sativa* and *O. minuta,* we first focused on the relationship between leaf anatomy and B1 resistance. Analysis of the infection of isolate PO6-6 on BC_2F_2 progeny, however, showed no apparent relationship between anatomical features and resistance. Work is in progress to determine whether reduced spore adhesion is transmitted to BC_2F_2 progeny and is correlated with B1 resistance. Furthermore, we are attempting to locate the "wild" resistance genes in F_2 progeny with *O. minuta* species-specific probes and rice chromosome-specific RFLP probes provided by S. Tanksley of Cornell University.

Conclusions and recommendations

Genetic analysis of *M. grisea* shows that multiple-gene traits are involved in pathogenesis. Current efforts in several laboratories will continue to generate new information on the genes that have strong effects on the interaction. Little is known, however, about minor genes that have cumulative effects on pathogenesis. While it is important to focus on genes conditioning cultivar specificity, we should extend our attention to traits such as aggressiveness and sporulation capacity that influence the overall epidemiological potential of the fungus. Knowledge of the genetic basis of such fungal attributes may help us understand the mechanisms of partial resistance in the host, which has been found to be agronomically important (Bonman and Mackill 1988). At the population level, our knowledge of how the rice crop affects pathogen virulence and pathogen composition is limited. Work on pathogen population biology has been initiated, but field experiments that consider the effects of the host population on the pathogen have yet to be designed.



3. Histopathological comparison of infection of B1 fungus isolate PO6-6 on *O. sativa* and *O. minuta.* a) Compatible interaction between rice variety CO 39 and isolate PO6-6. 1) At 24 h after inoculation (HAI), appressorium (App) was formed. 2) At 48 HAI, infection hyphae (IH) were formed from appressorium. 3) IH were established in host cells at 72 HAI. 4) Colonization of IH and necrosis of parenchyma cells (NPC) at 96 HAI. b) Incompatible interaction between *O. minuta* and isolate PO6-6. 1) At 24 HAI, infection peg (IP) was unable to penetrate host cell. 2) At 48 HAI, some IPs were able to penetrate bullifom cell. 3) At 72 HAI, IH were not able to establish in host cells and started to deteriorate. 4) Brown parenchyma cells (BPC) at 96 HAI.

Studies of host plant resistance have emphasized gene cloning (e.g., transposon tagging and RFLP mapping). Little effort has been made to delineate the steps leading to resistance. Work on the biochemical, physiological, and cytological bases of resistance can be complementary to the cloning efforts. Available NILs and wild-rice recombinant/translocation lines will be welcome tools for the analysis of resistance mechanisms.

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Discussion

Session 11: Molecular genetics of disease resistance

Q—*Shahjahan:* You mentioned that there is no apparent resistance to rice tungro spherical virus (RTSV). But we have found three varieties resistant to RTSV. If rice tungro bacilliform virus (RTBV) is able to transmit without the help of RTSV, and RTBV produces the disease, why are you doing research on RTSV and not RTBV?

A—*Hull:* I am most interested to hear about RTSV resistance. I understood that RTSV resistance is really tolerance in which the virus replicates at a low level. We should try for true immunity so that the virus cannot replicate, and then there will be less chance for variation to overcome the resistance, We are working on both RTSV and RTBV so that we can develop nonconventional resistance to both.

Q—*Potrykus:* Under what containment conditions are you allowed to work on these experiments in Great Britain?

A—*Hull:* We use containment category B. The agrobacteria are disarmed but we take the precautions dictated by the regulating authorities. If this technique of agroinfection of RTBV is to be used widely by plant breeders, there will probably have to be centers where the appropriate containment conditions are set up.

Q—*Bennett:* Can you comment on the view that the transgenic coat protein approach for virus resistance is less likely than the transgenic BT toxin/protein inhibition approach for insect resistance to select for disease agents capable of overcoming the resistance mechanism?

A—*Hull:* The evidence for work with coat-protein protection against tobacco mosaic virus is that the more the strain of the virus differs from that giving the coat protein protection, the less effective the protection. This is why we are looking at variations in RTSV and RTBV over Southeast Asia.

Q—*Bennett:* Is the elicitor employed in the PHG/TAG/CAD induction study derived from a bean or from a rice pathogen?

A—*Nelson:* It is from a soybean pathogen, *Pytophthora megasperma* var. *glycinea,* known to have a broad host range.

Q—*Bennett:* Have you attempted to study defense genes in transgenic rice callus, which is easier to produce than transgenic rice plants?

A—*Nelson:* No.

Q—*Potrykus:* (a) Is this “transgenic” work in tobacco? (b) Is there natural production of chitinase in rice leaves?

A—*Nelson:* (a) Yes. (b) We do not yet know, and western blot work is in progress.

SESSION 12

Transformation Techniques

Co-transformation of indica rice via polyethylene glycol-mediated DNA uptake

Jianying Peng, L.A. Lyznik, and T.K. Hodges

Protoplasts were prepared from cell suspension cultures of Oryza sativa L. (indica variety IR54) and transformed with two separate plasmids, pKAN and pPUR, containing the neo and gusA genes, respectively. Each gene was driven by the CaMV 35s promoter. Plasmid DNA uptake was mediated by polyethylene glycol. Transformation efficiency with the neo gene was 0.4-2.0 × 10^{-5}. About 25% of the calli containing the neo gene also contained the functional gusA gene. Southern blot analysis of DNA from callus indicated that both genes were stably integrated, intact as well as in rearranged forms, into the genome. Seven plants were regenerated from G-418-resistant calli and grown to maturity. Calli selected on kanamycin appeared incapable of regenerating plants. The plants regenerated from G-418-resistant calli were confirmed to contain the neo gene, but not the gusA gene. All transgenic plants were phenotypically normal; however, none set seed.

One strategy for genetic engineering of monocots is transformation of protoplasts by direct uptake of DNA and then regeneration of plants. During the past 5 yr, there have been several reports of successful plant regeneration of rice *Oryza sativa* L. from protoplasts of both japonica (Abdullah et al 1986, Fujimura et al 1985, Kyozuka et al 1987, Toriyama et al 1986, Yamada et al 1986) and indica varieties (Kyozuka et al 1988, Lee et al 1989, Wang et al 1989).

Transformation of japonica rice protoplasts was achieved by direct uptake of DNA into protoplasts mediated by either polyethylene glycol (PEG) (Zhang and Wu 1988) or electroporation (Shimamoto et al 1989, Toriyama et al 1988, Yang et al 1988, Zhang et al 1988), and transgenic plants were produced. Most authors used the neo gene, which encodes for neomycin phosphotransferase II, in combination with antibiotics to select transformed cells; however, Shimamoto et al (1989) and Matsuki et al (1989) used the *hph* gene product (aminoglycoside phosphotransferase type IV) to discriminate transformed cells. The transformation frequency was 0.4-36/10^5 protoplasts treated. For callus selection and plant regeneration of japonica genotypes transformed with the *neo* gene, Zhang et al (1988) used the antibiotic kanamycin; however,

Toriyama et a1 (1988) were unable to regenerate plants following the selection of kanamycin-resistant calli. These authors did regenerate five transgenic plants from G-418-resistant calli.

Although indica rice is one of the most important subspecies of *Oryza sativa* L. and a major food crop for millions of people, transformation has not previously been reported. Since we had developed the procedures for regenerating indica rice plants from protoplasts in our laboratory (Lee et a1 1989), we pursued transformation with both the *neo* and *gusA* genes using a PEG-mediated DNA uptake protocol in combination with selection of transformed cells with kanamycin or (G-418. We have produced transgenic callus containing the *neo* and *gusA* genes and transgenic plants with the *neo* gene, as evidenced by Southern blot analysis.

Materials and methods

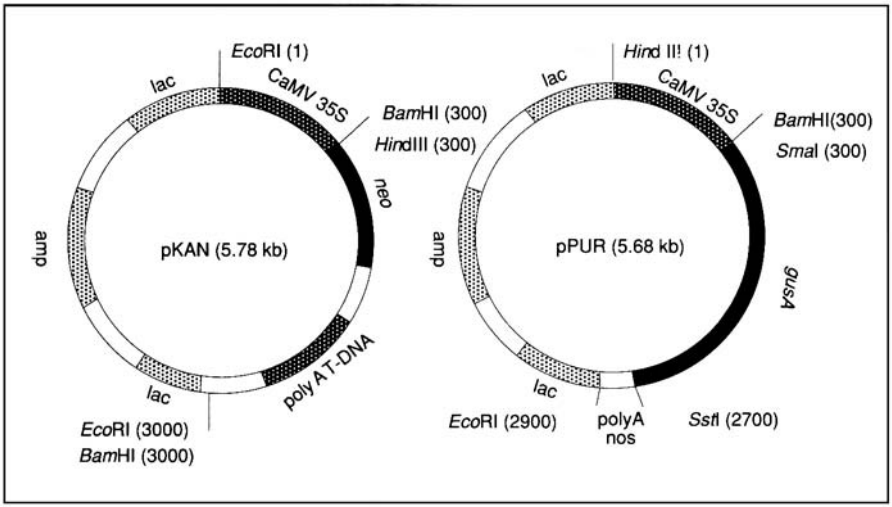
Seed of indica variety IR54 was obtained from the International Rice Research Institute. Immature embryos were excised 10-12 d after anthesis and used to initiate calli as described by Lee et a1 (1989).

Initiation of cell suspensions and isolation of protoplasts

Protoplasts were isolated from cell suspension cultures of *Oryza sativa* L. (indica variety IR54) as described by Lee et a1 (1989). Cell suspension cultures designated IR54-5 and IR54-6 were initiated from 4-wk-old calli derived from immature embryos in August 1988 and January 1989, respectively, and cultured in N6 medium (Chu et al 1975) supplemented with 4 mg 2,4-dichlorophenoxyacetic acid (2,4-D)/liter, 20 mM proline, and 3.0% sucrose. The cell cultures were maintained at 26°C in the dark on a shaker adjusted to 80 rpm. Procedures for protoplast regeneration were as described by Lee et a1 (1989), with modifications presented in the following sections.

Plasmid constructions and transformation protocol

Figure 1 shows the two plasmids used. pKAN contained the CaMV 35s promoter; the *neo* gene, which encodes neomycin phosphotransferase II (NPT II); and a T-DNA (orf 26) polyadenylation site. Expression of the *neo* gene in combination with the antibiotics kanamycin or (G-418 served as the system for selecting transformants. The other plasmid, pPUR, contained the CaMV 35s promoter, the *gusA* gene encoding ß-glucuronidase (GUS), and a *nos* polyadenylation site. The *Bam*HI fragment of pKAN was the hybridization probe used in Southern blot analysis. For transformation, protoplasts were suspended in CPW medium (Frearson et al 1973) at a density of 1-2 x 10^7 protoplasts/ml. One milliliter of protoplast suspension was mixed with 50-100 µg of each plasmid in TE buffer (Maniatis et a1 1982) and 1.1 ml 40% PEG in Krens' F solution (pH 7.0) (Krens et a1 1982). The temperature of the incubation mixture was raised to 45 ºC for 5 min. The mixture was then chilled on ice for 20 s and incubated an additional 25 min at 30 °C. The incubation mixture was diluted gradually with 30 ml of Krens' F solution over the next 30 min, and then protoplasts were pelleted at 100 g for 15 min.



1. Plasmid constructions used in transformation experiments.

Protoplast culture and observation

Protoplasts were suspended at a density of 2×10^6 /ml in Kao's protoplast regeneration medium (Kao 1977) as modified by Lee et al (1989). Two hundred microliters of protoplast suspension were plated onto Millipore filters (0.8 µm pore size, type AA) on top of modified Kao's medium (solidified with 0.8%, wt/vol, Sea Plaque agarose) containing IR52 nurse cells (Peng et al 1990). Plating efficiency (defined as number of microcalli per protoplast plated × 100) was scored 2 wk after culturing the protoplasts. Another method of protoplast culture was a "donut" culture method modified after Hanes et al (1989). A ring of nurse cells in modified Kao's medium containing agarose was formed just inside a petri dish leaving a "donut" hole in the center of the dish for holding a solution with protoplasts. The hole was molded by placing a bottle cap in the center of the dish prior to pouring the agarose. To keep the nurse cells from floating into the protoplast well, a second 1-mm layer of Kao's medium containing agarose without nurse cells was poured on top of and inside the first ring by using a smaller bottle cap as the mold. One milliliter of protoplasts at a density of 2×10^5/ml was cultured in each dish, and protoplast growth was observed with an inverted microscope.

Selection of transformed calli, plant regeneration, and growth of regenerated plants

The Millipore filters with protoplast-derived calli were transferred, 3 wk after transformation, to Murashige and Skoog's (1962) (MS) medium, to which was added 2 mg 2,4-D/liter. 0.5 mg cytokinin (either 6-benzylaminopurine or zeatin), 3.0% sucrose, and 100 mg kanamycin/liter or 15 mg G-418/liter. Six weeks after transformation, the kanamycin- or G-418-resistant calli were selected and transferred for another 2-wk

period to antibiotic-free Linsmaier and Skoog's (1965) medium supplemented with 2.0% sucrose and 0.5 mg 2,4-D/liter. The calli were then transferred to N6 regeneration medium containing 10 mg kinetin/liter, 0.1 mg naphthaleneacetic acid/liter, and 3% sucrose. Three weeks later, calli with shoots were transferred to hormone-free MS medium supplemented with 3.0% sucrose. All media were solidified with agarose at 0.6% (wt/vol) except for the initial protoplast culture medium, which was 0.8% (wt/vol). Regenerated plantlets were transferred to small bottles containing the same regeneration medium and cultured for 2 wk more, then transplanted into soil and grown to maturity in a growth chamber with 10-h light (at 28 °C) and 14-h dark (at 24 °C) periods.

Southern blot analysis, and GUS and protein assays

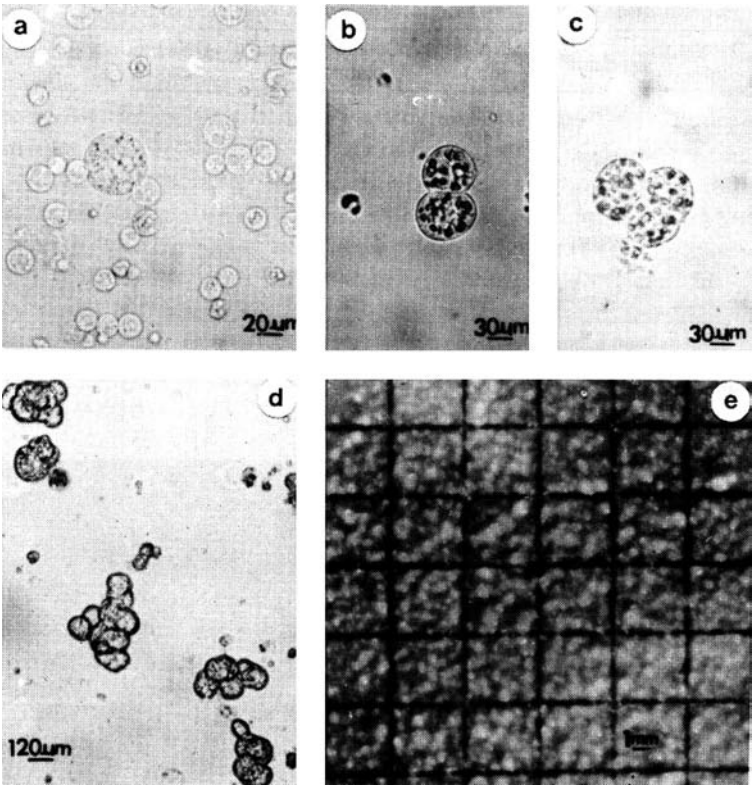
We used Mettler's (1987) protocol to isolate DNA from the leaves of the putatively transformed plants. Restriction enzyme digestion, electrophoresis, and Southern blot analyses were done according to Maniatis et al (1982). GUS assays were performed on extracts of selected calli and leaves of regenerated plants according to Jefferson (1987). Protein content was determined by the procedure of Lowry et al (1951).

Results

Cell suspension lines IR54-5 and IR54-6 went through typical cell suspension stages as described by Lee et al (1989). Three months after initiation, they started to release protoplasts when treated with cellulase RS and pectolyase Y23, but the yields were low, and callus formation from these protoplast cultures was very poor. High protoplast yields (1-5 × 10^7/g fresh weight of suspension cells) were obtained (Fig. 2a) after about 6 mo of liquid culture.

The standard feeder system used for protoplast cultures was not suitable for microscopic observation of the initial cell divisions and growth. By using the liquid "donut" culture system, the first and second cell divisions could be observed after 2-5 d (Fig. 2b,c) with the aid of an inverted microscope. Many multiple cell clusters were produced at 21 d (Fig. 2d). Likewise, protoplasts grew very well on Millipore filters on top of the feeder cells (Fig. 2e). Plating efficiency scored 2 wk after protoplast isolation was 0.13-1.17% over a wide range of plating densities (Table 1). Six-week-old embryogenic-like calli were transferred to regeneration medium, where they produced shoots in 3 wk and plantlets with roots in 6 wk. Plants were regenerated from 12-27% of the calli that were transferred to regeneration medium (Table 2). Both N6 and MS medium supplemented with either kinetin or benzylaminopurine supported plant regeneration (Table 2).

Putatively transformed calli were recovered from medium containing either 100 mg kanamycin/liter (Fig. 3a) or 15 mg G-418/liter (Fig. 3d), while the untransformed protoplast-derived calli did not undergo further growth and became necrotic when subjected to the same selection. The transformation frequency, defined as number of



2. Growth of IR54 protoplasts. a = freshly isolated protoplasts. b and c = first and second cell divisions, respectively. d = multiple cell clusters observed 21 d after isolation; see Materials and Methods for the liquid culture method. e = microcalli formation from protoplasts cultured on Millipore filters placed on top of a nurse culture.

Table 1. Plating efficiency of IR54-5 protoplasts when plated at different densities.

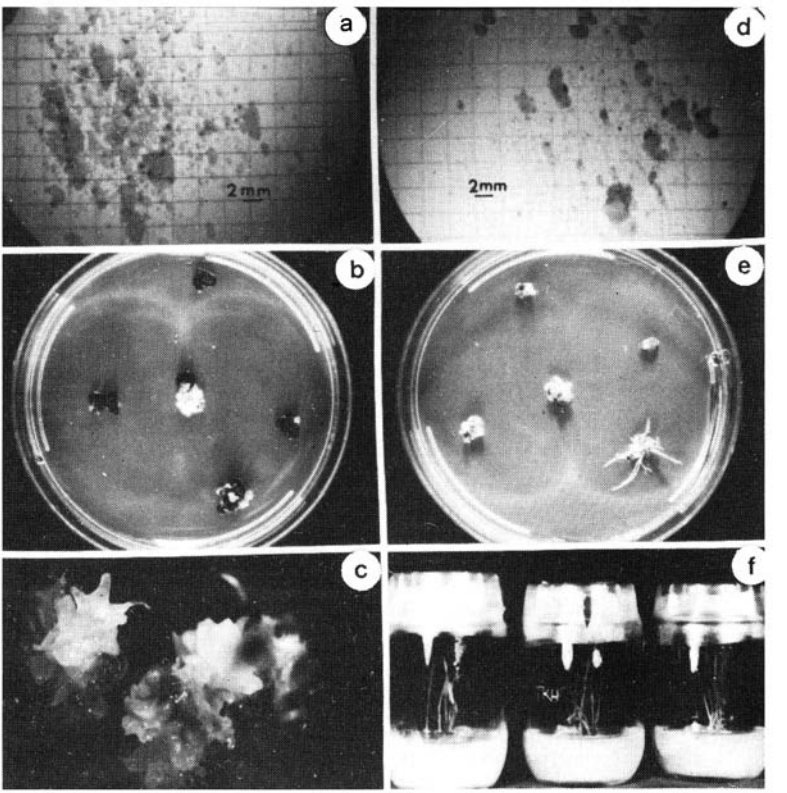
Plating density[a] ($\times 10^{-6}$/ml)	Mean no. of microcalli[b] per plate ± S.D.	Plating efficiency[c] (%)
0.1	25 ± 10	0.13
0.5	770 ± 54	0.77
1.0	2350 ± 208	1.17
2.5	4012 ± 468	0.80
5.0	5288 ± 490	0.53

[a] Volume = 0.2 ml/plate. [b] Number of microcalli were scored 2 wk after protoplasts were cultured on top of Millipore filters placed on medium containing IR52 feeder cells. [c] Plating efficiency (%) is defined as the number of microcalli after 2 wk of protoplast culture divided by total protoplasts plated × 100.

Table 2. Regeneration of plantlets from protoplast-derived calli of IR54.

Line	Duration (mo)	Medium[a]	Calli plated (no.)	Calli regenerating plantlets[b] (no.)	Calli producing plants (%)
IR54-5	6	N6(K)	105	25	23.8
		N6(B)	140	19	13.6
		MS(B)	85	10	11.8
IR54-5	8	N6(K)	80	16	20.0
		N6(B)	70	10	14.3
		MS(B)	100	17	17.0
IR54-6	10	N6(K)	150	40(125)	26.6
		N6(B)	150	21 (72)	14.0

[a]N6 or MS basal medium supplemented with cytokinin (10 mg/liter) as either kinetin (K) or benzylaminopurine (B). [b]Numbers in parentheses are total number of plantlets produced at 8 wk after calli were transferred to regeneration medium.



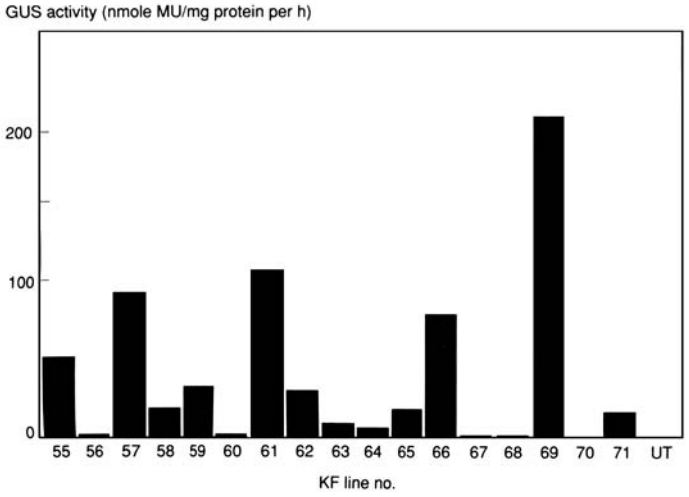
3. Selection of kanamycin- and G-418-resistant calli and regeneration of plantlets. a and d = kanamycin- and G-418-resistant calli 3 wk after transfer to antibiotics, respectively. b and e = kanamycin- and G-418-resistant calli 3 wk after transfer to regeneration medium, respectively, c = shoot-like structures developed from a kanamycin-resistant callus: no plants were obtained from kanamycin-resistant callus. f = plantlets produced from G-418-resistant calli.

antibiotic-resistant calli per 10^5 protoplasts treated, was from 0.4 to 2.0 in separate experiments. Southern blot analysis of DNA isolated from the kanamycin-resistant calli confirmed the presence of the *neo* gene in most of the selected calli (Peng et al 1990).

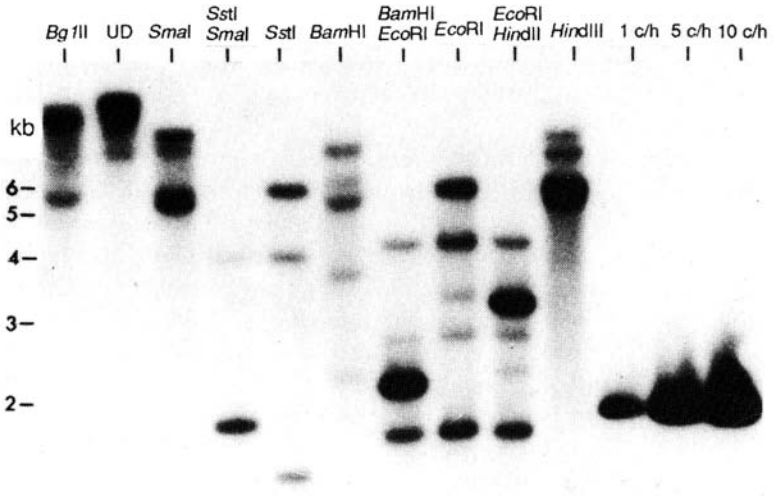
About 25-30% of the kanamycin-resistant calli expressed GUS activity above background levels (Table 3). Considerable variation existed in GUS activity in different kanamycin-resistant calli (Fig. 4). Southern blot analysis revealed the presence of the *gusA* gene in DNA isolated from these calli (Peng et al 1990). In line KF71, DNA was extracted from 5-mo-old callus, purified with CsCl centrifugation, and digested with various restriction enzymes (Fig. 5). Undigested DNA or DNA digested with *Bg*III (a restriction enzyme that does not cut pPUR) hybridized with the probe to give a smear in the high molecular weight region. This indicates the integration of *gusA* coding sequences into genomic DNA. However, an additional band was observed in the region of 5.0-6.0 kb when the DNA was digested with *Bg*III, suggesting the occurrence of at least 2 integration events. Digestions of the DNA with two restriction enzymes revealed the presence of DNA fragments containing homologous

Table 3. Percentage of kanamycin-resistant calli expressing GUS activity.

Experiment number	Kanamycin-resistant calli (no.)		%
	Assayed for GUS	Expressing GUS activity	
1	31	8	25.8
2	20	5	25.0
3	50	15	30.0



4. GUS activity in 17 kanamycin-resistant calli (KF55-71) assayed 3 mo after transformation. MU = 4-methylumbellifryl-b-D-glucuronide, UT = untransformed callus.



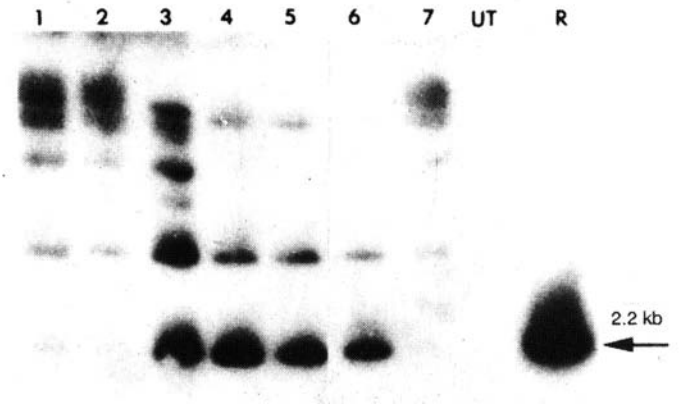
5. Southern blot analysis of DNA from kanamycin-resistant line KF71. Five µg CsCl-purified DNA was digested with restriction enzymes as shown on top of each lane, subjected to electrophoresis and Southern blotting, and hybridized with a ^{32}P-labeled *Bam*HI-*Sst*I fragment of pPUR. UD = undigested, 1, 5, and 10 c/h = 1, 5, and 10 copies of *Bam*HI- *Sst* I fragment per haploid genome.

regions to the *gusA* probe at the expected molecular weight regions. This indicates that at least one intact EcoRI- *Hin*dIII fragment of the pPUR plasmid had been integrated into the rice genome. Fragments smaller than 1.8 kb containing homologous regions to the probe in *Sst*I, *Sst*I/*Sma* I, or in all digestions involving *Eco*RI indicated the presence of a truncated *gusA* gene in the genomic DNA (the length of the *gusA* coding sequence is 1.8 kb). In addition, it appeared that rearrangements of the fragments as well as multiple insertions had occurred, since as many as five hybridization bands could be detected in some restriction digestions (*Eco*RI, *Eco* RI/ *Hin*dIII, *Bam*HI).

Our attempts to regenerate transgenic plants from kanamycin-resistant calli were unsuccessful. The kanamycin-resistant calli grew as undifferentiated cell masses in regeneration medium for 3-6 wk and then became necrotic (Fig. 3b). In rare cases, shoot-like structures developed, but they never grew into plants (Fig. 3c), and they eventually died.

G-418-resistant calli formed morphological structures resembling coleoptiles or shoots 3 wk after transfer to N6 medium (Fig. 3e). These structures developed into plantlets when transferred to MS medium containing no plant hormones. After 5 wk in MS medium (Fig. 3f), these plantlets were readily transplanted into soil and grown to maturity in environmental chambers.

Southern blot analysis of DNA isolated from leaf blades of seven plants, using the *Bam* HI fragment of the plasmid pKAN (Fig. 1) as the probe, confirmed the presence of the *neo* gene in the plants (Fig. 6). Four of the plants had a 2.2-kb band corresponding to the intact *neo* gene coding sequence, and all had bands corresponding to molecular



6. Southern blot of DNA isolated from leaf blades of plants 1 mo after transplanting to soil. Ten micrograms of leaf DNA digested with *Bam*HI from putatively transformed plants was loaded (lanes 1-7). Probe used was the *Bam*HI fragment of pKAN. UT = DNA from an untransformed plant, R = *Bam*HI fragment of pKAN loaded at 5 copies per rice haploid genome.

weights higher than 2.2 kb. However, GUS assays and Southern blot analyses of DNA from these plants revealed neither GUS expression nor the presence of the *gusA* gene. All seven transgenic plants reached maturity (Fig. 7) but failed to set seed. Several additional G-418-resistant plants are currently being grown to maturity and will be evaluated for seed set and for the nature and complexity of gene insertions.

Discussion

Cell lines IR54-1 and IR54-2, previously used for plant regeneration from protoplasts (Lee et a1 1989), declined in regeneration ability after 1.5 yr in culture. New cell suspension cultures were initiated from callus derived from immature embryos, and the results presented here provide evidence that protoplasts isolated from two newly established cell lines, IR54-5 and IR54-6, had similar plating efficiencies as the previous lines (Lee et al 1989) and were capable of regenerating plants. Thus, the declining regeneration potential of protoplasts obtained from suspension cultures as they become older is an ongoing problem that can be overcome by initiating new suspension cultures from primary callus of immature embryos. Because of the loss of totipotency of cell lines over time, a suitable cryopreservation method for regenerable rice lines, as described for regenerable maize lines (Shillito et al 1989), would be very useful.

The "donut" nurse culture setup permitted direct observation and scoring of the initial protoplast growth and cell division (Fig. 2b,c), which was difficult using the culture system that employed the Millipore filter on top of a nurse culture system that was used in most experiments. The "donut" setup would also be a useful technique for protoplast culture if nurse cells are required, but membrane filters can not be obtained.

7. Transgenic plants 70 d after transplanting.

The detrimental effects of kanamycin on plant regeneration from protoplast-derived calli reported here have been observed by others. Toriyama et al (1988) reported that calli selected on medium with kanamycin were not able to regenerate green plants but were able to do so when selected on medium with G-418. The fact that Zhang et al (1988) could not produce transgenic plants with kanamycin in the regeneration medium also indicates that kanamycin in some way interfered with plant regeneration processes. We also observed that G-418-resistant calli were capable of regenerating plants. Thus, either kanamycin or G-418 is effective for selection of transformed calli, but it appears that only G-418-selected calli of rice are capable of plant regeneration.

Co-transformation of the nonselectable *gusA* gene with the selectable *neo* gene was achieved in 25-30% of the kanamycin-resistant calli. This indicates the effective application of the co-transformation technique, which is in agreement with the results of Schocher et al (1986) for tobacco, Lyznik et al (1989) for maize, and Tagu et al (1988) for petunia. The *gusA* gene in one of the callus lines (Fig. 5) was found to be inserted into the genome intact, truncated, or in modified form. In the seven transgenic IR54 plants derived from co-transformation experiments and containing the *neo* gene, the *gusA* gene was absent. Shimamoto et al (1989) did obtain 1 transgenic japonica rice plant co-transformed with the *hph* and *gusA* genes. and Matsuki et al (1989) found that 2 of 26 *hph* transgenic plants were co-transformed with the *gusA* gene. However, in

another co-transformation study, Toriyama et al (1988) did not find the *gusA* gene in transgenic japonica rice plants, although the *neo* gene was present. In our study, it is not yet clear whether the lack of co-transformed plants was due to an insufficient number of plants studied, or to preferential regeneration of transformed as compared with co-transformed plants. This uncertainty should be clarified as more transgenic plants are produced during the next few months.

In summary, we have obtained transgenic indica rice plants from two experiments using a PEG-mediated transformation protocol in combination with techniques for developing cell suspensions, protoplast culture, G-418 selection, and plant regeneration. Stable co-transformation was obtained for the *neo* and *gusA* genes in callus, but transgenic plants contained only the *neo* gene. The *gusA* gene was integrated into the genome of callus cells intact as well as in truncated form or as rearrangements. Four of seven transgenic plants had the intact *neo* sequence as determined by Southern blot analysis. The transgenic plants looked morphologically healthy; however, they were sterile.

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Notes

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Efficient transformation of rice cells and production of transgenic rice plants

Y. Tada, M. Sakamoto, M. Matsuoka, and T. Fujimura

We developed a method for reproducibly obtaining transgenic rice at high frequency (10^{-4}) by electroporation. Co-transformation frequencies of the ß-glucuronidase *(GUS)* and hygromycin phosphotransferase *(HPT)* genes located on two separate plasmids were higher than 50%. Transgenic rice plants contained multiple copies of introduced genes integrated into their genomes in a complex manner. ß-glucuronidase enzyme activity by CaMV35S promoter was not proportional to gene copy number. Introduced *HPT* genes were detected and expressed in the progeny of transformants. The *GUS* gene linked to the rice light-harvesting chlorophyll a/b-binding protein (LHCP) promoter was also introduced into rice. Expression of the *GUS* gene with the LHCP promoter in transformed protoplasts and callus was much lower than that with the CaMV35S promoter. But in the leaf, the LHCP promoter was about 10 times more efficient than the CaMV35S promoter. *GUS* expression by the LHCP promoter was detected in leaves, stems, and glumes but not in roots.

Transgenic plants have been obtained by electroporation in Gramineae, including *Oryza sativa* (Shimamoto et al 1989, Toriyama et al 1988, Zhang et al 1988), *Zea mays* (Rhodes et al 1988), and *Dactylis glomerata* L. (Horn et al 1988), which are refractory to transformation by *Agrobacterium.* But the reported transformation frequencies of rice and the other Gramineae have been generally low, at 10^{-6}-10^{-5} transformants/electroporated cell (Dekeyseret al 1989, Hauptmann et al 1988, Shimamoto et al 1989, Toriyama et al 1988). We found high transformation frequencies of rice obtained by electroporation.

We introduced the ß-glucuronidase *(GUS)* gene linked to the CaMV35S promoter or the rice light-harvesting chlorophyll a/b-binding protein (LHCP) promoter, and the hygromycin phosphotransferase *(HPT)* gene linked to the CaMV35S promoter and analyzed the resultant transgenic rice plants. This is the first report of the introduction of an isolated rice promoter into rice.

Materials and methods

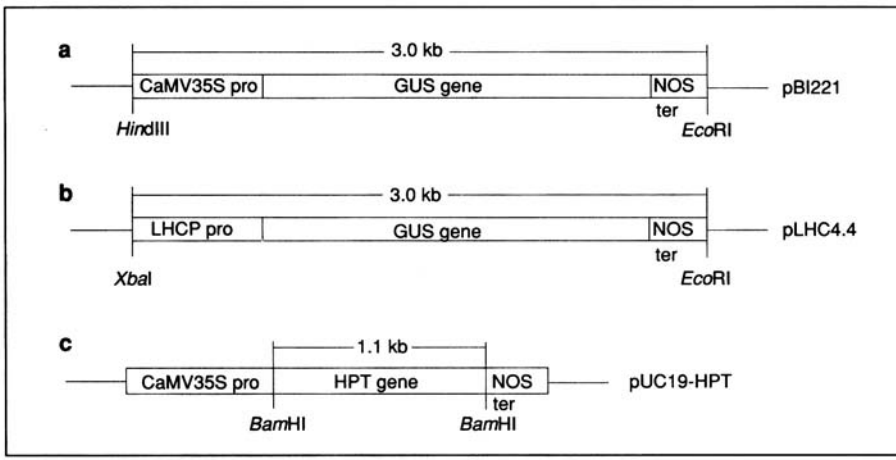
Protoplasts were isolated from cell suspensions of rice (Norin-8, Sasanishiki, or Nipponbare) and purified according to the method of Akagi et al (1989). Electroporated protoplasts were cultured as reported by Fujimura et al (1985).

Plasmids pBI221 (Fig. la) containing the *GUS* gene linked to the CaMV35S promoter (35S-GUS), pLHC4.4 (Fig. lb) containing the *GUS* gene linked to the rice LHCP promoter (LHCP-GUS), and pUC19-HPT (Fig. 1c) containing the *HPT* gene linked to the CaMV35S promoter (35s-HPT) were used.

Protoplasts (10⁶/ml) and plasmid DNA (0-10 µg/ml) were incubated on ice for 20 min and then transferred into an ice-cold electroporation chamber. An exponential electric pulse was applied to the chamber from a capacitor (880 µF). On the basis of preliminary transient expression experiments with the *GUS* gene (data not shown), an initial voltage of 475 V/cm with time decay constant ½T(defined here as time for decaying voltage to reach half of initial voltage) of 30.0 msec was chosen for all experiments described here. Electroporated protoplasts were incubated on ice for 20 min and collected by centrifugation. These protoplasts were resuspended at a density of 10⁶/ml in protoplast culture medium.

b-glucuronidase (GUS) activity was assayed in leaf extracts as described by Jefferson et al (1987). After 1 h incubation with the substrate 1 mM 4-methyl umbelliferyl glucuronide (4-MUG), the amount of product, 4-methylumbelliferon (4-MU), was determined by fluorometric assay. Expression of the *GUS* gene in callus or plant tissues was also detected by a staining method (Jefferson et al 1987) utilizing 5-bromo-4-chloro-3-indolyl- b-D-glucuronide (X-Gluc).

At day 14 of protoplast culture, hygromycin (50 =µg/ml) was added to the medium. After another 2 wk, the colonies were transferred to fresh medium containing



1. Plasmids pBI221 (a), pLHC4.4 (b), and pUC19-HPT (c). Only restriction sites used in this work are indicated.

hygromycin (50 µg/ml). After further culture, surviving colonies were transferred onto plant regeneration medium lacking hygromycin.

Plant DNA was extracted from leaves according to the method of Rogers and Bendich (1985). The purified DNA was digested with restriction enzymes, electrophoresed, and transferred onto nylon membranes. Hybridization was carried out using a nonradioactive, DIG-ELISA DNA labeling and detection kit (Boehringer Mannheim) or ^{32}P-labeled probes by random labeling and Hybond-N+ (Amersham).

Selfed seeds from each hygromycin-resistant plant were germinated under sterile conditions in hygromycin solution (20 µg/ml). After 10 d, surviving seedlings were counted and planted in soil.

Results

Transient and stable expressions of introduced genes were examined as follows:

Introduction of 35S-GUS or 35S-HPT gene

Initially, the GUS and *HPT* genes were introduced into rice protoplasts. After protoplast incubation for 48 h, the transient expression of the *GUS* gene was reproducibly detected (data not shown). Selecting for hygromycin resistance, we reproducibly got transformants at a high frequency (10^{-4}) (Table 1). Under our selection conditions, no colonies were formed after 2 mo of culture from protoplasts electroporated without DNA.

Co-transformation

The *GUS* and *HPT* genes were introduced simultaneously into protoplasts, and the resulting colonies were selected against hygromycin. Surviving colonies were assayed for *GUS* activity using X-Gluc to determine co-transformation frequency (Table 1). On the average, more than 50% of the hygromycin-resistant colonies exhibited GUS activity.

Table 1. Transformation and co-transformation frequencies of the *GUS* and *HPT* genes (5 wk after electroporation).

Experiment	Transformatior frequency[a]	pBI221 +pUC19 -HPT (µg/ml)	Hyg [Rb]	GUS+[c]	Co-transformation frequency (%)
1	1.33×10^{-4}	10+10	109	16	14.7
2	2.00×10^{-4}	10+10	12	8	66.7
3	Not tested	20+10	21	7	33.3
4	Not tested	20+10	175	76	43.4
5	2.57×10^{-4}	20+10	212	179	84.8
6	2.57×10^{-4}	20+10	36	20	55.6
7	2.62×10^{-4}	20+10	203	113	55.7

[a]Mean of 3 replications. [b]Hyg[A] = no. of hygrornycin-resistant colonies tested. [c]GUS+ = no. of colonies staining blue with X-Gluc.

Analysis of regenerated plants

One hundred eleven plants (23.0%) were regenerated independently from hygromycin-resistant calli (483). Leaves from each of these plants were tested for GUS activity by staining with X-Gluc, and 43 plants (38.7%) stained positively. All tissues examined from the 43 plants—roots, leaves, stems. glumes, and awns—stained positively. GUS activities were determined quantitatively by fluorometric assay of leaf extracts using 4-MUG (Table 2). GUS activities among nontransformant and non-stained plants were low (25-134 pmoles 4-MU/ min per mg protein), while those of stained plants were very high, though variable (1,497-10,531 pmoles 4-MU/min per mg protein).

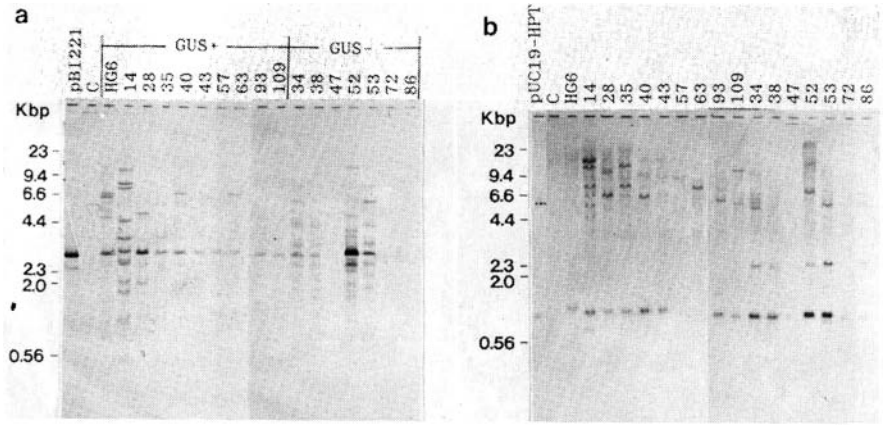
Total DNA was extracted from leaves of regenerated plants and digested with *Eco*RI/*Hind*III or *Bam*HI; and Southern blots were performed using the *GUS* or *HPT* structural gene sequences as probes (Fig. 2). Only HG43 displayed a single band corresponding to the intact CaMV35S promoter, the *GUS* structural gene, and the NOS terminator (3.0 kb). The other plants staining positively with X-Gluc contained multiple copies of the *GUS* gene, many not corresponding in restriction pattern to the initial gene construct. Of seven regenerated plants tested whose leaves did not stain positively with X-Gluc, four (HG34, HG38, HG52, and HGS3) nevertheless contained copies of the *GUS* gene.

The *HPT* gene displayed a complex integration pattern similar to that of the *GUS* gene. Most plants contained a 1.1-kb hybridizing fragment. which corresponded to the initial *HPT* gene construct. HG57 and HG63, however. contained only altered *HPT* gene sequences, which apparently expressed, as these were regenerated from hygromycin-resistant calli.

Table 2. GUS activity in leaf extracts from transgenic plants (35s-GUS).

Plant[a]	GUS activity (pmoles 4-MU/min per mg protein)	Plant	GUS activity (pmoles 4-MU/min per mg protein)
C[b]	134	HG36	4308
HG3	30	HG40	3093
HG47	25	HG42	3578
HG73	36	HG43	6842
HG100	107	HG57	5650
HG6	7154	HG63	98940
HG8	1921	HG67	4127
HG9	4509	HG68	2281
HG13	5639	HG75	6457
HG14	1497	HG78	1788
HG15	5798	HG81	7957
HG19	7005	HG93	10531
HG20	5874	HG105	3563
HG24	2559	HG107	4451
HG25	4033	HG109	6516
HG32	5430	HG111	7695
HG35	4154		

[a]Leaves of first 5 plants listed did not stain perceptibly with X-Gluc. The remaining plants all stained.
[b]Nontransformant.



2. Southern blot analysis of DNA extracted from plants transformed with pBI221 and pUC19-HPT. Numbers refer to individual regenerated plants. a) Total DNAs (5 µg) digested with *Eco*RI and *Hin*dIII were probed with GUS structural gene. X-Gluc stainable (GUS+) and nonstainable (GUS-) plants were used. Lane C = nontransformant. b) Total DNAs (5 pg) digested with *Bam*HI were probed with *HPT* structural gene. Lane C = nontransformant.

Although an accurate determination of introduced gene copy number cannot be made from this experiment, clearly GUS activity levels did not correspond simply to the copy numbers of foreign genes maintained in regenerated plant genomes. This lack of correspondence is demonstrated most clearly for regenerant HG93 (compare Table 2 with Fig. 2a).

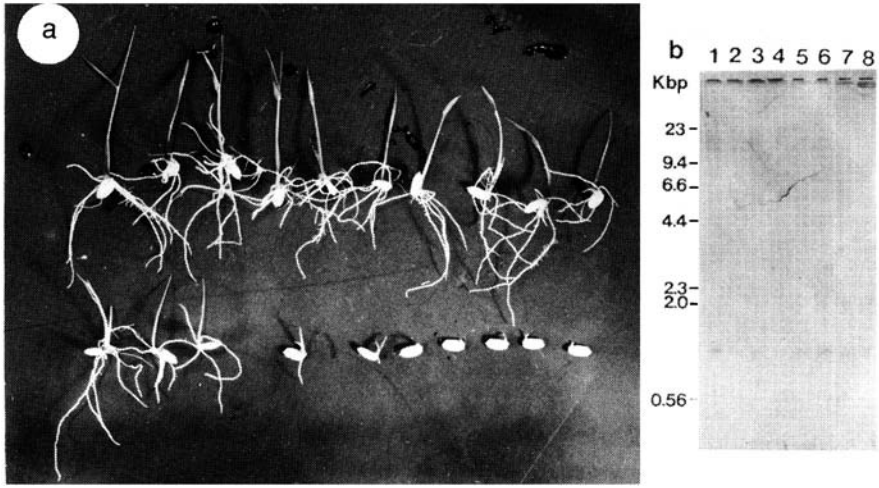
Transmission of expressed *HPT* gene among progeny

Selfed seeds from two hygromycin-resistant plants (S-1 and S-7) were germinated in hygromycin solution for 10d. Under this condition, radicles of nonresistant germinating seeds elongated very little and then died within 2 wk (Fig. 3a). The approximate ratios of the segregation of hygromycin resistance among the selfed seedlings of both transformants were determined (Table 3). DNAs isolated from leaves of S-7 and its hygromycin-resistant progeny were subjected to Southern blot analysis (Fig. 3b). A 1.1-kb fragment hybridizing with the *HPT* gene probes was observed in S-7 and all resistant progeny. These results demonstrate stable transmission and expression of the introduced *HPT* gene among transformant progeny.

Expression of LHCP-GUS gene

pLHC4.4 and pUC19-HPT were also co-transformed into rice. Transformation frequencies were determined by selection with hygromycin. They were 1.81-3.29 × 10^{-4} based upon initial electroporated protoplasts.

GUS expression in plants regenerated from randomly selected hygromycin-resistant calli was detected by leaf staining using X-Gluc. Fourteen positive plants were independently regenerated. In these plants, leaves, stems, and glumes stained positively, but roots did not. The GUS activities of leaf extracts from some of the plants,



3. Transmission of *HPT* gene to progeny. a) Seedling resistance to hygromycin. Selfed seeds of the hygromycin-resistant transformant S-7 were germinated in solution of 20 µg hygromycin/ml for 10 d. Figure shows 14 resistant and 6 sensitive seedlings. b) Southern blot analysis of DNAs from S-7 and its progeny. Total DNAs (5 µg) digested with *Bam*HI were probed with HPT structural gene. Lane 1 = S-7, lanes 2-8 = hygromycin-resistant progeny of S-7.

Table 3. Transmission of hygromycin resistance to progeny.

Seed	Seeds tested (no.)	Resistant seeds (no.)	Sensitive seeds (no.)
S-1 selfed	20	17	3
S-7 selfed	40	29	11

determined using 4-MUG, were about 10 times that using the CaMV35S promoter (Table 4).

The total DNAs extracted from leaves of some plants staining with X-Gluc were digested with *Xba*I and *Eco*RI, and Southern blot analyses were performed (Fig. 4). All plants displayed a 3.0-kbp band corresponding to the LHCP promoter, the *GUS* structural gene, the NOS terminator, and some bands of other size. LH2 containing several copies of the intact *GUS* gene showed very weak GUS activity (Table 4).

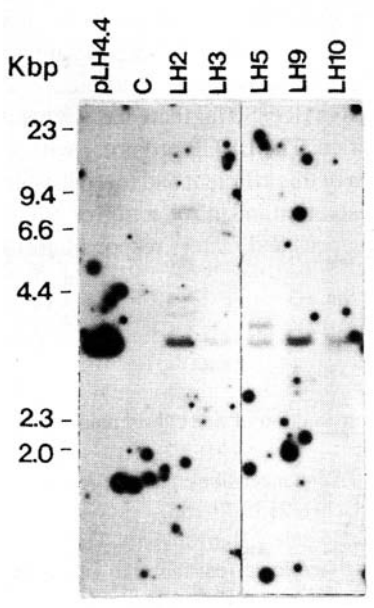
Discussion

We have reported efficient gene introduction into rice by electroporation. The frequency of plant regeneration from calli formed from electroporated protoplasts was as high as that from untreated protoplasts, and many fertile transgenic plants were obtained.

Table 4. GUS activity of leaf extracts from transgenic plants.

Plant	GUS activity (pmoles 4-MU/min per mg protein)
Control (nontransformant)	106
LH1[a]	68781
LH2[a]	516
LH3[a]	42423
LH4[a]	32138

[a]Transformed with LHCP-GUS.



4. Southern blot analysis of DNA extracted from plants transformed with pLHC4.4. Numbers refer to individual regenerated plants. Total DNAs (5 µg) digested with *Eco*RI and *Xba*I were probed with part of *GUS* structural gene. Lane C = nontransformant.

Co-transformation by electroporation has been reported by others (Shimamoto et al 1989, Wirtz et al 1987). In our work, some plants with both rearranged and apparently intact *GUS* sequences integrated in their genomes exhibited no *GUS* activity. This fact suggests that the actual co-transformation frequencies are higher than those in Table 1.

GUS gene expression under control of the CaMV35S promoter was detected in all organs of transgenic rice plants examined, in agreement with the report of Aoyagi et a1 (1988). Considerable differences in *GUS* activity were observed among transformants, perhaps reflecting position effects. In some cases, plants with apparently intact and multiple rearranged copies of the gene exhibited no activity above background: in other cases, plants with relatively few gene copies showed high expression levels. Disparities between gene copy numbers and expression levels were also reported by DeBlock et al (1987) and Streber and Willmitzer (1989).

In contrast to the report of Shimamoto et a1 (1989), we observed rather complex patterns of foreign gene integration into the genome. The reason for these differences is not apparent.

So far, foreign genes linked to the Cab promoters of pea (Simpson et a1 1986), wheat (Nagy et a1 1987), and petunia (Gidoni et al 1988) have been introduced into tobacco and expressed in a coordinated and tissue-specific manner in transgenic plants. In monocotyledons, these observations have not been extended, because no routine transformation system has been reported. Using our method, we found light-inducible and tissue-specific expression of the *GUS* gene linked to the rice LHCP promoter.

Dekeyser et a1 (1989) reported that the 2' promoter of octopine T-DNA was three to four times more efficient than the CaMV35S promoter. GUS activity under the LHCP promoter in leaves of transgenic plants (Table 4) was about 10 times that under the CaMV35S promoter, indicating that the LHCP promoter is the most efficient one that has been isolated, introduced into, and expressed in rice. So far there has not been a promoter capable of expressing foreign genes in rice. The LHCP promoter will be efficient for rice improvement. Practical exploitation of this efficient and reproducible transformation system for rice will depend upon the isolation and introduction of useful genes and upon a greater understanding of the sequences and factors responsible for tissue- and/or stage-specific expression.

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Notes

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Rice transformation, vectors, and expression of transferred genes

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S. Rueb, B.S. de Pater, and R.A. Schilperoort

During the last 3 yr, we have established technologies and acquired materials for genetic engineering of rice. Rice cell suspension cultures were established and used for protoplast isolation and direct DNA transformation. Hygromycin- or methotrexate-resistant rice calli were obtained, using constructs containing the genes for hygromycin or methotrexate resistance. Transformation was confirmed by Southern blot analysis. Regenerated transformed rice plantlets were obtained. The in vitro expression patterns of transferred genes were studied. *Agrobacterium* mediated transformation of rice cells, using young inflorescences and seedlings, is possible, although its applicability to genetic engineering must be evaluated further. Highly expressed tissue-specific and constitutive single-copy rice genes were isolated and characterized. One of these genes was subject to rearrangement in vivo and in vitro, suggesting the possible involvement of a transposon. The pollen tube pathway procedure for transformation of rice was repeated using our constructs; 276 flowers were treated. Ultimately, three plants with positive signals in Southern blot analysis were obtained.

Several japonica and indica varieties of rice (*Oryza sativa* L.) were tested as sources to initiate cell suspension cultures. The cultures were tested for protoplast isolation, regeneration of protoplasts into calli, and regeneration of calli into rice plantlets. After 6-9 mo of subculturing, only japonica varieties T309, Fujisaka 5, and Nipponbare displayed these desired traits, although the regeneration frequency was very low.

To preserve such useful cell lines, established procedures (Withers and King 1980) were optimized to store the lines in liquid nitrogen. In contrast to the cryopreserved cell lines, the original lines gradually lost their regenerative capacity upon subculture.

Cryopreserved rice cells remained viable for more than 18 mo. We expect that this period can be extended considerably.

Gene constructs

Regenerating rice protoplasts, cell suspensions, calli, germinating seeds, and 10-d-old seedlings were tested for susceptibility to antibiotics kanamycin, (G418, hygromycin, and methotrexate, and to the amino acid analogue homoarginine. Rice was found to be sensitive to hygromycin and methotrexate at moderate levels (20 and 0.5 µg/ml, respectively), and resistant to high levels of the other compounds tested.

We constructed vectors that could be used for both direct DNA transformation and *Agrobacterium*-mediated transformation. They were tested extensively in model plant systems using a range of *Agrobacterium* strains containing different wild type and helper Ti and Ri plasmids. Transgenic SR_1 tobacco plants, regenerated from transformed calli and containing 1-2 copies of transferred DNA, displayed high b-glucuronidase (GUS) activity (up to 70 nmole/min per mg extracted protein).

Direct DNA transformation of rice

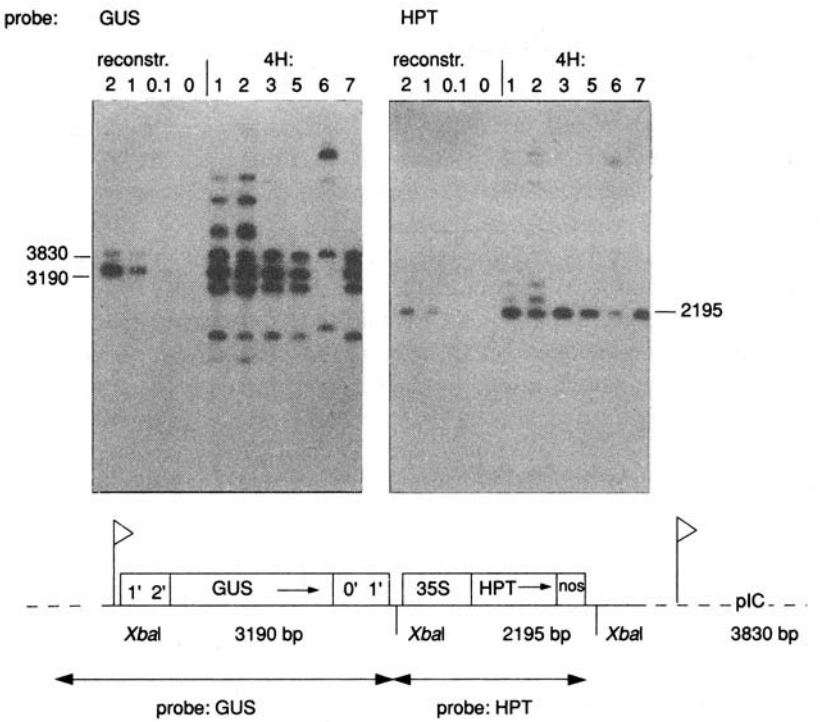
To obtain transgenic rice material, DNA was delivered to isolated rice protoplasts. In another experiment, DNA was delivered via the pollen tube pathway to sperm cells or egg cells during fertilization.

Transformation of rice protoplasts by electroporation and chemical procedures

In direct DNA transformation experiments, three constructs were used. Using the Leiden vectors HH271 and HH10mk, containing the hygromycin phosphotransferase (HPT) gene under control of the 35S CaMV promoter and the polyadenylation signal sequence of the nopaline synthase (NOS) gene (Van der Elzen et al 1985), hygromycin was used for selecting transformants. Methotrexate was used with the Monsanto vector pMON806 containing a dihydroxyfolate reductase (DHFR) gene under the same regulatory sequences as the HPT gene (Fig. 1,2).

Rice protoplasts were subjected to electroporation or a chemical procedure for direct DNA transformation (Krens et al 1982). The latter is less laborious, and more transformation experiments can be performed with samples from the same protoplast preparation. Both procedures consistently gave 15-20 transformants/10^6 treated rice protoplasts.

Optimal selection conditions (start of selection pressure and level of antibiotics used) were determined. We now routinely apply the first selection (50 µg hygromycin/ml, 0.5 µg methotrexate/ml) on day 10 after transformation. The selection pressure is increased to 100 µg hygromycin/ml after 24 d. Final selection is performed after 45 d (200 µg hygromycin/ml, 2.5 µg methotrexate/ml). Resistant rice calli survive on media containing 300 µg hygromycin/ml or 5 µg methotrexate/ml. The transformed state of the tissue was confirmed by Southern blot analysis and northern blot analysis of DNA and RNA extracted from cell suspensions initiated from the resistant calli (Fig. 1, 2). Intact resistance genes were present in all transformed tissues analyzed, showing that



1. Southern blot analysis of transgenic rice cell suspensions derived by chemical transformation of japonica (T309) protoplasts with HH271. For each cell line, 5 µg total DNA was digested with *Xba*I, electrophoresed, blotted onto Genescreen Plus, and hybridized. A pure HPT probe and a mixed *GUS*/pIC plasmid probe were used for analysis of hygromycin-resistant cell suspensions. reconstr. = 5 µg DNA isolated from nontransformed T309 suspensions, digested with *Xba*I and mixed with *Xba*I-digested HH271 plasmid DNA derived from a *dam Escherichia coli* strain. 2, 1, 0.1, or 0 copies of HH271 plasmid DNA were mixed per diploid T309 genome. m = marker. Sizes of hybridizing bands in bp are indicated. HH271 contains the reporter gene for GUS under control of the promoter of gene 2' and terminator sequence of gene 0. Genes 2', 0', and 1' are from the T_R-DNA of the octopine plasmid pTiAch5. In construct HH10mk the *GUS* gene is under control of the gene 1' promoter (box upstream of the *GUS* gene is inversed). Reading frames and regulatory sequences are indicated by boxes. Triangles indicate border sequences involved in the transfer of T-DNA from *Agrobacterium* to plant cells.

no false positive transformants escaped the selection procedure in the protoplast transformation experiments.

Expression of the HPT and DHFR selection genes gave rise to highly resistant calli. Northern blot analysis indicated high steady-state levels of their messenger RNAs (mRNAs), which are of the same magnitude as that found in transgenic tobacco plants grown under nonselective pressure. This indicates that the regulatory sequences of both genes work efficiently in the rice tissues analyzed (data not shown).

Intact *GUS* genes were present in the majority of the hygromycin-resistant tissues. However, the level of GUS mRNA was marginal, if any was detected. Clusters of 10-200 GUS-positive cells were sometimes observed in homogeneously transformed

callus by histochemical staining using X-Gluc (5-bromo-4-chloro-3-indolyl-b -D-glucuronide)(Rueb and Hensgens 1989). However, in 30% of the suspension cultures initiated from transformed callus tissue, GUS activity was present more consistently. The number of positive cells and the level of GUS activity were increased by adding 3×10^{-5} M 5-azacytidine to the medium. The maximum activity found thus far in transformed rice suspensions was 0.25 nmol/min per mg protein present in the extracts (Meijer et al 1991).

In contrast with the low and nonpersistent activity observed in in vitro tissues, rice plantlets regenerated from transformed tissues displayed high GUS activity in the leaves and roots, and especially in the trichomata and stomata (data not shown).

The observed reporter gene activity seems to depend on the developmental nature of the cells. This might be caused by the specific promoters (T_R genes 1' or 2') or terminator (T_R gene 0') used, although other factors cannot be excluded. Our observations indicate that an early selection for expression of introduced genes might lead to disposal of transformed material that could have expressed the desired trait upon regeneration.

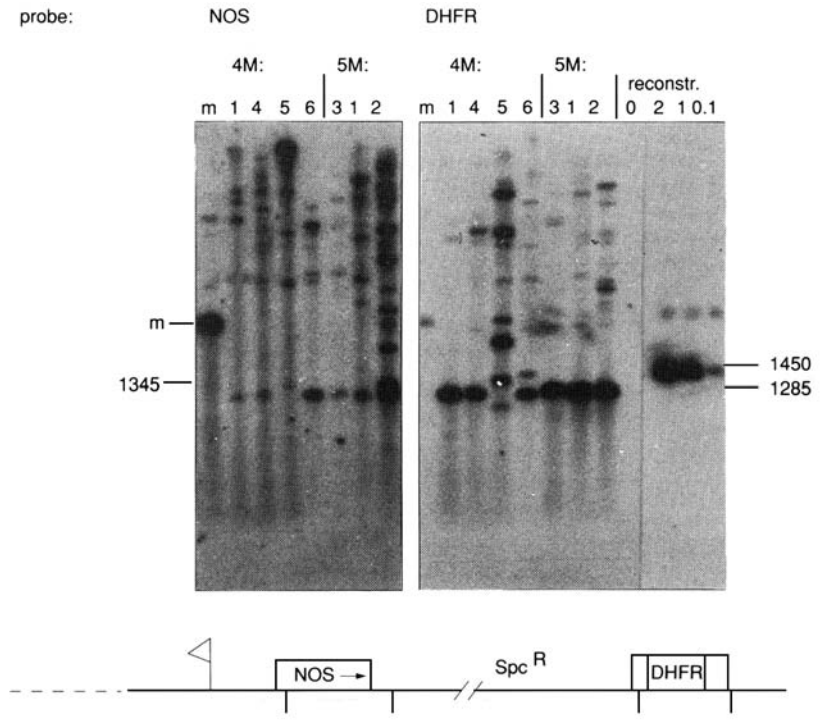
The high GUS activity observed in trichomata and stomata of regenerants indicates that the 2' promoter may be of potential use for the expression of resistance genes against diseases that occur by infection of these plant structures. Examples of these are bacterial leaf streak, narrow brown leaf spot, minute leaf and grain spot, sheath rot, and blast (Ou 1985).

Transformed rice tissues containing the pMON806 construct do not contain detectable NOS activity, nor detectable NOS mRNA. Southern blot analysis revealed that two ClaI-sites within the *NOS* gene are almost always methylated (Fig. 2), indicating inactivation by methylation. In this case the addition of 5-azacytidine did not lead to any detectable NOS activity or NOS mRNA.

Direct DNA transformation via the pollen tube pathway

Luo and Wu (1988) described the pollen tube procedure for the transformation of rice as simple and efficient. Using japonica variety T309 and the construct HH271 (GUS, HPT) in the plasmid pIC-20H, we repeated their experiment to compare it with other methods (see also Figure 1).

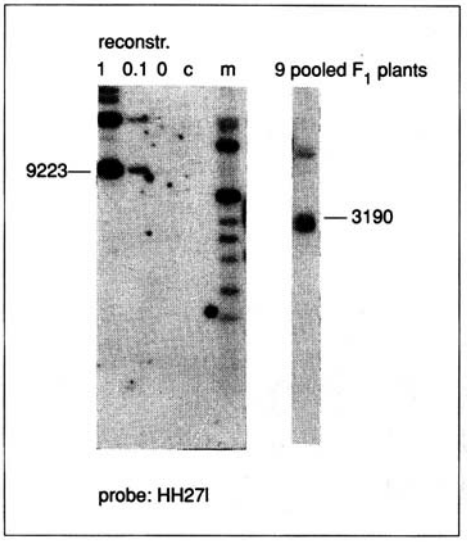
In total, 276 flowers were treated, 76 of which set seed that germinated. Leaves from 15 seedlings displayed 4-5 times more GUS activity than normally found in nontransformed rice seedlings. All 76 seedlings (defined as F_0 plants) were allowed to flower and form seed. Seeds from each plant were tested for GUS activity and the ability to germinate in the presence of hygromycin. Since the results were inconsistent, we analyzed the DNA from 12 F_0 parental plants by Southem blotting for the presence of foreign DNA. Two plants were positive, although no real discrete bands were observed. A third plant displayed a weak high molecular-weight band. DNA was isolated from pooled leaves of 9 F_1 plants derived from this third plant. DNA was digested with *Xba*I and analyzed by Southern blot analysis (Fig. 3). We concluded that the *GUS* gene is present intact and that bands corresponding to an intact *HPT* gene are



2. Southern blot analysis of transgenic rice cell suspensions derived by chemical transformation of japonica (T309) protoplasts with pMON806. For each cell line, 5 µg total DNA was digested with C*la*I, electrophoresed, blotted onto GenescreenPlus, and hybridized. The methotrexate-resistant suspensions were analyzed with a pure *NOS* and a pure *DHFR* probe (indicated by the horizontal bars). Note that these two probes share the *NOS* terminator sequences. The *DHFR* probe detects also the *NOS* gene band, although less efficiently. reconstr. = 5 µg control T309 DNA digested with C*la*1 and mixed with an isolated *DHFR* gene fragment (1450 base pairs). 2, 1, 0.1, and 0 copies per diploid T309 genome were mixed. m = marker. Sizes of hybridizing bands in bp are indicated. pMON806, from R. Fraley, Monsanto Co., St. Louis, MO, USA, contains the nopaline synthase gene itself (*NOS*) and the right border of the nopaline ti-DNA. Between the 2 genes, the bacterial gene coding for streptomycin resistance is present.

absent, thus explaining the hygromycin-sensitive phenotype of the F_1 seedlings. Histochemical staining using X-Gluc showed that six of these plants displayed very high GUS activities in the sheath pulvinus and lower parts of the leaf sheath and internode (data not shown).

It seems that, using this method, DNA indeed is integrated into the rice chromosomes and probably stably transmitted to the F_1. However, our data suggest that the original plants derived from the transformed egg cell were chimeric, because not all shoots of these plants were GUS positive, and only about 5% of the F_1 seedlings displayed GUS activity. In fact, no proof exists that the DNA transferred into the fertilized cell is integrated into the rice chromosomes at the unicellular phase.



3. Southern blot analysis of DNA extracted from pooled leaves of 9 F_1 rice plants derived from 1 F_0 plant transformed by plasmid HH271 via the pollen tube pathway. DNA was isolated according to Hensgens and Van Os-Ruygrok (1989). 5 µg DNA was digested with *Xba*I, separated on 0.8% agarose lxTBE gel, transferred to Genescreen-Plus, and hybridized with nicktranslated HH271. reconstr. = reconstruction in which 5 µg of non-transformed control T309 DNA was digested with *Xba*I and mixed with HH271 cut with *Bam*HI (gives the 9223 band) to give 1, 0.1, or 0 copies of HH271 per diploid T309 genome. m = marker DNA for size determination of fragments. Two hybridizing bands are visible in the pooled DNA. The 3190-bp band corresponds to the *Xba*I fragment containing the complete *GUS* gene (see also Figure 1). Note that plasmid DNA HH271 used in the transformation was methylated by the *dam* methylation system of *E. coli* on the first 2 *Xba*I sites.

Agrobacterium-mediated transformation of rice

Cells of monocots such as maize, asparagus, and narcissus can be transformed by *Agrobacterium,* as has been shown by sensitive biomonitoring systems like agroinfection (Grimsley et al 1986,1987) and by the detection of products produced by T-DNA-encoded enzymes (Graves and Goldman 1987, Hooykaas-Van Slogteren et al 1984). In agroinfection experiments, no selection for the transformed cell(s) is possible, thus limiting their application to genetic engineering.

We performed experiments using more conventional markers and showed that rice cells indeed can be transformed by *Agrobacterium.*

Inoculation of young seedlings

Six- to seven-day-old (2 cm high) rice seedlings were inoculated with different *Agrobacterium* strains to see whether the young meristematic tissue present within the coleoptiles could be used as a target for transformation and subsequent regeneration of transformed cells.

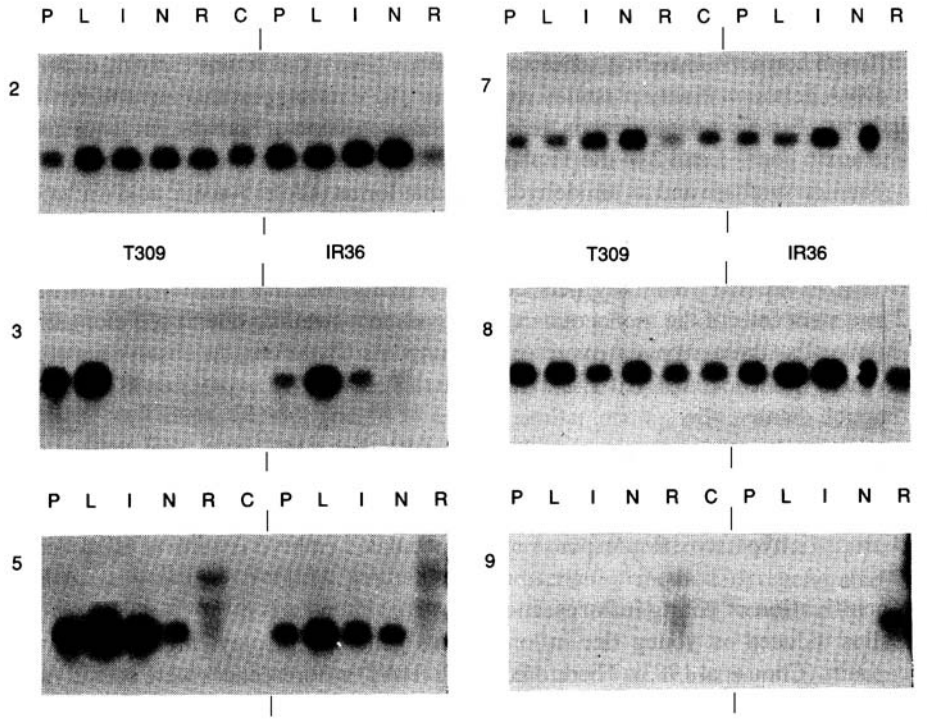
In a preliminary experiment we used deepwater variety Habiganj Aman I (HA I) seedlings and the *Agrobacterium rhizogenes* strain LBA9402. In some cases, roots with a phenotype similar to "hairy roots" developed from the inoculated wound that was made close to the coleorhiza just above the meristematic zone. Since this variety showed poor in vitro growth, experiments were repeated with japonica (T309) seedlings. With this variety, however, strong necrotic responses of the tissue surrounding the inoculation site were observed. To see whether necrosis could be prevented and roots or calli could be induced, the response of T309 seedlings inoculated with various

Agrobacterium strains was studied. Wild type strains, strains carrying mutations in different hormone-encoding genes and virulence genes, and strains carrying different T-DNA genes on binary plasmids were used. The data suggest that *Agrobacterium*-induced necrosis is dependent on the combined expression of both the virulence genes and auxin genes 1 and 2 of the Ti plasmid. These genes code for the enzymes indole acetamide synthase and an amidohydrolase that forms indole-3-acetic acid (Schroder et al 1984, Thomashow et al 1986, Van Onckelen et al 1986). Until now no data have been published indicating that auxin genes are expressed in Agrobacteria. P.J.J. Hooykaas (Leiden University, pers. comm.) demonstrated that transfer of genes 1 and 2 into stem cells of the monocot narcissus leads to a swelling due to cell elongation, showing that these genes are expressed in monocots. Therefore, the observed necrosis is probably due to transfer of T-DNA into the rice cells and its expression in these cells (unpubl. data).

Our results are in agreement with those of Raineri et al (1990), who showed that germinating rice embryos can be transformed by *Agrobacterium* and that this transformation is dependent on Ti-plasmid-encoded virulence genes. They also showed that a necrotic or hypersensitive response of the inoculated embryo is cultivar dependent.

Cocultivation of young inflorescences

Callus induced on young rice inflorescences generally displays high regenerative capacity (Chou et al 1983). Therefore, young (HAI) inflorescences were cocultivated with *Agrobacterium* LBA 4404 containing the binary plasmid HH27l carrying the HPT gene. In this procedure, panicles were harvested 10 d before tillering and cocultivated with agrobacteria (10^8 bacteria/ml) in minimal induction medium (Melchers et al 1989) for 30 min. Afterwards, the inflorescences were washed and incubated on different callus-inducing agar media (as described by Chou et al 1983 and Wang et al 1987). After 2 d, inflorescences were transferred to fresh medium containing cefotaxime at 100 µg/ml and vancomycine at 100 µg/ml to counterselect against agrobacteria. Furthermore, 10 µg hygromycin/ml was present to initiate a low level of selection for transformed rice cells. The selection pressure was raised stepwise, so that after 4 mo a final concentration of 100 µg hygromycin/ml was reached. Eighty-five hygromycin-resistant calli were tested for GUS activity by the histochemical assay. Twelve of these displayed a variable number of positive cells. For proof of transformation by DNA analysis, it was necessary to increase the biological mass by inducing cell suspensions from a number of these calli. Ultimately, three poorly growing suspensions were obtained. DNA analysis of this material showed that only one of these three lines contained DNA derived from the vector HH271 (data not shown). Unfortunately, the deepwater variety (HAI) we used showed poor in vitro growth, and the remaining nine hygromycin-resistant and GUS-positive calli could not be analyzed further. We are repeating this cocultivation experiment using young T309 inflorescences. We have shown that calli induced on the inflorescences of this cultivar retain their regenerative capacity during the first 4 mo of culture (unpubl. data).

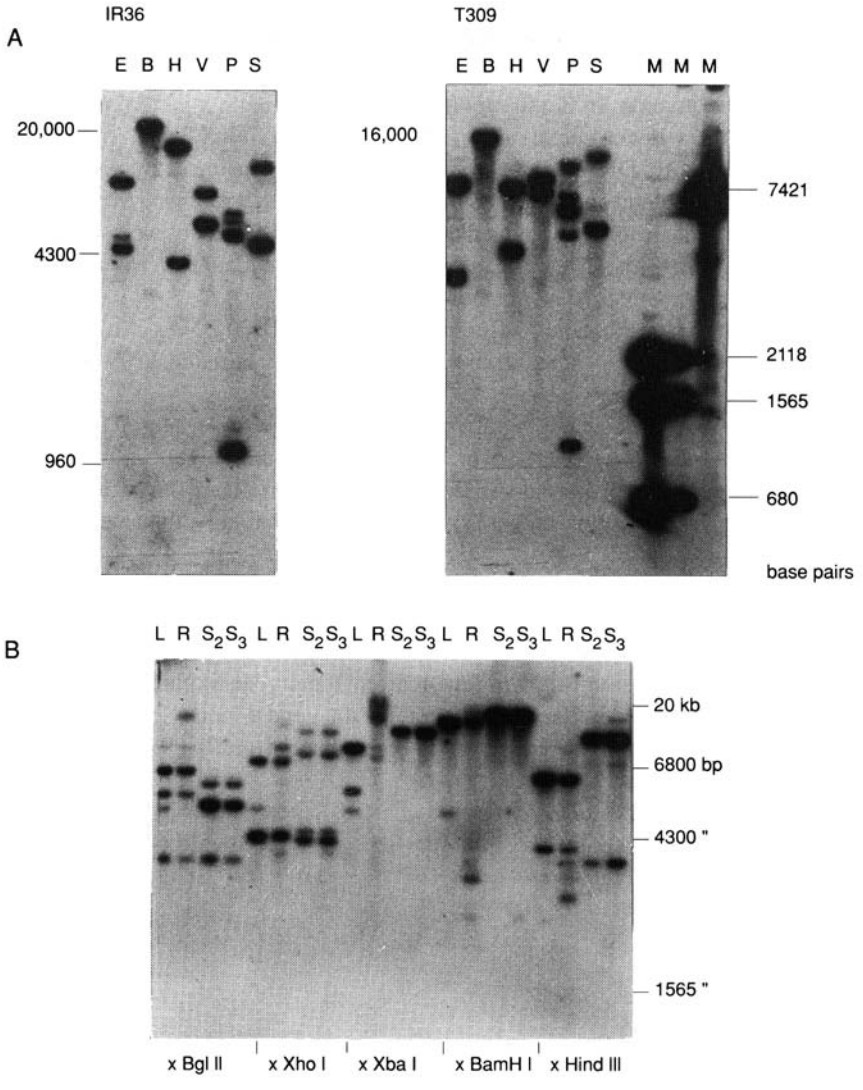


4. Tissue-specific and constitutive expression of 6 rice genes. Total RNA was isolated from panicles (P), leaves (L), internodes (I), nodes (N), and roots (R). Indica IR36 (right lanes) and japonica T309 (left lanes) plants were used. For T309, RNA was also isolated from cell suspensions (C). 5 µg RNA was separated on agarose gels, blotted onto Genescreen, and hybridized with the IR36 cDNA clones 2,7,3,8,5, and 9 labeled by nicktranslation.

Structure and expression of three low- or single-copy rice genes

For the practical use of rice genetic engineering, it is important to understand gene expression in rice as well as to have strong tissue-specific promoters and other regulatory sequences available. For this we have isolated and studied highly expressed tissue-specific single-copy genes from indica variety IR36.

By screening complementary DNA (cDNA) and genomic libraries, we obtained several rice genes that are expressed in a tissue-specific or constitutive manner. Three have been studied extensively. The complete nucleotide sequence of the cDNA and the genomic sequences have been determined (de Pater et al 1990). Transcription start sites were determined, and expression in the tissues was studied by northern blot analysis (Fig. 4). No homology was found with genes present in sequence data banks. The genes contain 2-4 introns and their mRNAs contain 100- to 300-nucleotide-long leader sequences. As a preliminary experiment, the three genes were transferred into tobacco to see whether the rice regulatory sequences were recognized in this dicot. No RNA



GENE 9 (RI)

5. Southern blot analysis of the root-specific gene *GOS9* in indica (IR36) and japonica (T309) plants and T309 cell suspensions. Total DNA was extracted from roots and green tissues of 10-d-old plantlets and from cell suspensions. 3 µg DNA was digested with *Eco*RI (E), *Bam*HI (B), *Hin*dIII (H), *Eco*RV (V), *Pst*I (P), or *Sac*I (S) (panel A). In panel B, results for 2 cell suspensions (S_2 and S_3), T309 leaves (L), and roots (R) are shown. Patterns of independently initiated suspensions were identical (S_3 was a gift from E.C. Cocking, University of Nottingham; S_2 was initiated at Leiden). After electrophoresis, DNA was blotted and hybridized with a ^{32}P-labeled cDNA clone in pBluescript corresponding to half of the second exon and the entire third exon of the gene. M = lambda DNA digests used as marker. For further details, see previous figures.

derived from the rice genes could be observed in the transformed tobacco tissue, thus hampering a detailed promoter analysis in this model plant.

In the analysis of the root-specific expressed rice gene 9. we encountered a very interesting phenomenon. In Southern blot analysis, the banding pattern observed for T309 plant DNA was completely different from the one found for IR36 plant DNA (Fig. 5). Furthermore, DNA isolated from T309 cell suspensions and that from T309 plants differed completely in hybridization pattern if cDNA 9 was used as a probe (Fig. 5). However, plant IR36 DNA and cell suspension T309 DNA showed identical hybridization patterns with cDNA 9 as the probe, and nonidentical patterns with cDNAs from four other rice genes (data not shown).

It is unlikely that the observed difference between the T309 DNAs is due to somatic variation, since the DNA isolated from four independently initiated T309 cell suspensions gave identical patterns.

The observed restriction length polymorphism may have been caused by the use of unpure batches of T309 seed. However, we think this is unlikely, because four independently isolated suspensions gave identical patterns. Similar differences have been observed with 15 restriction enzymes; it is therefore unlikely that methylation is involved.

If rice is not hemizygous for the gene 9 locus, its structure is different for both chromosomes between T309 and IR36 plant DNA and between T309 plant DNA and T309 cell suspension DNA. The data suggest a transpositional event within 20 kb of the gene. Gene conversion following the rearrangement can explain that both sister chromosomes display a similar organization for the gene 9 locus.

We are now comparing the cloned genomic sequences from T309 plant DNA, IR36 plant DNA, and T309 cell suspension DNA.

Recently, the function of the light-inducible rice gene 5 has become clear. The protein putative encoded by the rice gene has 86% homology with a 10.2-kDa photosystem I polypeptide from barley. The gene for this polypeptide has been designated as *psaH* (Okkels et al 1989).

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Evaluation of transformation techniques for monocots

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Since most cereal crop plants are not amenable to genetic transformation by Agrobacterium infection, efforts were made to design and optimize nonbacterial transformation techniques for monocots, using rice as a model system. In addition to the standard electroporation and polyethylene glycol-mediated protoplast transformation methods, attempts were made to transform embryogenic cells by liposome-mediated DNA introduction using lipofectin, a reagent capable of forming cationic-liposomes, and by microprojectile bombardment. Other methods—pollen tube and dry embryo soaking techniques—were analyzed and their potential for transformation assessed.

In the last decade the most significant advancement in crop improvement. complementing the conventional tools of plant breeding, has been the development and utilization of gene transfer technology. With these modern tools, genes of interest can be introduced stably into the plant genome, providing an important avenue to study gene regulation in transgenic plants. Moreover, this technology eliminates the barrier of sexual incompatibility to the genetic improvement of crop plants, because genes conferring important agronomic traits can now be isolated and transferred between unrelated plant species (Gasser and Fraley 1989). For stable introduction of foreign genes into plants. a very efficient system has been developed using the disarmed T-DNA plasmid of *Agrobacterium tumefaciens* (Hooykaas 1989). This approach, however, is limited to dicotyledonous plants. the normal hosts susceptible to this phytopathogen. Although a few noncereal monocot species have been reported to be amenable to this technique (Davey et al 1989), most are recalcitrant to *Agrobacterium* infection, and other methods must be employed. One such alternative approach combines cell culture techniques, i.e., the isolation and regeneration of protoplasts. with chemical and/or electrical shock methods of direct DNA uptake by these cells (Hain et al 1985, Lorz et al 1985, Yang et al 1988). This approach has been successfully used to obtain stably transformed, kanamycin-resistant plants of maize (Rhodes et al 1988) and rice (Shimamoto et al 1989, Toriyama et al 1988, Zhang and Wu 1988, Zhang et al 1988). Other more recent techniques for delivering exogenous DNA into

plant cells, especially into monocot species, include the use of high-velocity DNA-coated microprojectiles (Klein et al 1987, 1988; Mendel et al 1989; Wang et al 1988; Wu et al 1989), DNA imbibition of dry embryos (Topfer et al 1989), microinjection of DNA into single plant cells (Crossway et al 1986, Neuhaus et al 1987) or meristematic tissues (de la Pena et al 1987), and DNA introduction via the pollen tube pathway (Duan and Chen 1985, Luo and Wu 1988). Although all these non-*Agrobacterium*-mediated techniques have been successful to some extent in delivering foreign DNA into plants, no comparative study has been made of their routine application in producing transgenic monocots. Using as a model system rice, a species amenable to all these techniques, we present an assessment of the efficiency, ease, and reproducibility of these methods to deliver plasmid DNA into plant cells. The potential of these approaches in generating transgenic rice and other important monocots is also addressed.

Protoplast transformation

Young inflorescence tissues of several japonica rice lines were plated on Murashige and Skoog's (MS) medium (Murashige and Skoog 1962) containing 2 mg 2,4-dichlorophenoxyacetic acid/liter for callus induction. Among the lines tested—T309, Nipponbare, Minhekari, Calrose-76, and 77-170—77-170 yielded calli that maintained a relatively long duration (>2 yr) of totipotency. Suspension cultures were obtained by culturing embryogenic calli (compact, yellow. and opaque clusters) in AA_2 medium as suggested by Thompson et al (1986). In view of the documented sensitivity, ease, and versatility of assaying b-glucuronidase (GUS) enzyme activity in plant tissues (Jefferson et al 1987), *GUS* reporter gene-based vectors were used in all studies reported here. Protoplasts were transformed using 3 vectors containing the *GUS* gene under the control of the maize ADH promoter (1.7-kb fragment containing the 0.7-kb first intron), CaMV 35S promoter (0.8 kb), or rice glutelin gene (Okita et al 1989) promoter (1.8 kb). All three promoter fragments were able to drive *GUS* gene expression in rice protoplasts when introduced by either electroporation or polyethylene glycol (PEG) treatment. As judged by transient expression analyses, the CaMV 35S promoter was about five times as effective as the glutelin promoter. Transient expression of the *GUS* gene under the ADH promoter, though comparable to the CaMV 35S promoter, was dependent upon anaerobic conditions during protoplast culture.

For optimal DNA uptake by electroporation, 0.6-1.0 × $l0^6$ protoplasts were carefully suspended in electroporation buffer (as described by Fromm et al 1985, except that it contained 10% glucose instead of mannitol) and mixed with 50 µg plasmid DNA in 1.0 ml total volume. The protoplast solution was then subjected to an electrical pulse of 650 V/cm^2 using an old sparky type capacitor unit (Fromm et al 1985). The electric shock resulted in about 30-40% cell mortality. The basic protocol for PEG-mediated DNA uptake used in this study was essentially as described by Yang et al (1988). One ml of F medium containing 0.6-1.0 × 10^6 protoplasts was mixed with 50 µg plasmid DNA, followed by thedropwiseaddition of 1.2 ml of 40% (wt/vol)PEG-

4000 solution. After 30 min of incubation at 25 °C, the protoplasts were washed 4 times with modified F medium (Yang et al 1988) and twice with Kao's protoplast regeneration (KPR) medium (Thompson et al 1986). Direct comparison of the transient expression levels exhibited by protoplasts transformed with these vectors indicated that electroporation was at least twice as effective as PEG in facilitating DNA uptake. Similar observations on the effectiveness of electroporation over the PEG method have been reported by Yang et al (1988). In another approach, plasmid DNA was introduced into plant cells via cationic-liposome carriers prepared with lipofectin, an effective transfecting agent for animal cells (Felgner et al 1987). Rice cells (0.2×10^6 in 200 µl KPR medium) were incubated with a premixed solution of 25 µl lipofectin and 25 µl DNA (10 µg in sterile water) at 25 °C for 30 min. The protoplasts were then washed four times in KPR medium before culture in the same medium. Plant cells treated under these conditions exhibited about one-third the GUS activity of PEG-treated cells. A detailed study to further optimize the conditions for liposome-mediated DNA introduction into plant cells is in progress.

For plant regeneration studies, protoplasts were cultured in KPR medium (Thompson et al 1986) with 0.4% agarose. Our routine plating efficiency was 20-40% for control cells and 10-18% for electroporated protoplasts. The plating efficiency was found to be dependent mainly on the cell line, growth stage of the cell suspension when harvested for protoplasting, and physical characteristics (uniform size and dense cytoplasm) of the protoplasts. Protocalli obtained from the cultured protoplasts (4-5 wk) were tested for GUS activity by nondestructive enzyme assay (Gould and Smith 1989) and by histochemical staining (Jefferson et al 1987). Putatively transformed calli were cultured on regeneration medium, MSD-4 (Thompson et al 1986), or hormone-free MS medium to obtain plantlets. More than 50 plants obtained by this technique are presently being analyzed for spatial and tissue-specific expression and inheritance of the introduced gene.

Since this technique involves the transformation of plants starting from individual cells or protoplasts, it is the most reliable method so far for generating true transgenic plants rather than chimeras. The main difficulties in this approach are the establishment of a totipotent suspension culture and the elucidation of culture conditions for regeneration of transformed protoplasts into plants—tedious and time-consuming processes. Once the protoplast-to-plant system has been defined for the monocot species of interest, however, this technique appears to be the only presently feasible approach to obtain a sufficient number of transgenic plants for genetic analysis.

Introduction of DNA into plant cells by high-velocity microprojectiles

Highly embryogenic calli obtained from young inflorescences, young embryos, or anthers of rice (varieties T309 and 77-170) were bombarded with tungsten particles coated with the *GUS* reporter gene vectors described above. Despite efforts to optimize the conditions for DNA coating of the microprojectiles and their subsequent bombardment, as suggested by Klein et al (1988), the number of *GUS* expression units on the

rice callus plates was very poor (maximum 34). The number of expression units in different experiments varied perhaps because of the inability to obtain uniform dispersion patterns and precise targeting of the microprojectiles. A few potentially transformed calli exhibiting GUS activity were regenerated into plants, and their genetic analysis is under way. This technique was also utilized in unsuccessful attempts to introduce DNA vectors into highly embryogenic calli of wheat anthers. The failure with wheat was probably due to the very compact and brittle texture of the anther callus, which is apparently refractive to microprojectile entry. Studies are in progress to optimize the bombardment conditions and to alter the wheat callus texture so that it is more conducive to microprojectile entry.

Although the microprojectile-mediated DNA transfer technique suffers from some disadvantages, i.e., low transformation frequency and expensive instrumentation, it is relatively easy to perform and does not involve any tedious cell culture or protoplasting steps. This approach has already yielded some stable transformants in dicots (Christou et a1 1988, Klein et a1 1987, McCabe et al 1988) and could also be a promising approach for monocots, especially for those plants where a protoplast regeneration system is not available.

Pollen tube-mediated DNA introduction

In another attempt to transform rice and wheat plants, the pollen tube pathway technique described by Luo and Wu (1988) was employed. Stigmas of rice and wheat flowers pollinated for 6-8 h were cut with a sterile razor blade, and a DNA solution containing the *GUS* reporter gene was applied to the decapitated florets. Histochemical staining for GUS activity (Jefferson et a1 1987) in seeds obtained from the DNA-treated rice florets showed that in some instances seed tissues could be transfected with exogenous DNA. Approximately 10% of the seed samples exhibited GUS activity, but only in small patches distributed over the seed coat. Less than 1% of the seeds showed stain in the embryo region. The potentially transformed rice seeds are being analyzed for the stable integration of plasmid DNA in the F_1 and F_2. Despite several attempts, this technique has not been successful with wheat, as no-trace of GUS activity was evident in the embryo regions.

Thus it appears that this approach, though simple and easy, may not be applicable to all monocots. Although the pollen tube method does not involve tissue culture expertise or expensive instrumentation, the frequency of seed set on decapitated stigmas is very poor (~10%). Moreover, only about 10% of the progeny display any evidence of DNA uptake, as judged by histochemical staining methods, and an even smaller percentage of seeds exhibits *GUS* gene expression in embryonic tissues. The stable integration of DNA in these seeds is yet to be confirmed. However, if the DNA introduced by this technique were stably integrated and expressed faithfully in subsequent generations, it would be an inexpensive method for obtaining transgenic rice plants, especially with indicas and those varieties where protoplast regeneration is too cumbersome.

Introduction of foreign DNA into dry viable seed embryos

Embryos of dry seeds have deformed membranes (Webster and Leopold 1977) and are capable of importing foreign DNA (Hess 1969,1970; Ledoux and Huart 1969; Ledoux et al 1971, 1974; Soyfer 1980) Recently, Topfer et al (l989) demonstrated that DNA-soaked embryos of both monocots and dicots exhibited transient expression of an introduced *Npt* II gene. Based on DNA uptake by dry seed embryos, we assessed the utility of such a system for transforming rice, wheat, and barley seed embryos. Using the more sensitive and versatile *GUS* reporter gene, we observed that 20% dimethyl sulfoxide (DMSO, a membrane-permeabilizing agent recommended by Topfer et a1 1989) enhanced the ability of dry embryos to take up DNA and hence to express the *GUS* gene. The level and frequency of GUS activity observed, however, were highly variable in the DNA-imbibed seeds. Moreover, this chemical, at such high concentrations, was found to be deleterious to seed germination (unpubl. data). Lower concentrations (5-15%) of DMSO, though less toxic, showed hardly any effect on the capacity of dry embryos to take up DNA. To improve and optimize this technique, we tested the membrane-permeabilizing agents PEG, polybrene, and diethyl amino ethyl sephacel-dextran. PEG was the least toxic and most effective transfecting agent. With this improved technique, DNA-treated rice and barley embryos exhibited uniform expression in most embryonic tissues, as judged by histochemical staining of GUS activity (Jefferson et a1 1987). Although Topfer et al (l989) reported the expression of imbibed DNA in wheat embryos, we did not observe this phenomenon with the varieties we tested. We believe that DNA introduced into dry seed embryos with this simple procedure has the potential to be stably incorporated into the genomes of at least some embryo cells. Culture and regeneration of such transformed cells under appropriate selection conditions should result in true transgenic plants. Currently, we are optimizing the conditions for this technique so that it can be extended to other cereal crop plants.

Conclusions

We evaluated several approaches for introducing foreign DNA into plant cells and for assessing their feasibility for generating transgenic rice and other important cereal crop plants. It appears that, although laborious and time-consuming, obtaining plants from selected transformed protoplasts remains the best approach. The frequency of stable transformants obtained (0.01-0.02%; Yang et al 1988) can be further improved by optimizing the DNA entry (PEG, electroporation, liposome, etc.) and protoplast regeneration conditions. This technique has already proven successful in producing transgenic rice and maize plants (Davey et a1 1989). Since a system of protoplast regeneration to plants is now established for wheat (Harris et a1 1988), it should not be long before transformation of this important cereal crop can also be accomplished by this approach.

The use of DNA-coated microprojectiles in introducing foreign genes into plant cells has proven successful for obtaining stable transformants of a number of dicots.

A preliminary report on rice transformation (Wu et al 1989) suggests that this technique may have utility for monocots, especially for species that lack a well-defined protoplast regeneration system. The main shortcoming of the method is the very low frequency of transformation due to variability in the dispersion pattern and lack of control in targeting the microprojectiles. Introducing DNA via the pollen tube pathway, though straightforward, yields at best a low frequency of putative transformed embryos, and the technique may not be extendable to other important cereal crops like wheat. Another approach for obtaining transgenic monocots, where a protoplast regeneration system is not well defined, could be the dry embryo soaking technique (unpubl. data). Work is in progress to assess the frequency of stable transformants, if any, obtained by this procedure.

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Transgenic rice plants produced by direct uptake of the d-endotoxin protein gene from *Bacillus thuringiensis* into rice protoplasts

H. Yang, S.D. Guo, J.X. Li, X.J. Chen, and Y.L. Fan

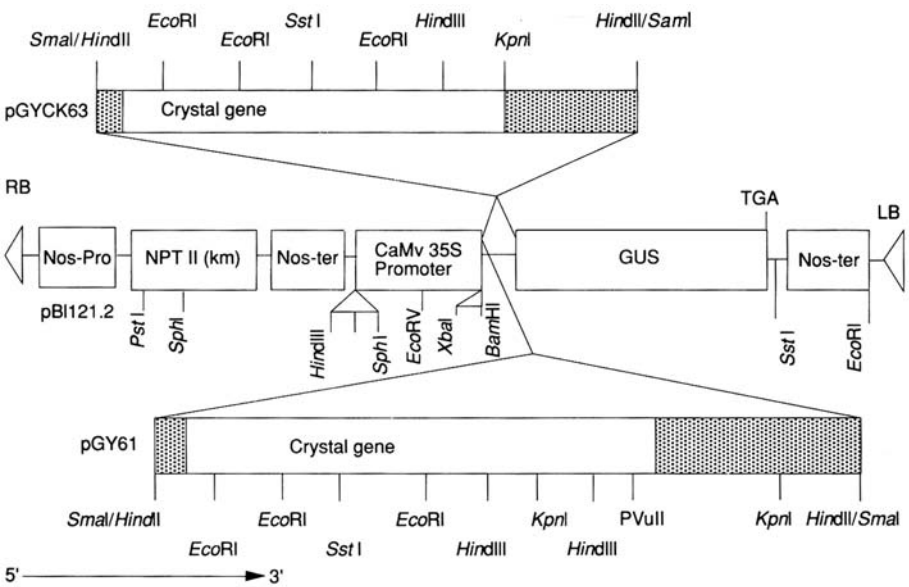
Transgenic rice plants containing the d-endotoxin protein gene from *Bacillus thuringiensis* were produced under laboratory and greenhouse conditions. Plasmids pGY61 and pGYCK63 carry a chimeric gene consisting of the d-endotoxin protein gene fused with the *GUS* gene and the NOS polyadenylation region. The GUS gene is downstream of the *B. thuringiensis* d-endotoxin protein gene, and shares the same promoter and terminator. Rice protoplasts were isolated from cell suspension culture. The plasmids were delivered into protoplasts by the polyethylene glycol method. The callus derived from the transformed protoplasts had high b-glucuronidase (GUS) activity. The *GUS* gene was expressed in the rice callus, suggesting that *B. thuringiensis* d-endotoxin protein gene was translated in the rice callus. Furthermore, green rice plants were regenerated from the protoplast-derived transformed callus, and the presence of the d-endotoxin protein gene in the rice genome was confirmed by DNA hybridization (Southern blot). In addition, high GUS activity was found in the transgenic rice plants, demonstrating that the *GUS* gene is expressed and providing evidence of the expression of the d-endotoxin protein gene in rice plants.

Rice, one of the most important crops in the world, is damaged by some Lepidopteran insects such as *Chilo suppressalis,* with great production losses. *Bacillus thuringiensis* is considered an effective agent for controlling these insects. In the last several years, d-endotoxin protein genes have been isolated, cloned, sequenced, and transferred in some dicots such as tobacco and tomato (Fischhoff 1987, Vaeck et al 1987) in the USA and in Belgium, Recently, transgenic rice plants containing genetic marker genes were obtained in several laboratories (Zhang and Wu 1988, Zhang et al 1988). An (1989) transferred the nitrate synthetase gene into rice cells. Until now, no work on transforming agriculturally important genes into rice protoplasts has been reported. This paper presents the transformation of the d-endotoxin protein gene from *B. thuringiensis* into rice protoplasts and the expression of this gene in rice plants.

Materials and methods

The following procedures were employed:

- *Subcloning of the* d*-endotoxin gene in the plant expression vector.* The d-endotoxin protein gene was isolated from *Bacillus thuringiensis* Aizawai 7-29. The complete structural 3.8-kb gene fragment and its 2.4-kb deletion gene fragment were inserted in plant expression vector pBI121.2 and fused with the *GUS* gene in the vector, resulting in recombinant plasmids pGY61 and pGYCK63, respectively (Fig. 1).
- *Establishment of rice cell suspension.* A cell suspension using variety Taipei 309 was established according to the method of Thompson (1986). The mature embryo was induced in embryogenic callus and shaken in AA2 liquid medium for 6 mo. The cell suspension was subcultured every week until the embryogenic cell suspension was obtained.
- *Isolation and culture of rice protoplasts.* Rice protoplasts were isolated by the method of Thompson (1986). Protoplasts thus isolated were stained with fluorescence diacetic acid (FDA). Cell survival and damage to cell membranes were observed under the fluorescence microscope. After heat shock, the cells were counted and embedded in Kao's protoplast regeneration (KPR) agarose medium with density of 3×10^5/ml and cultured at 28 °C in the dark.
- *Transformation of rice protoplasts.* Transformation of the d-endotoxin protein gene into rice protoplasts was carried out by the method of Krens (1982). The plasmid DNAs pGY61 and pGYCK63 were delivered into rice protoplasts at 20



1. Restriction map of recombinant plasmid.

μg DNA/10^6 protoplasts, and the plasmid-treated protoplasts were embedded in KPR agarose medium and cultured at 28 °C in the dark.

- *Detection of ß-glucuronidase activity in rice tissues.* According to Jefferson's (1987) method the callus and plants derived from plasmid-treated protoplasts were extracted in the extraction buffer and reacted with 4-methyl umbelliferyl glucuronide (Sigma M-9130). Then fluorescence of the reaction product was measured by fluorimeter under a 360-nm excitation spectrum and a 452-nm emission spectrum. The standard curve of μmoles 4-methyl umbelliferone (4Mu) (Sigma 1508) against fluorescence density was made under the same conditions. The corresponding product produced in the reaction buffer (that is ß-glucuronidase [GUS] activity of the specimen) was found from the standard curve.
- *Regeneration of the transformed rice protoplasts.* The protoplast-derived microcolonies grew to 1 mm in diameter and were transferred into differentiation medium (Abdullah et al 1986). One month later, shoots and roots came out, and the setups were transferred into the light. When the plantlets were 10 cm high, they were transferred to normal soil. The plants grew well if the temperature was kept at 28-32 °C and the relative humidity at 60-70%, and if the proper fertilizers were supplied.
- *Confirmation of transgenic rice plants.* Transgenic rice plants were confirmed by Southern (1975) blot. When the plants grew up, 1.5 g of leaves was used for DNA extraction according to the method of Dellaporta et a1 (1983). The plant DNA was digested with restriction enzyme *Hin*dIII. The 3.0-kb *Hin*dIII fragment of the δ-endotoxin protein gene was labeled with ^{32}P as a probe. DNA hybridization was done by the Southern method.

Results and discussion

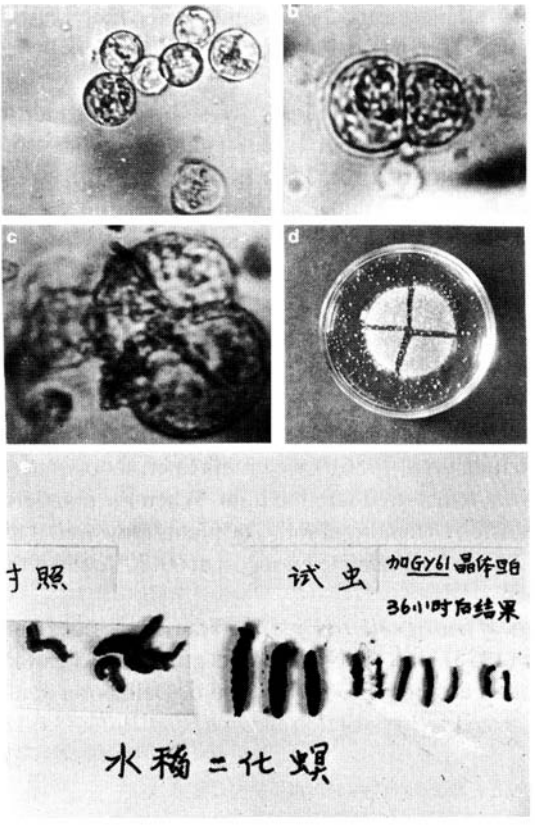
We transferred the *B. thuringiensis* insecticidal crystal protein gene into rice protoplasts and successfully obtained transgenic rice plants.

Isolation and culture of rice protoplasts

The rice protoplasts isolated from cell suspension culture (Fig. 2a) numbered 5×10^7/g fresh cells. More than 90% of the protoplasts were viable, as shown by staining with FDA. The purified protoplasts were embedded in KPR agarose medium. The first division was observed after 3-5 d of isolation (Fig. 2b). The second division of most cells was observed on the 7th to 10th day of isolation (Fig. 2c). Plating efficiency was about 1%.

Transformation of δ-endotoxin protein gene from *B. thuringiensis* into rice protoplasts

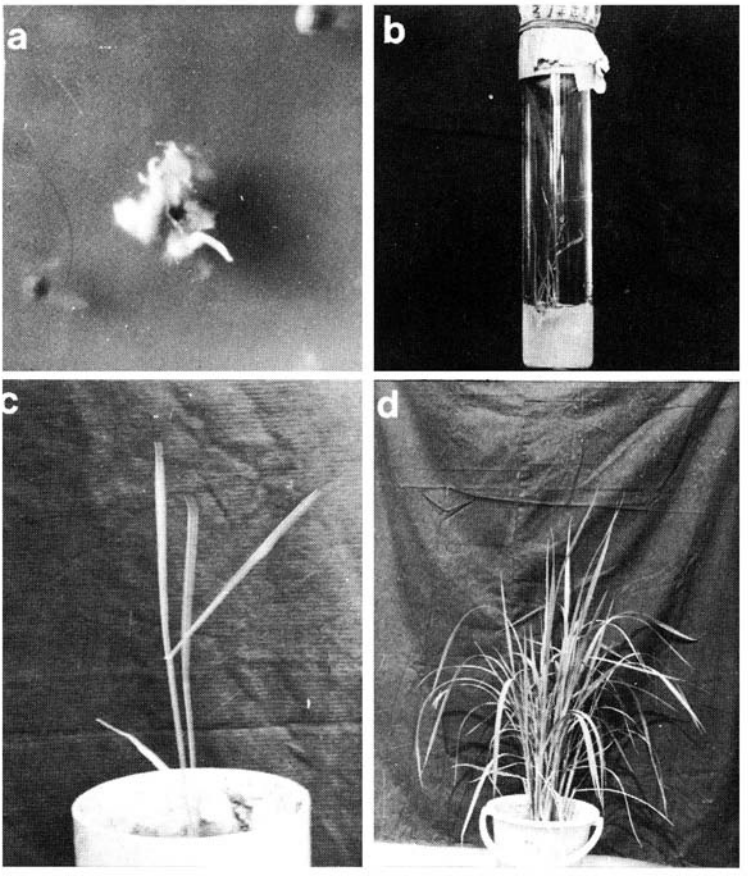
Recombinant plasmids pGY61 and pGYCK63 were transferred into rice protoplasts separately. pGY61 contained the complete δ-endotoxin protein gene fragment (3.8 kb),



2. a) Protoplasts isolated from rice suspension. b) First division of rice protoplasts. c) Second division of rice protoplasts. d) Microcolonies derived from rice protoplasts. e) Biotest of transformants of recombinant plasmid. (From Yang et al 1989)

and pGYCK63 contained the deleted d-endotoxin protein gene fragment (2.4 kb). In both plasmids, the d-endotoxin protein gene and the *GUS* gene were fused as a fusion gene. The *GUS* gene was downstream of the d-endotoxin protein gene. They shared the same CaMV 35s promoter and NOS terminator (Fig. 1). This construct offered a convenient way for detecting expression of the d-endotoxin protein gene in rice tissues. Bioassay of the two *Escherichia coli* clones (containing recombinant plasmids pGY61 and pGYCK63, respectively) for *C. suppressalis* showed their larvicidal activity against *C. suppressalis* (Fig. 2e).

The two plasmids were introduced into rice protoplasts by the polyethylene glycol method. Among the calli derived from treated protoplasts, 15 clones were selected randomly for detecting GUS activity. The GUS activity in 3 of 15 clones was 2120 pmoles 4-Mu/min per g fresh weight, whereas that of the control callus derived from untreated protoplasts was 60 moles 4-Mu/min per g fresh weight. The GUS activity of transformed callus was 15 times higher than that of the control, suggesting that the *GUS* gene was expressed in the transformed rice callus. The absolute transformation



3. a) Shoot and root differentiated from callus derived from rice protoplast. b) Small plantlet regenerated from transformed rice protoplast. c) Green plant seedling regenerated from plasmid-treated rice protoplast in soil. d) Green plant regenerated from transformed rice protoplast. (From Yang et al 1989)

frequency was 2×10^{-4} based on the number of protoplasts treated. The relative transformation frequency was 20% based on divided cells (microcolonies).

Regeneration of transformed rice protoplasts

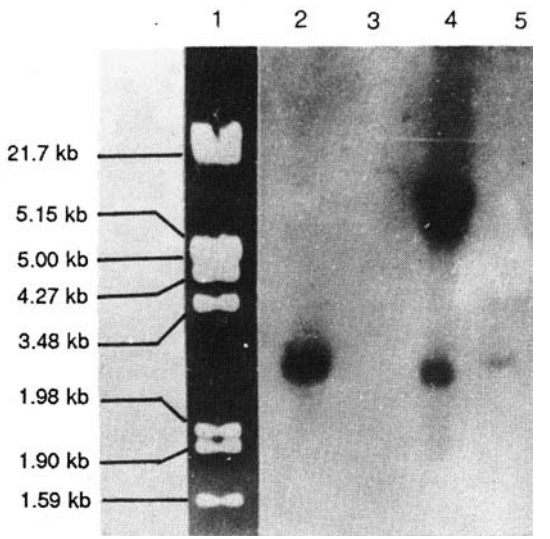
The microcolonies (Fig. 2d) derived from treated protoplasts differentiated shoots and roots (Fig. 3a). They developed green plantlets after being transferred to the light (Fig. 3b). When the plantlets grew to about 10 cm, they were transferred to normal soils (Fig. 3c). The plants grew well in the soil (Fig. 3d) after fertilizer application.

Confirmation of transgenic rice plants

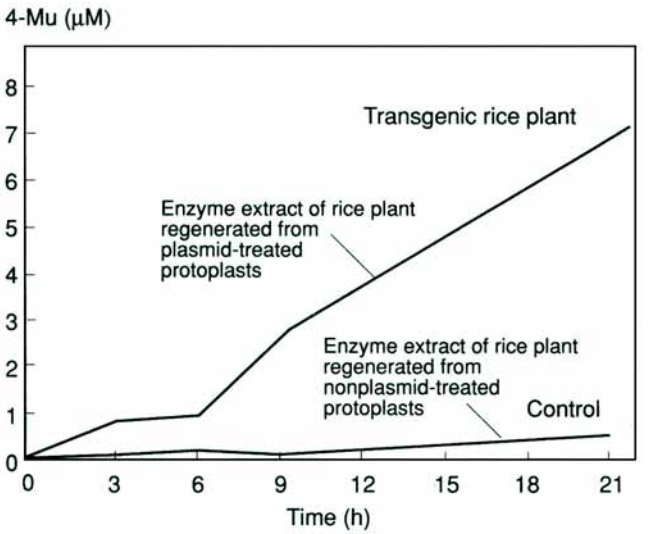
Transgenic rice plants were confirmed by Southern blot and analysis of GUS activity.

The plant DNA regenerated from plasmid-treated rice protoplasts was digested with *Hin*dIII and transferred into nitrocellulose membranes. The 3.0-kb *Hin*dIII fragment of the d-endotoxin protein gene was labeled with ^{32}P as a probe and hybridized with digested rice plant DNA (Fig. 4). Of 10 rice plants, 2 showed positive results,

suggesting that the d-endotoxin protein gene had been integrated in the rice genome. Furthermore, GUS activity was detected in plants regenerated from the plasmid-treated protoplasts. Among the two plants showing positive results in the Southern blot test,



4. Southern blot analysis of genomic DNA from transgenic rice plants. Lane 1 = l DNA digested with *Eco*RI/*Hin*d III, Lane 2 = DNA of pGYCK63 digested with *Hin*d III, Lane 3 = DNA from nontransformed protoplast-regenerated rice plant, Lanes 4, 5 = DNA of rice plants regenerated from plasmid-treated protoplasts.



5. GUS activity of transgenic rice plant.

one showed high GUS activity (Fig. 5). 4-Mu accumulation increased as the reaction time increased in the transformed rice plant derived from plasmid-treated protoplasts. On the other hand, the control plant regenerated from nonplasmid-treated protoplasts did not show any significant increase of 4-MU, suggesting that the *GUS* gene had been expressed in the transgenic rice plants. As mentioned, the *GUS* gene was fused with the d-endotoxin protein gene and downstream of the *B. thuringiensis* gene. The expression of the *GUS* gene offered evidence for the expression of the d-endotoxin protein gene in rice plants.

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Inheritance of a foreign gene in transgenic indica rice plants

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The efficient improvement of rice requires a system of transforming a desired cultivar with a defined foreign gene and obtaining the inheritance of that gene in the progeny. Direct gene transfer techniques that are potentially applicable to all plant species have been developed using a few dicot species, as well as rice (japonica) as model systems. However, genetic transformation of indica rice is not yet available, as this crop is recalcitrant in its tissue-culture response. We developed a protocol for efficient regeneration of fertile transgenic indica rice plants from protoplasts; they were transformed using a bacterial gene (*hph*) conferring resistance to hygromycin B under the control of the 35S promoter of cauliflower mosaic virus, and polyethylene glycol treatment. Transmission of the foreign gene in the progeny was confirmed by Southern blot analysis and enzyme assay. We also demonstrated the efficient splicing of dicot intron in rice. As any gene can be easily and efficiently cotransformed with a selectable marker gene, the way should be open now for the transfer of agronomically more interesting genes in indica rice.

Rice *Oryza sativa* L. is the world's most important crop (Khush 1984). Indica rice varieties feed more than 2 billion people, predominantly in Third World countries (Swaminathan 1982). Despite great efforts using a variety of approaches, it is still a problem to obtain fertile transgenic cereals (Potrykus 1990). Dividing cereal protoplasts from which plants can be regenerated are isolated from embryogenic cell cultures (Vasil 1988). Such cultures are normally derived from immature embryos. We have developed an embryogenic suspension culture from immature pollen grains (Datta et al 1990a) of indica rice Chinsurah Boro II. Plants can subsequently be regenerated from protoplasts of these cultures (Datta et al 1990b). Here we report transformation of indica rice and transmission of the transgene to the progeny.

Materials and methods

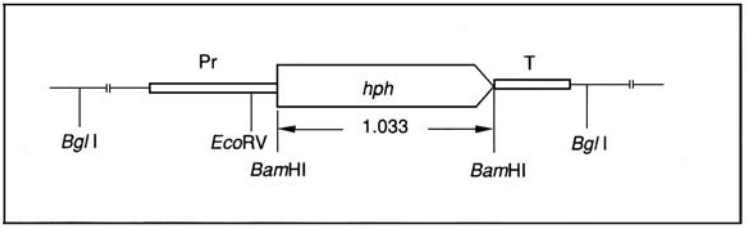
Experiments were carried out with indica rice variety Chinsurah Boro II (IRRI Act. 11484, supplied by the International Rice Research Institute, Philippines, and the Rice Research Station, Chinsurah, West Bengal, India). Growth of donor plants, culture of microspores, embryogenic cell suspensions, and protoplasts have been described earlier (Datta et al 1990a.b).

Plasmid construct

Plasmid pGL2 was obtained by cloning the *hph* gene as a *Bam*HI fragment derived from plasmid pGL88 (Blochinger and Diggelmann 1984) into the *Bam*HI site of plasmid pDH51 (Pietrzak et al 1986). The transforming DNA used for direct gene transfer was cut with *Bgl*I, releasing the fragment shown in Figure 1. Plasmid pGL2 contains a single *Eco*RV site in the promoter region of the gene. Carrier DNA was prepared by dissolving calf thymus DNA (Sigma) sheared to an average size of approximately 4 kb in water and filter sterilization.

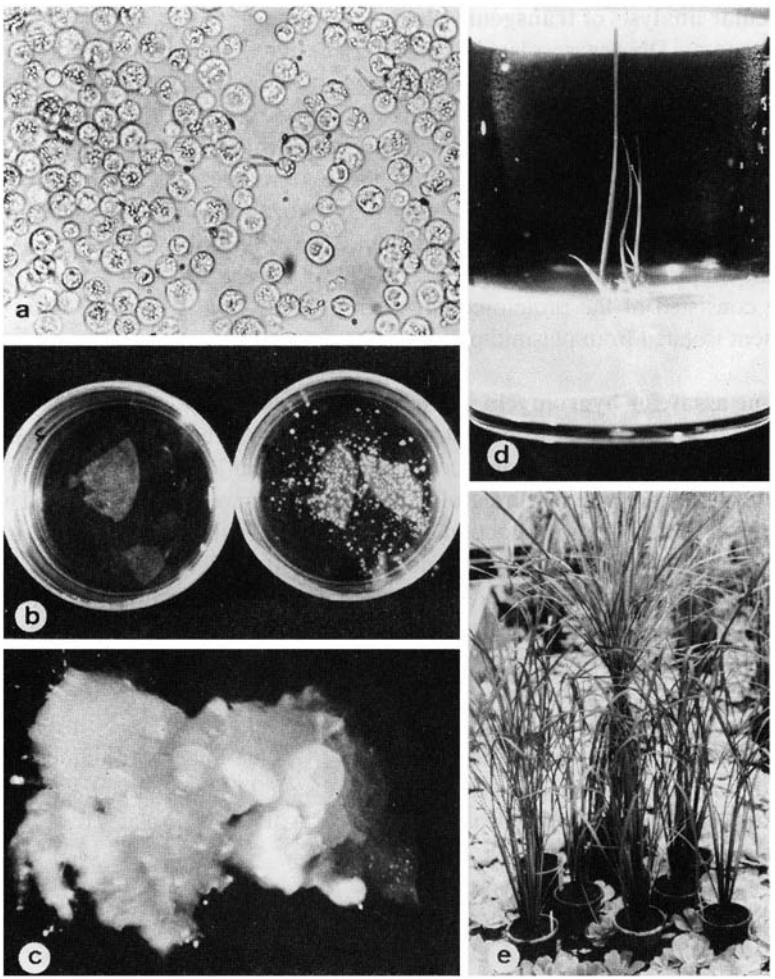
Protoplast culture, transformation, and production of transgenic plants

An embryogenic cell suspension (ECS) was obtained from dividing microspores and maintained for more than 1 yr under 24-h diffuse light (7 µE/m^2 per s at 80 rpm) in a medium described by Datta et a1 (1990a). The culture was composed of clusters of small, cytoplasm-rich cells and required subculturing every 5 d. Protoplasts were isolated from 5- to 10-mo-old ECS cultures as described by Datta et al (1990b). Aliquots of 0.4 ml of the protoplast suspension were mixed with *Bgl*I-digested pGL2 plasmid DNA (6 µg) and calf thymus carrier DNA (28 µg). Immediately after mixing the protoplasts with DNA, 0.4 ml of polyethyleneglycol (PEG) solution (molecular weight 6000, Merck, 40% wt/vol) was added dropwise, and the mixture of protoplasts, DNA, and PEG was incubated at 20 °C for 10 min (Datta et al 1990c). Protoplasts were then diluted with 8 ml of washing solution (0.4 M mannitol, 0.1% 2-morpholino-ethanesulfonic acid, pH 5.6) slowly and centrifuged to remove the PEG. They were cultured as described by Datta et al (1990c). At day 14 after protoplast isolation,



1. Plasmid construct. Part of plasmid pGL2 containing bacterial gene *hph* for hygromycin phosphotransferase under control of expression signals of 35S transcript of CaMV. For transformation experiments, plasmid pGL2 was cut with *Bgl*I. releasing the fragment shown. The fragment contains a single *Eco*RV site within the promoter (Pr) region. T = terminator (after Datta et al 1990c).

hygromycin B was added to the medium to a final concentration of 25 µg/ml. Further development of hygromycin-resistant colonies and plant regeneration through embryogenesis (Fig. 2b-e) were followed as described by Datta et al (1990c). Regenerants were transferred to potting compost and adjusted to greenhouse conditions. In the greenhouse, the plants grew to maturity and set seeds.



2. Regeneration of transgenic fertile indica rice plants from protoplasts. a) protoplasts isolated from 5-mo-old embryonic cell suspension; b) hygromycin-resistant clones, proliferating only from protoplast populations that were treated with plasmid pGL2 and PEG (right dish) and not in control treatment (left dish): c) resistant clone developing somatic embryos: d) differentiation of multiple shoots with roots in the light: e) one primary transgenic plant with seeds and several offspring thereof (partly from Datta et al 1990b, c).

Progeny test for resistance to hygromycin

Seeds derived from self-pollinated plants were surface-sterilized in 1.8% (vol/vol) sodium hypochlorite and washed extensively with sterile distilled water. Murashige and Skoog's (1962) (MS) medium without hormones and vitamins, either liquid or solidified with 0.8% agar, and containing 40 µg hygromycin B/ml, was used for seed germination. Seeds were incubated for 14 d under light (24 $\mu E/m^2$ per s) for a 16-h photoperiod at 24 °C.

Molecular analysis of transgenic plants

Total genomic DNA was isolated from leaf tissue of hygromycin-resistant and control plants. The leaves were freeze-dried and ground to powder in a mortar. DNA was extracted using the cetyltrimethylammonium bromide method (Murray and Thompson 1980). Three micrograms of genomic DNAs were digested with *Bam*HI or *Eco*RV restriction enzymes. Following electrophoresis through 0.8% agarose, DNA was transferred to Hybond-N nylon membranes. Hybridizations were done according to the instructions of the manufacturer (Amersham). The radioactive probe was prepared by the random primer method using (α-^{32}P)dATP (Feinberg and Vogelstein 1983). The probe consisted of the protein-coding region of the *hph* gene (1.033-kbp *Bam*HI fragment isolated from plasmid pGL2).

Enzyme assay for hygromycin phosphotransferase

The enzyme assay was carried out as described by Carbanes-Bastos et a1 (1989), modified according to a personal communication from R.D. Shillito (CIBA Geigy, Research Triangle Park, North Carolina, USA). Leaves were frozen in liquid nitrogen and ground in a mortar in extraction buffer (0.05 M Tris-HCl, pH 7.0, 10% glycerol, 0.1 mM phenylmethyl sulphonyl fluoride) at 100–200 mg tissue/100 µl in the presence of acid-washed sea sand at 4 °C. The samples were then centrifuged at 14,000 r/min for 5 min at 4 °C, and the supernatant was used for the reaction. The enzyme reactions were carried out in 10-ml volumes containing 50 mM Tris-maleate, pH 7.0,50 mM $CaCl_2$, 0.05 mM ATP, 0.4 ml (γ-^{32}P) ATP (10 mCi/ml; 3000 Ci/mmol), 62 µg hygromycin B, and 5.6 µl crude extract. Reactions were carried out with and without hygromycin. Incubation was for 30 min at 37 °C. One-microliter aliquots from the reaction mixtures were applied to a PEI-cellulose F TLC plate (Merck), which was developed in 50 mM sodium formate/formic acid at pH 5.4. The plates were dried prior to autoradiography.

Results

Reliable, efficient plant regeneration from protoplasts is possible only when an embryogenic cell suspension can be established and maintained.

Protoplast culture, transformation, and regeneration

Protoplasts were isolated from 5- to 12-mo-old cultures by incubation in an enzyme mixture that yielded an average 4×10^6 protoplasts/g of cell suspension culture (Fig.

Table 1. Recovery of transgenic indica rice plants from protoplasts treated with plasmid pGL2 and PEG.[a]

Experirnent no.	Antibiotic	Concen-tration (µg/ml)	Plasmid used	Protoplasts used (no. × 10^6)	Hygromycin-resistant clones (no.) at 28 d	Colonies tested (no.) for regene-ration	Plants regenerated (no.)	
							Green	Albino
1	Hygromycin	25	pGL2	6.5	200	60	nr	nr
2	Hygromycin	25	pGL2	8.0	350	280	73	16
3	Hygromycin	25	pGL2	8.5	116	22	4	nr
4	Hygromycin	25	pGL2	5.8	28	nt	nt	nt
C1	-	-	-	8.0	2260[b]	430	25	2
C2	Hygromycin	25	-	8.0	nr	nr	nr	nr

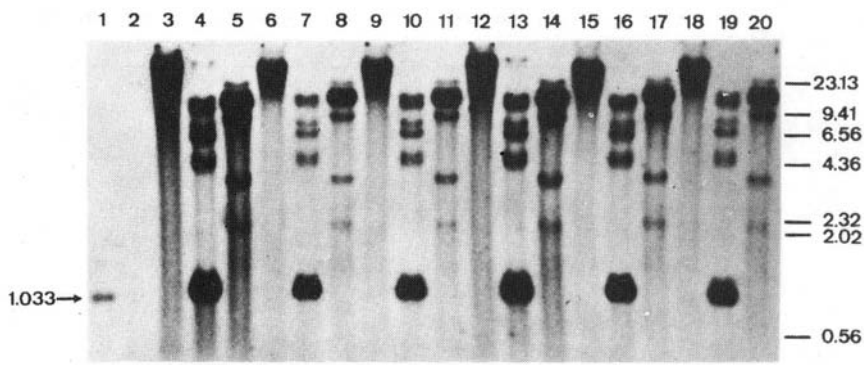
[a]nr = no response, nt = not tested, C1 = control without DNA and selection, C2 = control without DNA but with selection. [b]Number of colonies without selection.

2a). No undigested clumps of cells were detected in the protoplast preparations. However, a few spontaneously fused protoplasts were observed. Nurse cultures or feeder layers were not required for culturing isolated protoplasts, for division, or for plant regeneration (Fig. 2b-e). PEG-mediated transformation was performed as described under Materials and methods.

Treating the protoplasts with DNA and PEG 6000 for a short time (10 min) was important for a high frequency of protoplast survival, division, and subsequent plant regeneration. To allow for selection for transformed cell clones, plasmid pGL2, which carries the *hph* gene under the control of the 35s promoter and polyadenylation signal of CaMV, was used (Fig. 1). The functional *hph* gene confers hygromycin resistance to transformed cells. Hygromycin-resistant cell clones were transferred to semisolid N6 medium (Chu et al 1975) containing 25 µg hygromycin B/ml, 1 mg 2,4-dichlorophenoxyacetic acid/liter, and 0.3% agarose to allow for continued proliferation. No clones developed in the control samples (Fig. 2b, left dish). After 2-4 wk, compact embryogenic clones were transferred to modified MS culture medium without hygromycin B, and somatic embryos developed (Fig. 2c). All cultures to this point were kept in the dark. Transfer of somatic embryos to hormone-free, modified MS medium under light led to the outgrowth of multiple shoots and roots (Fig. 2d). Plants regenerated from hygromycin-resistant clones grew to maturity in the greenhouse and set seeds (Fig. 2e). They resembled control plants regenerated from untreated protoplasts or grown from seeds. Of 77 plantlets recovered, 24 were grown to maturity (Table 1).

Molecular data: Southern blot analysis

Total genomic DNA was isolated from 10 hygromycin-resistant primary regenerants and analyzed by Southern blot (Southern 1975). In all cases, hybridization with a radioactively labeled probe specific for the protein coding region of the *hph* gene demonstrated that this gene had integrated into the genome (data not shown). Figure 3 shows the Southern blot data for one representative primary transgenic plant (lanes

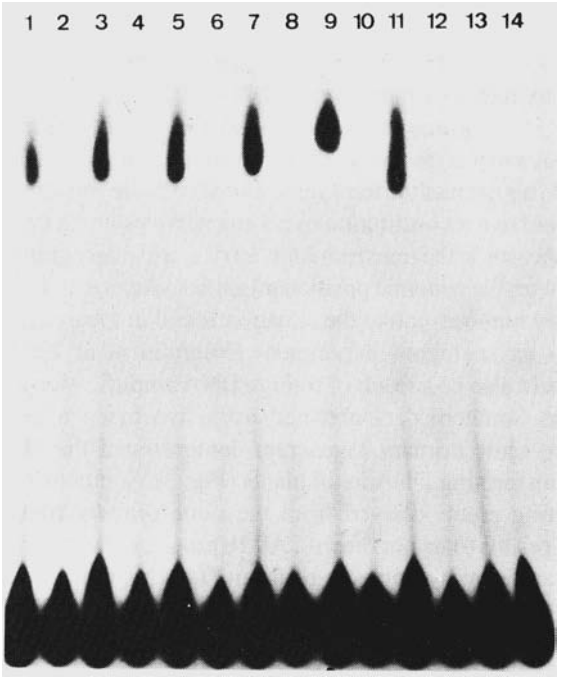


3. Southern blot analysis of transgenic plants and offspring. Southern blot data are presented for 1 representative primary transgenic plant (lanes 3-5), and 5 offspring of this plant (lanes 6-8, 9-11, 12-14, 15-17, 18-20). Lane 1 represents a 3-copy reconstruction of plasmid pGL2 cut with *Bam*HI; lane 2 contains DNA from a control rice plant, also cut with *Bam*HI. The samples are arranged in groups of 3 each. The first lane of each triplet (3, 6, 9, 12, 15, 18) contains undigested DNA, the second lane (4, 7, 10, 13, 16, 19) contains DNA restricted with *Bam*HI (releasing a 1.033-kb fragment characteristic for the protein-coding region of the gene; the third lane (5, 8, 11, 14, 17, 20) contains DNA restricted with *Eco*RV (yielding border fragments between transforming DNA and host DNA). The size markers in kilobase pairs are derived from lambda DNA digested with *Hin*dIII (Datta et al 1990c).

3-5), and for five independent offspring thereof (lanes 6-20). The integration pattern of the transgene in the offspring was identical to that of the parental plant. In all plants, hybridization of undigested DNA with the *hph*-specific probe showed that the transgene had integrated into the genome (Fig. 3, lanes 3, 6, 9, 12, 15, 18). After restriction digestion with *Bam*HI, the expected 1.033-kb fragment characteristic of the coding sequence of the *hph* gene was observed (Fig. 3, lanes 4, 7, 10, 13, 16, 19; compare with Fig. 1). Digestion with *Eco*RV, which cuts once within the plasmid pGL2, produced junction fragments within the transforming DNA or with the host genome (Fig. 3, lanes 5, 8, 11, 14, 17, 20). There was no hybridization with control material (Fig. 3, lane 2).

Enzyme assay

Evidence demonstrating the presence of the transforming DNA and resistance to hygromycin B was complemented by enzymatic proof that the gene was functional; specific phosphorylation of the antibiotic was observed with the use of protein extracts prepared from transgenic plants (Fig. 4). The plants taken for the enzyme assay were identical to those taken for the Southern blot analysis (Fig. 3). The assay for hygromycin phosphotransferase activity was positive for the primary transgenic regenerant (Fig. 4, lane 1) and the five offspring plants analyzed (Fig. 4, lanes 3,5,7,9,11). The enzymatic evidence was also confirmed by the growth of (selfed) seedlings derived from transgenic plants of 40 µg hygromycin/ml. They all developed into healthy plants under these conditions. So far, 31 seeds derived from 5 primary transgenic plants tested have all been found hygromycin-resistant, whereas 42 seeds from protoplast-derived control plants were sensitive. Control plants germinated poorly, developed brown roots, ceased to grow, and died.



4. Enzyme assay for hygromycin phosphotransferase. Plants taken for the enzyme assay were identical to those taken for Southern blot analysis shown in Figure 3. Autoradiogram shows labeled ATP at the start in all lanes and labeled hygromycin B in lanes 1, 3, 5, 7, 9, and 11. Even numbers represent enzyme reactions carried out without hygromycin B: odd numbers represent reaction in presence of hygromycin B. Lanes 1 and 2 contain crude protein extract derived from primary transgenic plant; lanes 3-12 contain extracts derived from 5 offspring of same primary plant: lanes 13 and 14 contain extracts isolated from untransformed control rice plant (Datta et al 1990c).

Discussion

Significant progress has been made in direct gene transfer in japonica rice. Transformation of this crop has been made by electroporation (Shimamoto et al 1989, Toriyama et al 1988, Zhang et al 1988) and by PEG (Zhang and Wu 1988). Dicot intron splices efficiently in rice (Peterhans et al 1990). We have demonstrated that rice plants (both japonica and indica) can be obtained from protoplasts without any nurse or feeder cultures, in contrast to earlier reports (Lee et al 1989, Shimamoto et al 1989). Moreover, nurse culture often reduces the growth of the transformed colonies (Zhang et al 1989). Heat-shock treatment (5 min at 45 °C) applied to the recipient protoplasts prior to addition of the plasmid followed by PEG, reported to be beneficial for rice transformation by Zhang and Wu (1988) and Zhang et al (1988), did not improve the transformation frequencies in our experiments with indica rice (data not shown). Microspore-derived embryogenic cell suspensions are, in our experience, a good source for reproducible production of transgenic colonies (Table 1). Transgenic plants obtained from protoplast-derived clones via somatic embryogenesis resembled seed-derived plants.

Southern blot data are presented for one representative primary transgenic rice plant and five offspring of this plant. The data demonstrate integration of the transforming plasmid DNA into high-molecular-weight DNA, presence of the expected 1.033-kb *Bam*HI fragment in the primary transgenic plant and offspring, and hybrid fragments between rice DNA and transforming DNA. From the comparison of the intensity of the 1.033-kb *Bam*HI fragment of the transgenic plants with the corresponding fragment of the control plasmid in a reconstitution experiment, we estimate that 50-100 copies of the plasmid are present in the transformants. In rice, weak recognition of the promoter sequence or unfavorable genomic position might be compensated for by accumulation of high gene-copy number, unlike the situation found in tobacco, which had a lower copy number in an analogous experiment (Negrutiu et al 1987). However, this accumulation could also be a result of foreign DNA amplification after its integration into the genome. Southern data obtained from five independent offspring plants derived from the same primary regenerant demonstrated the identical integration pattern of the transforming DNA in all plants (Fig. 3). Southern blot analysis of five additional offspring plants derived from the same primary transformant revealed exactly the same results (data not shown). All 10 progeny plants derived from the same primary regenerant were hygromycin resistant. Two hypothesis could explain these data, the first on the basis of a hemizygous primary transformant having integrated the transforming DNA into two or more independent genomic loci as described for tobacco (Potrykus et al 1985). In this case, however, a genotypic segregation of the hybridizing bands resolved on the Southern blots should be expected in the offspring plants, but was not observed (Fig. 3). Alternatively, since the starting material for protoplast isolation and transformation consisted of a microspore-derived cell suspension, we suggest that the fertile primary transgenic plants were homozygous, i.e., spontaneous diploidization of the protoplast-derived clones occurred after DNA integration into the genome. The lack of segregation of the integration pattern of the foreign DNA in the offspring indicates that the primary transformant is indeed homozygous. Therefore, information concerning the number of independent genomic integration sites cannot be obtained from analyzing the first self-pollinated R_1 progeny. Further genetic analyses of the transgenic plants should clarify the number of genomic loci for the transgene.

Resistance to hygromycin B is based on inactivation of the antibiotic via the transfer of the g-phosphate from ATP (Blochinger and Diggelmann 1984). The enzyme activity was absent in control rice tissue and present in both transformed leaves of the primary regenerant described here and in progeny plants thereof (Fig. 3). These data show that these plants not only carry the transgene but also express it.

The splicing mechanism in higher plants has not been investigated in great detail. Moreover, because of the difficulty of transformation in monocot species, expression studies of most plant genes and intron splicing have been carried out in transgenic dicots. Fortunately, the most important monocot crop species, rice, can now be used for this kind of research. A construct pAH containing the npt II coding region fused to the CaMV promoter and polyadenylation signals and interrupted by a modified intron 3 sequence of the soybean phaseolin gene has been used for the study of intron splicing

in rice (Peterhans et al 1990, Slighton et al 1983). Transformation was carried out using protoplasts isolated from ECS of microspore origin of japonica rice variety Yamabiko treated with pAH and PEG (Peterhans et al 1990). Molecular data including Southern and northern blot analyses, RNase protection, and SI mapping of npt II RNA splicing in kanamycin-resistant rice clones and comparing with tobacco in analogous experiments suggest the following:

- The phaseolin (dicot) intron splices in rice with high efficiency and complete accuracy not distinguishable from that seen in tobacco.
- The mean number of hybrid gene copies integrated into the rice genome averages 5- to 10-fold higher than in tobacco, and a lower level of transcript is observed in both transient and stable expression experiments.

Conclusion

We have reported a simple method for transformation in an important food crop, indica rice, and transmission of the foreign gene to the progeny. Experiments with intron splicing (dicot intron) clearly show that a monocot, particularly rice, can recognize heterologous gene expression signals. As cotransformation has been established as an efficient and routine procedure for the recovery of plants transgenic for nonselectable genes (Schocher et al 1986), it should now be feasible to approach the production of indica rice plants transgenic for agronomically more interesting genes, such as the genes conferring resistance to insect pests as available from the *Bacillus thuringiensis* system (Delanny et al 1989). We consider this a challenge worth accepting.

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Notes

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Polygenic transformation of rice using microprojectile-delivered DNA

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Rice roots cultured on Murashige and Skoog salts and vitamins, supplemented with 100 mg myo-inositol/liter, 100 mg thiamine/liter, 200 mg casein hydrolysate/liter, 3% sucrose, 1-10 µM 2,4-dichlorophenoxyacetic acid (2,4-D), and 1-10 µM naphthaleneacetic acid (NAA) were transformed with *Gus* and *aro A* genes. Root sections (1-5 cm long) were bombarded sequentially with tungsten particles coated with plasmid DNA containing these genes. Bombarded roots were assayed for ß-glucuronidase (GUS) expression the next day. The remaining root sections were induced to form somatic embryos. The optimal auxin concentration for the initiation of rice embryos and the development of pro-embryos was 10 µM NAA combined with 10 µM 2,4-D. Normal somatic embryos showing distinct coleoptile and coleorhizae were formed from pro-embryos that were transferred to embryo development medium containing 1 µM kinetin. DNA was extracted from somatic embryos, and Southern blot filters were probed with the *Gus* or *aro A* probe. Positive signals were detected with both probes. Several somatic embryos were germinated in a medium without growth regulators, and the rice plantlets were acclimated and grown in the greenhouse. GUS expression in the leaves of several regenerated plants confirmed the transformation events. However, Southern analyses on leaves remain to be completed.

Genetic engineering of cereals has been difficult because of the inefficiency of *Agrobacterium*-mediated transformation of monocots (Potrykus 1990). Lately, transformation of protoplasts and regeneration of plants have been reported in wheat, rice, and maize (Harris et al 1988; Rhodes et al 1988a, b; Toriyama et al 1988; Wang et al 1989; Zhang et al 1988). Both electroporation and polyethylene glycol-mediated transformation of protoplast, as well as combinations thereof, have also been successfully achieved (Fromm et al 1985, Junker et al 1987, Lorz et al 1985, Negrutiu et al 1987, Potrykus et al 1985, Uchimiya et al 1986, Yang et al 1988).

A successful, stable transformation requires an efficient, reliable, and repeatable regeneration system. In addition, a reliable transformation experiment requires two regeneration parameters. First, a transgenic plant must originate from a single cell to

avoid chimeric transformants, which may prevent transfer of the transforming DNA into the plant progenies. Second, a transgenic regenerant should not go through the callus stage prior to regeneration, to avoid somaclonal variations that are associated with callus growth and redifferentiation. Therefore, direct somatic embryogenesis via a single cell origin is the most desirable regeneration system for transformed cells and tissue.

Rice plants have been regenerated via somatic embryogenesis from callus cultures of mature seeds (Jones and Rost 1989, Raghava Ram and Nabors 1985, Siriwardana and Nabors 1983), immature panicles (Ling et al 1983), root-derived cell suspensions (Abe and Futsuhara 1986), and protoplasts (Abdullah et al 1986). Rice plants have also been regenerated through organogenesis from callus cultures developed from mature seeds (Fatokun and Yamada 1984), seedlings (Henke et al 1978), immature embryos (Lai and Liu 1986), pollen grains (Mukherjee 1972), anthers (Raina et al 1987, Torrizo and Zapata 1986, Tsay et al 1986), young inflorescences (Chen et al 1985), and protoplasts (Coulibaly and Demarly 1986, Yamada et al 1986). Direct somatic embryogenesis without an intervening callus phase has been reported in several monocot and dicot species (reviewed by Raghavan 1986, Williams and Maheswaran 1986). However, direct embryogenesis from rice root explants has never before been reported.

Here we report direct somatic embryogenesis from rice roots, and the tungsten-mediated polygenic DNA transformation, regeneration, and development of plants.

Materials and methods

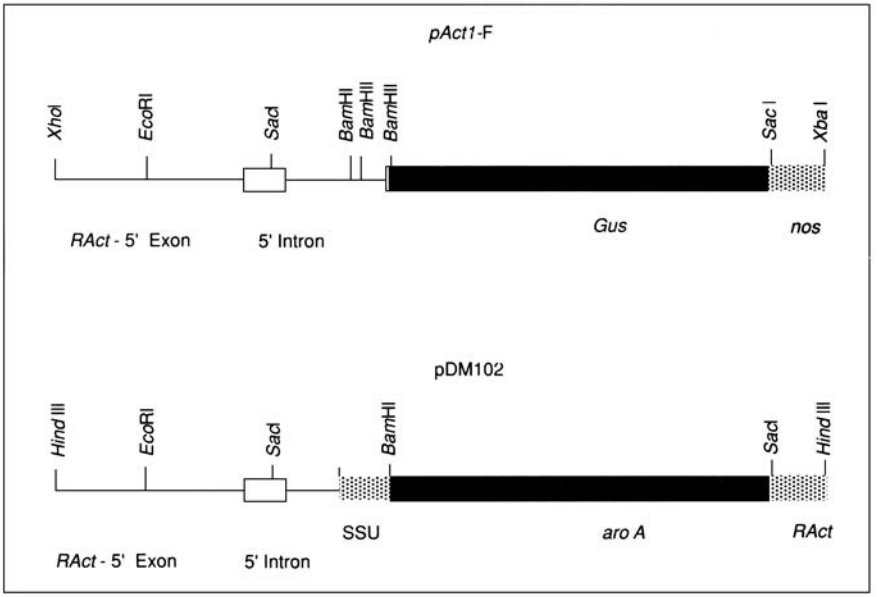
The plant material and reagents for culture, embryogenesis, and transformation were prepared as described below.

Seed germination and treatment of roots

Dehusked S-201 rice seeds were surface-sterilized for 30 min in 50% commercial bleach (2.7% final concentration of sodium hypochlorite), soaked in sterile distilled water for 2 h, and rinsed 3 times in sterile water. Then the seeds were cultured in petri dishes containing a germination medium (modified Murashige and Skoog's [1962] medium [MMS] containing 100 mg myo-inositol/liter. 100 mg casein acid hydrolysate/liter, 200 mg thiamine/liter, and 3% sucrose). The MMS medium was adjusted to pH 5.7, gelled with 0.7% phytagar (Gibco), and autoclaved prior to distribution into sterile petri dishes. The cultures were incubated in the dark at 24-25 °C for 7 d. Root explants (13-2.0 cm) were excised from germinated seeds 7 d after initial seed culture, then bombarded with tungsten particles coated with plasmid DNA harboring either a *Gus* gene or an *aro A* gene (Fig. 1).

Plasmid adsorption to tungsten microprojectiles, and root bombardment

Two plasmids were used in this experiment: 1) pAct1-F containing the *Escherichia coli Gus* gene (McElroy et al 1990), and 2) pDM102 containing a glyphosate resistance



1. Restriction maps of the plasmids. p*Act*I -F codes for ß-glucuronidase, and pDM102 codes for a mutated 5-enolpyruvyl-shikimate-3-phosphate synthase conferring glyphosate resistance. Both plasmids use a rice actin promoter and other 5' sequences as described under Materials and Methods. pDM 102 also has soybean SSU (small subunit of rubisco gene) 5' leader sequences in frame with the *aro A* gene for correct chloroplast targeting of the protein product. The 3' terminator in p*Act*I-F is a *nos* (from nopaline synthase) gene of Ti plasmid from *Agrobacterium.* In pDM102 the 3' terminator sequences are from the rice actin gene.

gene (*aro A*) from *Salmonella* (Fig. 1). Both plasmids contain a rice actin promoter, 5'intron, and 5' noncoding exon before the coding sequence. p*Act*I-F has the *E. coli Gus* gene followed by the nopaline synthase (nos) terminator. pDM102 has the same 5'sequences as pActI-F, but also has the soybean rubisco small subunit leader before the *Salmonella aro A* gene, which is followed by the rice actin 3' terminator region.

The plasmid DNA was adsorbed to the tungsten particles essentially as reported earlier (Cao et al 1990). The DNA-carrying tungsten particles were delivered to the root cells using a DuPont Biolistic PDS-100 system.

Embryo initiation, development, and germination

The bombarded roots were transferred to petri dishes containing embryo initiation medium (Sticklen 1991) having 10 µM naphthaleneacetic acid (NAA) and 10 µM 2,4-dichlorophenoxyacetic acid (2,4-D). The cultures were incubated in the dark for 6 wk at 24-25 °C. Pro-embryos were transferred to MMS medium with or without 1.0 µM kinetin. Cultures were incubated for 3 wk under cool white fluorescent light (60 µE/m^2 per s) at 24-25 °C. Mature somatic embryos were transferred to petri dishes containing MMS medium. These cultures were also incubated for 3 wk under the same light and temperature conditions we described.

Assay for GUS expression and integration of DNA in genome

Tissue assayed for b-glucuronidase (GUS) expression was monitored essentially as described by Wang et al (1989) by soaking tissue in the assay buffer 1 d after bombardment for 1-2 d in the dark and scoring the appearance of blue coloration in the tissue.

DNA was isolated from somatic embryos by freezing in liquid nitrogen, then pulverizing to a fine powder in a precooled mortar before extraction. Powdered material was extracted in cetyltrimethylammonium bromide buffer (Murray and Johnson 1980, Rogers and Bendich 1988), then incubated at 60 °C for 1 h and cooled to room temperature. After two or three extractions with chloroform-isoamyl alcohol, the aqueous phase was ethanol-precipitated, the DNA was dried and dissolved in TE (10 mM Tris [pH 8] 1 mM [ethylenedinitrilo] tetraacetic acid). The DNA solution was restriction-digested with appropriate enzymes, fractionated on 1% agarose gels, and Southern (1975) blotted to Nytran filters. The filters were probed with $^{a\,32}$P dCTP-labeled isolated DNA fragments containing either the *Gus* gene or the *aro A* gene, which had been subjected to the random primed labeling method (Feinberg and Vogelstein 1983). Probed filters were washed at high stringency and autoradiographed on Kodak X-Omat AR5 film.

Plantlet acclimation and growth

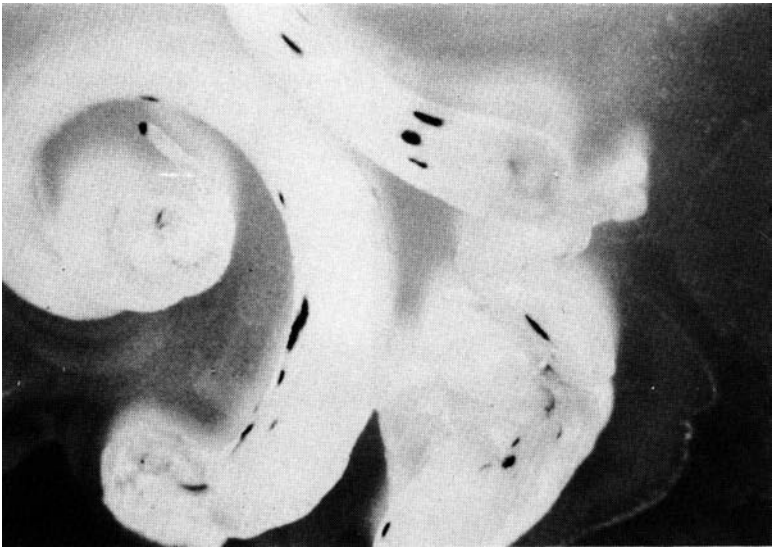
When plantlets were 1-2 cm long, they were transferred to 20-cm culture tubes containing MMS medium and incubated for 3 wk under cool white light (60 μE/m^2 per s) at 25 °C. Plantlets (15-20 cm) were transferred to small clay pots (7.5 cm diameter) containing peat-perlite (1:1 ratio). Pots were enclosed in plastic bags, watered daily, and incubated under light (60 μE/m^2 per s) for 2 wk. To acclimate the plants to lower humidity, a small hole (0.3-0.5 cm diameter) was made in each plastic bag every other day for 10 d. The acclimated plants were transferred to larger clay pots containing equal parts of peat-perlite-clay and were grown in a rice greenhouse at 30 °C and 80% relative humidity.

Results

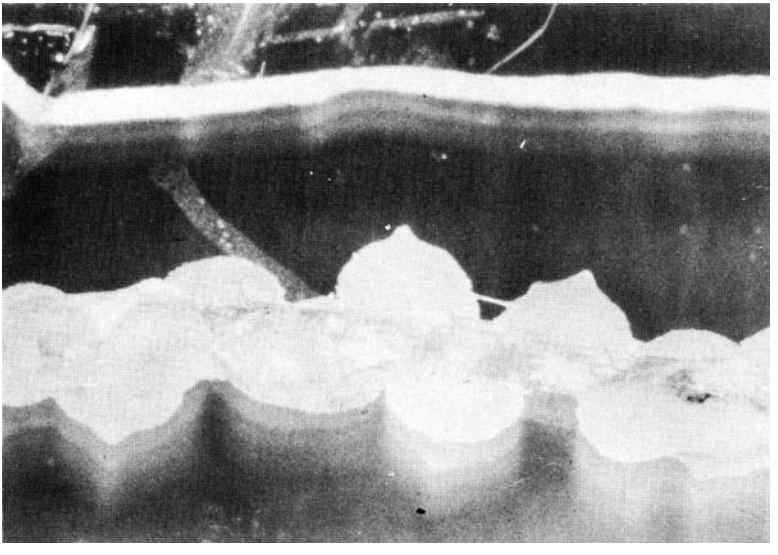
Distinct patches of blue coloration, indicative of positive expression of GUS activity, were seen in tissue after proper incubation time (Fig. 2), indicating successful transient expression of the transferred gene.

Globular pro-embryos were produced 7 d after rice roots were cultured in the embryo initiation media (Fig. 3). Some globular structures became malformed. It was not clear whether these malformed structures were abnormal or grafted embryo structures. Nevertheless, they were eliminated from the experiment.

Numerous pro-embryos were produced from each root section (1.0- 1.5 cm long) 6 wk after initiation of root cultures. Transfer of the pro-embryo-bearing root cultures into flasks containing liquid medium, followed by agitation (110 rpm) for 72 h, facilitated the separation of the pro-embryos. All roots cultured in medium containing

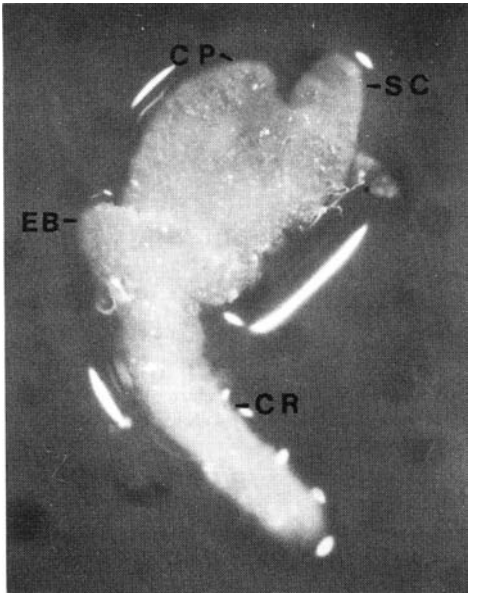


2. Transient GUS expression in root sections 1 d after bombardment. Note the patchy dark staining, which is actually deep blue. The blue coloration is due to the release of the indole dye following the cleavage of substrate X-gluc (5-bromo-4-chloro-3-indolyl-ß-D-glucuronidic acid).



3. Section of a control root (top) and root section from which several globular pro-embryos are emerging (bottom).

auxin produced globular structures. However, medium containing the combination of 10 μM 2,4-D and 10 μM NAA was optimal for producing these structures. Large numbers of them developed into normal somatic embryos containing distinct coleoptile, coleorhizae, scutellum, and epiblast (Fig. 4) 3 wk after their transfer to embryo development media.



4. Differentiated somatic embryo from a globular pro-embryo produced from the treated root sections. Note coleoptile (CP), coleorhiza (CR), epiblast (EB), and scutellum (SC).

5. Mature rice plants regenerated from somatic embryos like the one shown in Figure 4.

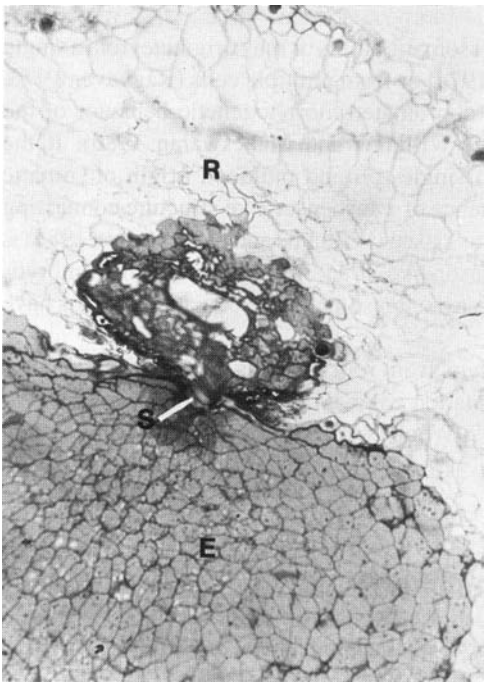
Several somatic embryos germinated and produced green plantlets when the cultures were maintained under light intensity of 60 μE/m^2 per s for 4-6 wk. All the germinated embryos that were subcultured in culture tubes (1 per tube) grew into 15- to 20-cm-tall plantlets within 2 wk in culture. Dozens of these plantlets (15-20 cm long) transferred to a clay-peat-perlite mix all survived acclimation. They grew into 1.0- to 1.3-m-tall plants within 75 d in a greenhouse (Fig. 5). Microscopic observation of the cross-sections of the rice mature root cultures containing early stages of embryogenesis showed that these somatic embryos originated directly from the rice root explants with a suspensor-like structure (Fig. 6).

Integration of the foreign DNA (*Gus* and *aro A* genes) is proved by the positive signal in autoradiograms of Southern blots of DNA extracted from somatic embryos after 2 mo in culture (Fig. 7). Similar analyses will be performed with mature plants.

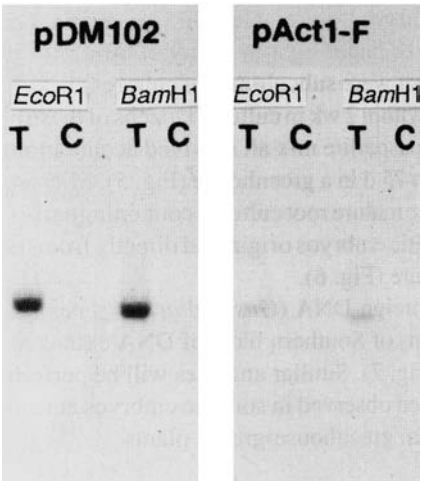
GUS activity has been observed in somatic embryos at later stages of development as well as in leaves from greenhouse-grown plants.

Discussion

We demonstrated the successful transformation of rice by two genes using a tungsten-mediated microprojectile delivery method coupled with direct somatic embryogenesis of bombarded tissue.



6. Cross-section of a root showing a somatic embryo attached to root via a suspensor-like structure. R = root.



7. Autoradiographs of Southern blots from pro-embryos regenerating from bombarded root sections. The extracted DNA was cut with 2 restriction enzymes (*Eco*RI and *Bam*HI). Note the clear signals in lanes containing DNA from transformed (T) tissue compared with lanes containing DNA from untransformed control (C) tissue. Probes were made from gel-purified gene fragments. The presence of 1 band, larger than the total plasmid, suggests a single integration site in the host tissue.

This research also confirms previous reports that auxin, specifically 2,4-D, plays an important role in conferring embryogenic competence in in vitro cultures of gramineous species (Botti and Vasil 1984). Direct somatic embryos may originate from a single cell (Haccius 1978, Street and Withers 1974) or from multiple cells (Raghavan 1976, Tisserat et a1 1979), depending on the coordinated morphogenetic behavior of the neighboring cells in the explant tissue (Williams and Maheswaran 1986). If the hypothesis that the distinction between single-cell and multicell origin of somatic embryos is based on the presence or absence of a suspensor-like structure connecting the single cell origin embryo to the explant tissue (Williams and Maheswaran 1986) is reliable, the presence of narrow suspensor-like structures connecting the embryos produced from rice root tissue (Fig. 6) may suggest that these somatic embryos have single-cell origin.

Direct regeneration of somatic embryos from root explants of rice without callus formation may prove ideal for transformation when a microprojectile bombardment method (Cao et al 1990, Klein et al 1987, Wang et al 1989) of gene introduction into rice cells is used.

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State of the art in gene transfer to cereals

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Agrobacterium tumefaciens provides a routine and efficient gene transfer system for a variety of plant species, but it apparently does not function with cereals. Alternative approaches for gene transfer to cereals have been demonstrated to function: direct gene transfer into protoplasts, biolistics, and microinjection. Transgenic cereals have so far been recovered only from direct gene transfer. Viral vectors, agroinfection, liposome injection, and electrophoresis show promise, although transgenic plants have not been recovered yet. No proof for integrative transformation is available so far from pollen transformation, pollen tube pathway, pollen maturation, incubation of dry seeds, incubation of tissues, liposome fusion with tissues, macroinjection, laser treatment, or electroporation into tissues, and it is difficult to envisage how these approaches could ever produce transgenic cereals. This paper discusses 1) why *Agrobacterium* does not function with cereals, 2) what merits and disadvantages we see for the methods that function, 3) what possibilities we foresee for some of the other approaches, and 4) why we do not expect the remaining ones to be successful.

Gene transfer to cereal crops is, despite intensive experimental efforts over many years, still a problem and limits application of plant biotechnology in this important group of plants. Some transgenic and fertile rice plants have been recovered from incubation of protoplasts in DNA solutions (direct gene transfer), but this technique is too complicated for routine plant breeding. The first fertile transgenic maize plants recovered from biolistic treatment have been reported at recent meetings, but this technique is far too inefficient for applied projects, and it is difficult to see how it could be made more efficient. Therefore, there is still an urgent need for the development of alternative techniques.

Agrobacterium tumefaciens

The soil bacterium *Agrobacterium tumefaciens* has the capacity to transfer DNA that is flanked by two border sequences of the Ti-plasmid to plant cells, where this DNA

integrates into the host genome. This biological phenomenon has been elucidated in detail and has been exploited for the development of efficient, simple, and routine gene transfer protocols. *Agrobacterium*-mediated DNA transfer functions in vivo and in vitro with plants, seedlings, tissue explants, cell cultures, and protoplasts and has led to the regeneration of numerous transgenic plants from a variety of species (Rogers and Klee 1987, Zambryski et al 1989).

Agrobacterium could be a perfect general vector for gene transfer to plants if it functioned with all plant species. Unfortunately, it does not. Many important crop plants, especially the cereals, do not respond to this natural vector. What, then, is the problem between *Agrobacterium* and cereals? It was believed until recently that there was no interaction between cereal cells and *Agrobacterium,* and that consequently there was no DNA transfer. However, elegant experiments have shown that, most probably, *Agrobacterium* is well able to transfer DNA into cereal cells (Grimsley et al 1987).

Why, then, have no transgenic cereals been recovered so far from the numerous treatments of cereal tissues with engineered *Agrobacterium?* The lack of cooperation obviously lies on the cereal side and probably has a biological basis that cannot be altered: *Agrobacterium* requires for its function a specific state of competence in the plant cell. This state is normally built up in wound-adjacent cells as a reaction to the "wound response" in dedifferentiating differentiated cells (Potrykus 1990a,b). Differentiated cereal cells apparently lack the wound response and consequently the capacity for dedifferentiation. Therefore, even if DNA could be transferred into wound-adjacent cells in cereals, and even if it integrated, this would not lead to transgenic cell clones because the wound-adjacent cells die and do not proliferate.

Functional gene transfer methods

The three methods that function are direct gene transfer into protoplasts, biolistics, and microinjection.

Direct gene transfer into protoplasts

Because we anticipated problems between *Agrobacterium* and cereals, we tried to develop a gene transfer protocol that is independent of any biological vector. This was possible on the basis of protoplasts. Plants can be regenerated from isolated, single plant cells without cell walls—the protoplasts (Potrykus and Shillito 1986). Protoplasts can be isolated from a great variety of plant tissues. As protoplasts are surrounded by only a simple plasma membrane, it was expected that genes could be introduced directly with straightforward physical treatments. This was indeed the case (Paszkowski et al 1984), and within a few years the basic method was optimized such that transgenic plants could be produced with great efficiency either with electroporation (Shillito et al 1985) or with simple chemical treatments (Negrutiu et al 1987). These plants showed perfect Mendelian inheritance of the foreign genes (Potrykus et al 1985) and stabilities comparable to those of original plant genes. This method, called direct

gene transfer, also enabled efficient cotransformation (Schocher et al 1986) and gene targeting (Paszkowski et al 1988).

Cereal protoplasts could be transformed early on, but it took a few years more until transgenic cereals could be regenerated (Rhodes et al 1989, Shimamoto et al 1989). There is probably no limitation concerning gene transfer to protoplasts, and plants that can be regenerated from protoplasts can therefore most likely be transformed. To date, plants have been regenerated in about 150 species (Roest and Gilissen 1989); in additional species, cell cultures have been recovered from protoplasts.

Is this the solution to the problem, then? Unfortunately, it is probably not. Plant regeneration from protoplasts is a very delicate and often unreliable process, depending upon too many parameters that are not under experimental control.

Particle gun or biolistics

The biolistics method has been developed from the rather surprising idea of bypassing all biological complications by shooting genes glued to heavy particles into plant cells (Klein et al 1987). The explosive force of gunpowder or heated water is used to accelerate large numbers of small metal particles into target tissues. This method has yielded evidence for transient expression of marker genes. The first case of regeneration of transgenic plants as a consequence of biolistic treatment of the shoot came with soybean (McCabe et al 1988). Since then, numerous laboratories, using biolistic devices in large-scale attempts, have found that this technology is not as efficient as expected. In fact, it is rather inefficient as far as integrative transformation is concerned.

What about its function with plant species that cannot be transformed with other methods? It has been stated that the only transgenic cereals come, so far, from protoplasts and direct gene transfer. And it is indeed surprising that it has taken so long for the first proven case of a transgenic cereal recovered from biolistic treatment to be reported. (The first cases of transgenic maize will probably soon appear in the literature.)

Why, however, is the biolistic approach so inefficient, and why has it taken so much effort to produce the first transgenic cereals? I see several possible causes, and it will be difficult to alter them experimentally:

- The frequency of integrative transformation depends upon the concentration of the transforming gene; if this drops below a critical level, the transformation frequency is close to zero. It may be difficult to deliver enough DNA on a particle, or the DNA may be released too slowly from the particle.
- Recovery of a transgenic plant requires that the foreign gene is transported into and integrates in a cell that is competent for integrative transformation and for clonal propagation and regeneration. Such cells are probably extremely rare in cereals.
- Even if successful, integrative transformation in one cell of a multicellular structure will yield a transgenic chimera, and if no secondary morphogenesis from and selection for those transgenic cells is easily possible, whether or not a transgenic sector will contribute to the “germ line” will depend on fortune.

I am therefore skeptical that biolistics can be developed into an efficient routine technique for every plant species.

Microinjection

Microinjection initially looked very promising but has encountered surprising problems. Three years ago microinjection of a marker gene into microspore-derived pro-embryos produced transgenic chimeras (Neuhaus et al 1987). Microinjection uses microscopic devices and microinjection capillaries to deliver defined volumes of DNA into defined cells without impairing their viability. We have made every effort to repeat this with cereals and to extend it to zygotic pro-embryos. We believed that the key problems would be related to the production of enough microspore-derived pro-embryos in our laboratory from the main cereals, and to the isolation of and plant regeneration from very young zygotic pro-embryos. Both problems were solved, and many hundreds of microspore-derived pro-embryos of maize, wheat, rice, and barley and of zygotic pro-embryos from these cereals have been microinjected and regenerated; many thousands of sexual offspring have been analyzed for the presence of the foreign gene. Surprisingly, we have seen no case where we can prove that the foreign gene would have been inherited by the offspring.

What is the problem, then, with this approach? One problem is certainly (as with the biolistic approach) the chimeric nature of putative transgenic plants, which makes transmission to offspring a chance event. We must also consider the possibility that cereal pro-embryos and meristems may not contain many cells competent for integrative transformation.

The key problem for the development of an efficient gene transfer method for cereals may concern not the method of DNA delivery, but the availability of competent cells. This is where protoplasts probably have one of their great advantages over all other systems: protoplast isolation shifts potentially competent cells into the competent state.

Other approaches

As none of the four approaches discussed so far has led to a routinely applicable gene transfer method, and as all of them have inherent problems, it is worth discussing briefly the other approaches that have not yielded proof of integrative transformation even with easier plant systems. Before doing that, we must define what constitutes "proof of integrative transformation."

Many years of experience with artifacts in numerous laboratories have shown that genetic, phenotypic, or physical data alone are not acceptable. Proof requires 1) serious controls for treatments and subsequent analysis; 2) tight correlation between treatment and predicted results; 3) tight correlation between physical and phenotypic data; 4) complete Southern analysis containing the predicted signals in high-molecular-weight DNA hybrid fragments between host DNA and foreign gene and in the complete foreign gene, plus evidence for the absence of contaminating DNA fragments; 5) data

that allow discrimination between false positives and correct positives in the evaluation of phenotypic evidence; and 6) if possible, correlation of the phenotypic and physical evidence with transmission to sexual offspring as well as genetic and molecular analysis of offspring populations. Judged on this basis, none of the following approaches has, so far, provided proof for integrative transformation.

DNA and RNA viral vectors

Replacement of an unessential viral gene by a selectable marker gene in cauliflower mosaic virus can be used to produce, on viral infection, turnip plants that contain and express the marker gene throughout the plant (Brisson et al 1984). It was hoped that this principle could be extended to many genes and viruses to make use of the multicopy situation. Viral genomes, however, are apparently so tight that they do not easily tolerate foreign DNA. Since normal cloning technology can be also applied to RNA viruses, this far larger group of plant viruses is open to a series of interesting engineering approaches (Ahlquist and French 1988). However, viral vectors will probably not contribute much to the production of transgenic plants unless experimental conditions are discovered that induce integration of viral genomes into host genomes.

Agroinfection

The DNA transfer mechanism of *A. tumefaciens* can be used for the transfer of virus genomes into plants where mechanical virus infection is not possible (Grimsley et al 1986). The virus then spreads systematically throughout the plant. This amplification of a single DNA transfer event has been used to study whether or not *Agrobacterium* can deliver DNA into cereals. Systemic spread of a maize DNA virus in maize following agroinfection showed that this is indeed the case (Grimsley et al 1987). It was later shown that there is not much difference in the efficiency of DNA transfer between dicots and monocots.

Was this, then, the long expected proof that *Agrobacterium* is also a viable vector system for genetic engineering of cereals? Probably not. Using an engineered virus carrying a foreign gene, one could spread this gene in the individual agroinfected plant. As the virus is excluded from the meristems, it is excluded from transmission to the offspring. As the virus does not integrate into the host genome, there is little chance that it integrates into the genome of a cell of the "germ line" or one of the rare cells competent for transformation and regeneration. The only chance for integration of the foreign gene would be in wound-adjacent cells, which are neither competent nor viable.

Liposome injection

Microinjection into differentiated cells can easily deposit DNA into the vacuole, where it is degraded. Lucas et al (1990) thought of using the vacuole for DNA delivery into the cytoplasm. Microinjection of liposomes into the vacuole leads to fusion with the tonoplast, thereby releasing the contents of the liposome into the cytoplasm, as demonstrated with cytoplasm-activated fluorescent dyes. The activity of injected DNA

has still to be shown. This method, though elegant, probably has no advantage over straightforward microinjection, especially for producing transgenic cereals. Cereals regenerate only from meristematic cells, which do not have large vacuoles.

Electrophoresis

Radioactive-labeled marker gene solutions have been electrophoresed across the shoot meristem area of barley seeds (Ahokas 1989). I have shown by autoradiography that the label moves along the cell wall. This cell wall labeling may be due to breakdown products. If DNA could indeed travel such long distances along cell walls, it could possibly also move across cell walls. This would be an important finding. So far, there is no proof for integrative transformation by this method.

Pollen transformation

In the early 1970s, experiments were undertaken to test whether or not pollen could be used as a vehicle for the delivery of foreign genes into sexual offspring (Hess 1987). Since then, numerous laboratories have performed experiments with increasing sophistication and application of molecular techniques. Although several interesting phenotypes have occurred in the offspring, there is no proven case of gene transfer. There are, however, many clearly negative results from large-scale experiments in experienced laboratories. If it were possible to transform a zygote by incubation of germinating pollen in DNA solution, this would be an ideal method. But because more than 15 yr of experimentation has not produced a single proven transgenic offspring, the chances that this will be developed into an efficient method are rather small.

Pollen tube pathway

It was tempting to test whether pollen tubes could be used for gene delivery to the zygote. Applications of marker gene solutions to cut pistils a few hours after pollination in rice *(Oryza sativa)* led to kanamycin-resistant offspring (not a reliable marker) and Southern data that unfortunately did not prove that integrative transformation had occurred (Luo and Wu 1988). Again, if this worked, it would represent an ideal method. However, it is obviously rather difficult to reproduce the data, and there are several biological problems that interfere with the experimental design (e.g., callose plugs within the pollen tubes, nucleases, cell wall adsorption, synergids, DNA transport), so it would be surprising if this approach did work.

Pollen maturation

Three key problems may have prevented pollen transformation so far: the cell wall, nucleases, and the intense heterochromatic state of sperm cells. The latter two problems may be overcome by the approach of in vitro maturation (Alwen et al 1990). Immature microspores can be matured to functional pollen in vitro. If genes could be transferred at the microspore stage, they might have a chance for integration.

Incubation of dry seeds

The most recent experiments have used dry cereal embryos separated from the endosperm at the scutellum, thereby creating a large wound site. Incubation in viral or nonviral DNA solutions yielded evidence for transient expression of marker genes and recombination of viral DNA (Töpfer et al 1989). Regeneration of plants from these embryos could lead to transgenic cereals; a large number of offspring are under study. There is so far no proof of integrative transformation, and the chances for transgenic cereals are extremely small; use of viral DNA may lead to systemic spread, which will not lead to integration; nonviral DNA has virtually no opportunity to reach competent cells that will contribute to shoot regeneration. The best this DNA can do is to reach some of the wound-adjacent cells, but these will not proliferate, and they die.

Incubation of tissues or cells

Many approaches have brought seedlings, organs, tissues, cells, or cell cultures of numerous plant species into direct contact with foreign DNA and defined marker genes. Treatments have also used open plasmodesmata or loosened cell wall structures. Some treatments ensured that competent cells were available at sufficient frequency. Even in experiments that would have recovered extremely rare events of integrative transformation, not a single proven case of integrative transformation occurred. Experiments relying on the passage of functional genes across cell walls have very little chance of success, not only because the cell wall is a perfect barrier against large DNA molecules, but also because it is an efficient trap. Even if there were occasional transfer, other negative factors would come into play: 1) DNA might become attached to the cell walls, 2) transport across further cell walls might occur, 3) there might be no mechanism for DNA transport, and 4) competent cells must be reached. The combination of several low-frequency events would cause problems even if one step occasionally worked (Potrykus 1990a,b).

Liposome fusion with tissues and protoplasts

Fusion of DNA-containing liposomes with protoplasts is an established method for producing transgenic plants (Deshayes et al 1985). It has, however, no obvious advantage over direct gene transfer. DNA-containing liposomes have also been applied to various tissues, cell cultures, and pollen tubes, with the rationale that liposomes might help transport the DNA via plasmodesmata or directly across the cell wall. Liposomes can carry small dye molecules into cells within tissues via fusion with the plasmalemma (Gad et al 1988). There is, however, no proof of transport and integration of marker genes. As plasmodesmata are sealed off immediately on wounding, this route is not open, even for small liposomes; impregnation of the cell wall with phospholipids seems not to change its barrier function.

Macroinjection

Use of injection needles with diameters greater than cell diameters leads to destruction of the cells. DNA integration would require that the DNA move into wound-adjacent

cells and, therefore, all problems previously discussed apply. The most promising data so far were reported in an experiment where a marker gene was injected into the stem below the floral meristem of rye *(Secale cereale)* (De la Peña et al 1987). Hybridization to the marker gene and enzyme assays with selected sexual offspring yielded strong indicative evidence. Unfortunately, it has not so far been possible either to reproduce these data in several large-scale experiments with other cereals or to establish proof with the original material. It is also very difficult to understand how the DNA could reach the sporogenic cells in this experimental design, as DNA would have not only to reach neighboring cells but also to travel across many layers of cells.

Microlaser treatment

A microlaser beam focused into the light path of a microscope can be used to burn holes in cell walls and membranes (Weber et al 1988). It was hoped that incubation of perforated cells in DNA solutions could serve as a basis for a vector-independent gene transfer method into walled plant cells. No conclusive data are available on DNA uptake, and there are problems with adsorption of exogenous DNA to cell wall material, even before it can be taken up. As microinjection and biolistics definitely transfer DNA into walled plant cells, the microlaser would offer advantages only in very specific cases where the two other techniques were not applicable.

Electroporation into tissues

Discharge of a capacitor across a cell containing protoplasts and DNA solution is a routine method for DNA uptake as well as for stable integrative transformation (Fromm et al 1986, Shillito et al 1985). Electroporation has also been applied to a variety of walled plant cells and tissues. The results so far confirm that cell walls are very efficient barriers against DNA molecules of the size of a functional gene.

Summary

Gene transfer to plants is routine and efficient by *A. tumefaciens* for plant species with a prominent wound response, and by direct gene transfer for species that can be regenerated from protoplasts. Transgenic cereals (rice, maize) have so far been recovered exclusively by direct gene transfer applied to protoplasts. This method is not applicable to all varieties of all plant species. The challenge, therefore, is to develop a routine gene transfer method that achieves integrative transformation with cereals and other important crop plants, or with any given plant species and variety. Two approaches have the best potential: biolistics and microinjection into zygotic pro-embryos. The other approaches, however, might still prove of interest.

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Discussion

Session 12: Transformation techniques

C—*Brar:* It is highly encouraging to see the progress made in protoplast regeneration and production of transgenic indica rices. The sterility of the transgenic plants could be due to high copy number of the foreign gene. However, the transgenic plants need to be examined to discover whether they are female sterile or not as a result of kanamycin selection.

C—*Hodges:* We currently have 50- to 60-mm transgenic plants that are in various stages of development. We will be analyzing these for seed set and both male and female fertility. We are reasonably certain that the sterility is not due to the G-418 treatment, but more likely due to the *neo* gene itself.

Q—*Hull:* Was the sterility in the *NPT-II*-transferred plants male sterility? If so, could not transfunction with *NPT-II* be used to produce male sterile rice?

A—*Hodges:* We were able to pollinate the plants with nontransformed pollen in only one case. In that situation, we did obtain eight seeds, suggesting that we are indeed dealing with a male sterile problem. We will address this as more transgenic plants reach flowering. I suspect there may be an easier way to develop male sterile rice.

Q—*Finch:* (a) What is the frequency of plant regeneration from IR54 protoplasts? (b) What are the relative and absolute transformation frequencies obtained in your direct DNA uptake work?

A—*Hodges:* (a) When embryonic-like calli are separated from the rest of the callus, about 20% of these calli produce plants. (b) The absolute transformation efficiency is about 10^{-4}. As for relative efficiency, we plate 400,000 protoplasts/petri plate and obtain 5-10 kanamycin- or G418-resistant calli. Of them, 90% contain the *neo* gene based on Southern analysis.

Q—*Ray Wu:* You mentioned that the light-harvesting chlorophyll a/b binding protein (LHCP) promoter is 10 times stronger than the 35S promoter so that the LHCP promoter is the strongest known promoter for rice transformation. We found that the rice actin-1 gene promoter system is about 100 times stronger than the 35S promoter. Thus, the actin-1 gene promoter is probably 10 times stronger than the LHCP promoter. What do you think?

A—*Tada:* Although it is difficult to compare the efficiency of tissue- or stage-specific promoters, the calculated ß-glucuronidase activity with the LHCP promoter is much the same as the activity with the actin-I gene promoter that was reported in your paper. The LHCP promoter can be said to be one of the most effective promoters for rice.

Q—*Fujimura:* The efficiency of pollen division is very high. How do you culture indica rice pollen? What is the source material for protoplast isolation—primary callus or subcultured callus?

A—*Datta:* Sometimes efficiency is high, perhaps depending on the donor plant. No special method was followed. The medium we described in the recent Plant science (1990; 67-83-88) paper achieved a response in japonica rice every other day, while a similar response happened once in 15 days in "indica" rice. Our source material for protoplast isolation was a subculture.

Q—*Narayanan:* You have used microspore-derived suspension cultures for protoplast isolation and regeneration. Did you find any haploids or other ploidy variants among the regenerants?

A—*Datta:* I have not checked the ploidy levels. However, we observed many sterile plants including aberrants with or without transformation, perhaps because of aneuploidy.

Q—*Hodges:* (a) How many R_0 transgenic fertile plants have been obtained? (b) How many independent suspension cultures have been developed that will actually regenerate plants?

A—*Datta:* (a) I obtained 24 mature plants grown in the greenhouse; 10 were fertile in the R_0. (b) Several independent embryogenic cell suspension cultures that were obtained produced regenerable protoplasts and plants. However, transgenic R_0 plants were obtained from one line.

Q—*Gupta:* Seeds derived from transgenic plants do not show segregation and show high copy number. This indicates that the gene was incorporated at the haploid level. Since you have used anther-derived cells, where only 30-35% of the cells are haploid, how do you explain this contradiction?

A—*Datta:* The ploidy levels were not checked before or after the transformation experiments. The results do not show any contradiction. Only one pattern of foreign gene integration was observed in the selected R_0 plant and in the 10 progeny obtained from this primary transgenic R_0 plant. The possible explanation is that protoplast transformation had taken place at the haploid level. Of course, gene integration at more than one locus cannot be ruled out. Further genetic analysis is required.

Q—*Slamet:* Is Chinsurah Boro II a type I indica according to the Glaszmann classification? What was the culture density used? In the japonica system I worked on, the nurse culture is not essential if protoplasts are plated at more than 3×10^5, but it is essential if protoplasts are plated at less than 10^5.

A—*Datta:* I am not sure about the classification. Chinsurah Boro II may be a type II indica, but nevertheless it is definitely an indica rice. The culture density was 1.5×10^6/ml. Certainly, you require a suitable density of protoplasts for regeneration. But if it is optimized and works well, why should you use a nurse culture? I do not require a nurse culture to regenerate protoplasts.

Posters

Characterization of a repetitive hypermutable region in *Oryza sativa*

T.E. Bureau, G.S. Khush, and S.R. Wessler

Several waxy *(wx)* mutants were obtained from a wide hybrid cross between cultivated rice (*Oryza sativa)* and a distantly related wild species, *O. officinalis.* The mutants segregated for the *wx* trait in a non-Mendelian fashion, reverting back to wild type at high frequency. Analysis by Southern blot hybridization indicated no transposable element in or flanking the rice *wx* gene. A polymorphic methylation domain was localized approximately 2-4 kb upstream from the 5' end of the rice *wx* gene.

A novel BstN1 restriction site was located in an intron of the revertant wxgene. The occurrence of the site is due to an A-G base substitution. After examining the same region in the *O. sativa* progenitor and in several mutant and revertant lines, other base modifications were localized within the same intron (with one exception). This hypermutable region spans 121 bp and contains 10 base modifications. The hypermutable region is highly repeated in the *O. sativa* progenitor genome and is absent in the 0. *officinalis* progenitor and other *Oryza* spp. genomes. A mechanism similar to gene conversion may be involved.

Notes

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Isolation and characterization of rice CC genome-specific sequences

A.S. Reddy, F. Cordesse, and M. Delseny

Repetitive DNA sequences may be present as tandem arrays, dispersed elements, or displaced members of tandem arrays in the eukaryotic nuclear genome. In most cases these highly repeated DNA sequences are genome- or species-specific. To develop genome-specific molecular markers and to analyze divergence among species or accessions of the CC genome, we isolated two tandemly repeated (clones: pEO38 and pSO271) and one dispersed (clone: pBO3) DNA element from *Oryza officinalis* (Acc. no. W1278). Hybridization of rice genomes with these DNA clones indicated that the families of repeats are completely absent in the AA, BB, EE, and FF genomes. All three clones were sequenced, and the detailed sequence comparison indicated that a region of about 80 bp is homologous within the repeat elements of pEO38 and pSO271. However, pBO3 showed no cross hybridization with either pEO38 or pSO271 even under low stringency conditions.

The pEO38 and pSO271 sequences were highly represented in *O. eichingeri, O. collina,* and almost all accessions of *O. officinalis,* whereas the pBO3 sequence was less abundant in *O. collina* and completely absent from *O. eichingeri.* The amphidiploid genomes BBCC and CCDD were also analyzed using these clones, and in all three repeats we failed to detect homologous sequences in the CCDD genome, pEO38 was very low, whereas pBOnt in the BBCC genome. The copy numbers of these sequences showed variability within accessions of the CC genome.

Work is in progress to identify more CC genome-specific sequences and to explain the phylogenetic relationships within the CC genome for identifying subgroups, if any.

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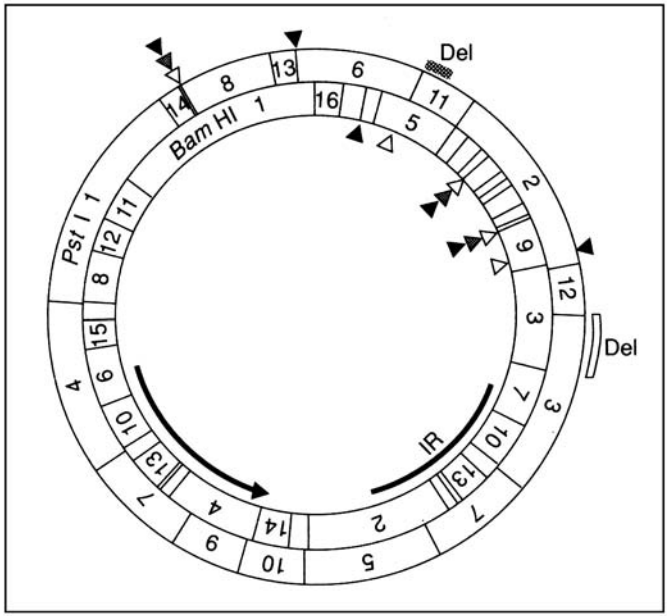
Comparative studies of the structure of chloroplast DNA from four *Oryza* species: cloning and physical maps

A. Kanno, K. Hattori, and A. Hirai

Knowledge of the ancestors and the phylogenetic relationships of cultivated rice is important for rice breeding. Information on chloroplast DNA (ctDNA) restriction patterns and detailed analyses of rearrangements and deletions of ctDNA among closely related species can provide phylogenetic and evolutionary indications. We have analyzed ctDNAs in four species of Oryza: cultivated rice *O. sativa* (AA genome) and three wild species- *O. punctata* (BB), *O. officinalis* (BBCC), and *O. australiensis* (EE). The complete nucleotide sequence of *O. sativa* ctDNA has been determined, and restriction pattern analysis has been applied to *Oryza* species.

We constructed clone banks of the ctDNA from three species. Chloroplast DNAs were prepared from green leaves, digested with restriction enzymes, and cloned in lambda phage vector (Lambda Fix) and plasmid vector (pUCI9). We analyzed the ctDNA libraries by using ctDNA fragments of *O. sativa* as probes, and we constructed physical maps (*Bam*HI and *Pst*I cleavage maps) of them.

The restriction endonuclease (*Bam*HI and *Pst*I) patterns of ctDNAs from the four *Oryza* species are very similar, and their total sizes are in close agreement with 134,525-bp *O. sativa* ctDNA. But there are some gains or losses of *Bam*HI and *Pst*I sites in comparison with *O. sativa,* probably the result of base substitution. Small deletions, localized near the junction, were discovered between an inverted repeat and the large single-copy region in *O. punctata* and near the *atp*I gene in *O. officinalis.* Base substitutions and deletions are localized between *rbc*L and *psb*A in the large single-copy region (Fig. 1), which suggests that this area is a hot spot of ctDNA alteration from *Oryza.*



1. Restriction map of *O. sativa* ctDNA and comparison with *O. punctata* (open), *O. officinalis* (shaded), and *O. australiensis* (black). Triangles = alterations of restriction sites, rectangles = deletions of fragments.

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Evolution of the intergenic spacer region of ribosomal DNA in cultivated and wild rice species

Y. Sano, R. Sano, and H.-Y. Hirano

Discrepancies in *Oryza* species recognition would originate from the paucity of species-specific characteristics even if internal isolation barriers were effective among taxa. An interbreeding complex between wild and cultivated rice species also makes species boundaries unclear, giving rise to controversy over phylogenetic relationships. Our recent work on ribosomal DNA (rDNA) spacer-length variation revealed that the intergenic spacer regions differ greatly among reproductively isolated taxa with respect to length as well as sequence (Sano et al 1989, Sano and Sano 1990). Among 243 accessions from 2 cultivated rice species and their wild relatives with the AA genome, 18 spacer-length variants were detected. Restriction enzyme maps showed that the length heterogeneity in Asian cultivars *(O. sativa)* and their wild progenitor *(O. rufipogon)* results from the number of repetitions of short repeated sequences in the intergenic spacer region. However, these repetitions detected in the *O. sativa-O. rufipogon* complex do not explain length variations among distantly related taxa, suggesting that different mechanisms must be involved in length variation among reproductively isolated taxa.

Ribosomal DNA is organized as families of tandemly repeated genes, which may comprise the nucleolar organizer (Nor) regions (Appel and Honeycutt 1986). Extensive sequence divergences observed in the intergenic spacer region confirmed rapid change in the Nor region in the evolution of rice. In addition, the number of chromosomes carrying Nor regions varies among different species or taxa (Selim 1930), suggesting differential expression of ribosomal RNA genes in rice. Southern blot analysis revealed that the African cultivated species *O. glaberrima* carries rDNA repeats at a single locus, but at least some accessions of Asian wild and cultivated forms carry rDNA repeats at two unlinked loci. The duplicated loci associated with rDNA repeats may be related to the high degree of heterogeneity observed in Asian wild and cultivated forms compared with that of African forms. Furthermore, Asian cultivars such as the indica and japonica types tend to carry homogeneous rDNA repeats although their wild progenitor frequently carries heterogeneous repeats (Table 1). No difference in heterogeneity within an individual was found between annual and perennial forms in *O. rufipogon,* suggesting that a high degree of homogeneity is not necessarily related to a high degree of inbreeding. Rice materials may offer a good opportunity to look into the evolutionary significance of rDNA repeats in relation to their dynamic changes in the genome.

Table 1. Number of accessions carrying homogeneous and heterogeneous rDNA repeats within individuals of Asian cultivated rice and its wild progenitor.

Species	Type	Accessions (no.)		rDNA diversity (H)[a]
		Homogeneous	Heterogeneous	
O. sativa	Indica	26	3	0.85
	Javanica	17	7	1.22
	Japonica	22	0	0.96
	Total	65	10	1.32
O. rufipogon		47	30	2.21

[a] Diversity (H) was computed as $H = -\sum_i p_i \ln p_i$ where p_i stands for frequency of *i*th spacer-length variant. Homogeneity: c^2 sat-ruf = 12.772** (df = 1); c^2 types = 8.787* (df = 2). * and ** show significance at the 5% and 1% level, respectively.

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Cytoplasmic and nuclear DNA differentiation in cultivated rice species

T. Ishii, T. Terachi, N. Mori, and K. Tsunewaki

To obtain new information at the molecular level on phylogenetic differentiation in cultivated rice species, two experiments were carried out.

First, restriction fragment length polymorphism was analyzed in *Oryza sativa* and *O. glaberrima* chloroplast (ct), mitochondrial (mt), and nuclear DNA. Two *O. sativa* japonica cultivars (J1 and J2), two javanicas (Jv1 and Jv2), four indicas (I1, I2, I3, and 14), and two *O. glaberrima* cultivars (G1 and G2) were studied. Their chloroplast DNA (ctDNA), mitochondrial DNA (mtDNA), and total DNA were examined by restriction endonuclease analysis and Southern hybridization analysis as shown in Table 1. Based on the results, dendrograms showing the phylogenetic relationships among their ctDNA, mtDNA, and nuclear DNA genomes were drawn (Fig. 1). Differentiation at the molecular level of three organelles (chloroplast, mitochondrion, and nucleus) appears to have proceeded synchronously for the most part. However, its magnitude was largest in the nuclear genome.

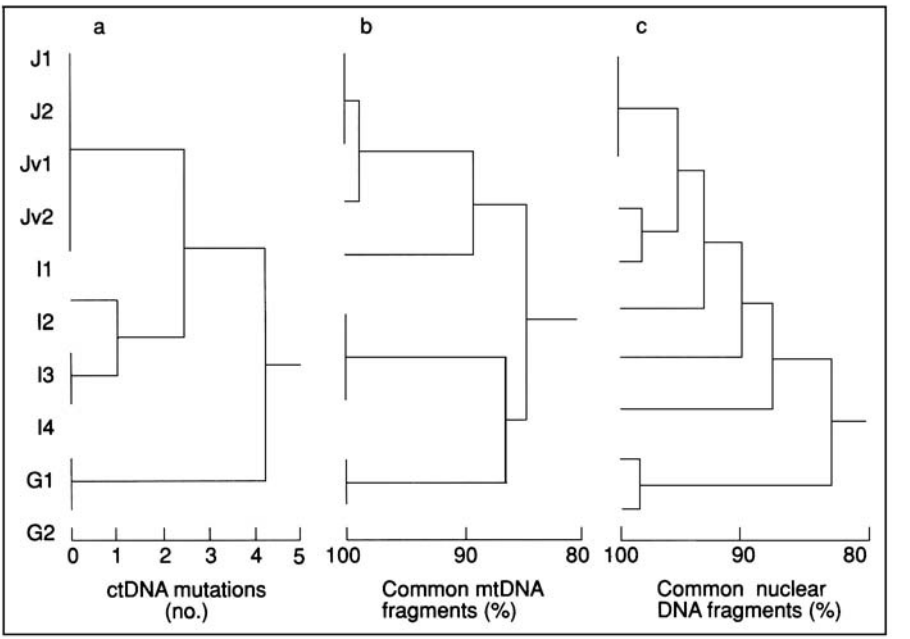
Second, restriction endonuclease analysis was performed on ctDNA from *O. sativa* cultivars collected in Asian countries. Chloroplast DNAs of 68 *O. sativa* cultivars from 15 Asian countries, for which isozyme data are available (Glaszmann 1985), were subjected to restriction endonuclease analysis with 6 enzymes: *Eco*RI, *Hin*dIII, *Pst*I, *Pvu* II, *Sma* I, and *Xho* I. Based on the restriction fragment patterns, they could be classified into five types (1, 3, 10, 11, and 12). Using Southern hybridization analysis, the number and nature of the mutations detected between them were determined, and the five chloroplast genome types were divided into two groups: japonica (types 1, 11, and 12) and indica (types 3 and 10) chloroplast genomes. However, no distinct geographical differentiation was observed in the chloroplast genomes. On the other hand, Glaszmann (1985, 1987) carried out isozyme analysis using the same materials, dividing them into six enzymatic groups: two major (groups I and VI), two minor (II and V), and two satellite (III and IV) groups. Based on analogy with other classifications, he considered groups I and VI as indica and japonica types, respectively. Table 2 shows the correspondence among Glaszmann's enzymatic groups and the present chloroplast genome types. The japonica isozyme group (VI) is associated with the japonica chloroplast genome, whereas the indica isozyme group (I) contains both japonica and indica chloroplast genomes.

O. sativa japonicas (including javanicas) and *O. glaberrima* show little variation in ctDNA, mtDNA, and nuclear DNAs. However, *O. sativa* indicas show wide differentiation. Similarly, the indica isozyme group reveals wider chloroplast genome differentiation than does the japonica group.

Table 1. Methods used for chloroplast DNA, mitochondrial DNA, and nuclear DNA analysis.

DNA analyzed	Method of analysis	Enzyme used	Index of differentiation
Chloroplast	Restriction endonuclease analysis	*Eco*RI, *Hin*dIII *Psf*I	Number of mutations
Mitochondrial	Restriction endonuclease analysis	*Bam*HI, *Hin*dIII *Psf*I, *Pvu*II, *Xho*I	Percentage of common fragments
Nuclear	Southern hybridization analysis[a]	*Hin*dIII, *Pst*I	Percentage of common fragments

[a]18 independent clones of nuclear DNA were used as probes.



1. Clustering by UPGMA (unweighted pair-group method using arithmetic averages) method (Sneath and Sokal 1973) of 10 rice cultivars, based on number of ctDNA mutations (a), percentage of common mtDNA fragments (b), and percentage of nuclear DNA fragments (c), showing their genetic relationships. J =japonica, Jv = javanica, I = indica, G = *O. glaberrima.*

Table 2. Correspondence among 6 enzymatic groups and 5 chloroplast genome types of 68 *O. sativa* cultivars.

Enzymatic group	Japonica chloroplast genome			Indica chloroplast genome		Total
	1	11	12	3	10	
I	5	1	0	19	0	25
II	5	0	0	8	0	13
III	1	0	0	1	0	2
IV	0	0	0	0	1	1
V	8	0	2	0	0	10
VI	13	0	0	0	0	13
—	2	0	0	2	0	4
Total	34	1	2	30	1	68

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Physical mapping of rice restriction fragment length polymorphism clones

J.P. Gustafson, C.L. Mclntyre, and J.E. Dille

The development of C-banding and in situ hybridization techniques has allowed the identification of chromosomes and the location of large inserts of alien gene complexes in many plant species. Previous work (Butler et al 1989) showed that hybridization of unique DNA sequences was not possible using slides of normal squash preparations. The present study analyzed the use of protoplast preparations and in situ hybridization with biotin to physically map rice restriction fragment length polymorphism (RFLP).

The rice RFLP clones used in this study were RG-83, RG-139, RG-322, RG-182, RG-207, RG-13, RG-233, and RG-316 (McCouch et al 1988) obtained from S. Tanksley of Cornell University.

The protoplast technique (Dillé et al 1990) used allowed for the production of metaphase cells free of cellular debris. The in situ hybridization technique allowed for the detection of low-copy and unique-sequence DNA probes (Gustafson et al 1990).

The arm lengths and the lengths from the centromere to the hybridization site were measured on a television monitor with calipers. The arm ratios were compared with the early metaphase chromosomes shown by Kurata (1986).

A total of 1088 metaphase cells were analyzed; approximately 11% exhibited hybridization. Hybridization was seldom seen on prometaphase spreads and was observed on only one of the two chromatids.

The hybridization sites for RG-322 and RG-83 relative to the centromere were determined; the entire linkage group appeared to be located on the short arm of chromosome 2. This was confirmed by mapping RG-139 from the center of the linkage group to a position between the ends of the group on the short arm.

RG-207 was located near the centromere on the long arm, while RG-13 was positioned on the short arm of chromosome 5. An unlinked RFLP on chromosome 5 (RG-182) was also mapped because it was located on chromosome 5, but it was not genetically linked to the other linkage group. The hybridization site was distal to RG-207 on the long arm.

RG-316 and RG-233 were located at the ends of the largest linkage map for chromosome 1, The linkage map spanned the centromere and covered the majority of the physical length of the chromosome.

The fact that the linkage maps for rice chromosomes 1 ,2, and 5 do not coincide with the entire length of the chromosomes is not surprising. It is widely known that

correlations between centimorgans (cM) and chromosomal distance can vary widely. What is surprising is the apparent size of the chromosome regions not covered by the linkage groups. Are these regions areas of low recombination, and hence the present linkage groups cover the rapidly recombining areas? Do these regions contain low levels of polymorphism such that DNA clones isolated from them do not show RFLP? To what extent are chromosomal length, DNA, and recombinational length related? These are questions for further study.

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Efficient introduction of foreign gene into partially digested small cell groups of rice by polyethylene glycol

Jinshui Yang, Koulin Ge, Yunzhu Wang, and C.C. Tan

Procedures for rice protoplast culture have been improved, but the frequency of protoplast regeneration is still low (Coulibaly and Demarly 1986). Damage to the protoplasts by polyethylene glycol (PEG) and electroporation prevents more transformed calli from forming clones (Zhang and Wu 1988). We wished to develop and establish a better method for gene transfer of rice cells.

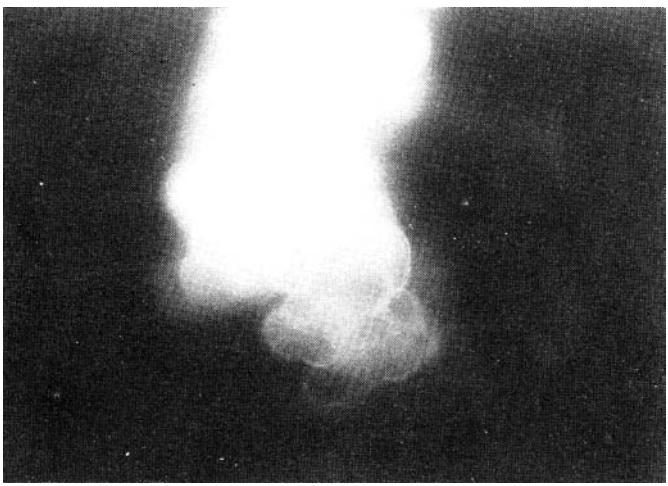
Embryo-derived calli subcultured on solid medium for 4 mo were transferred to N6 liquid medium supplemented with 2 ppm 2,4-dichlorophenoxyacetic acid (2,4-D) and cultured on a rotary shaker at 120 rpm. Suspension cultures of cell lines having 50-100 cells per group were established for gene transfer.

Each suspension culture was filtered through a 300-µm nylon mesh and transferred to new liquid medium. After 3 d of culture, about 0.5 g of culture was added to 10 ml of filter-sterilized enzyme solution (1% cellulase RS-10, 0.5 Macerozyme R-10, 2% hemicellulase, 7.7% mannitol CPW salts [27.2 mg KH_2PO_4/liter, 101 mg KNO_3/liter, 1.48 g $CaCl_2.2H_2O$/liter, 240 mg $MgSO_4.7H_2O$/liter, 0.16 mg KI/liter, 0.025 mg $CuSO_4.5H_2O$/liter]; pH 5.6) and digested at 26°C. After a given digestion time (Table 1), the mixture was sieved by 160-µm nylon mesh to remove the bigger cell groups. The partially digested cell groups were then separated from the protoplasts by a 40-µm metal mesh and collected with CPW solution (CPW salts, 7.7% mannitol, pH 5.6). The plasmid pBI121 (Clontech) harboring the ß-glucuronidase (*GUS*) gene was used for gene transfer (Jefferson et al 1986). Partially digested cell groups were suspended in 1 ml of CPW solution and incubated at 45°C for 10 min, followed by 0°C for 20s, and brought to 25 °C. Plasmid pBI121 (1 5 µg/ml), carrier calf thymus DNA (50 µg/ml), and 1 volume of 40% PEG-6000 (MW) solution were added sequentially at 15-min intervals. The mixture was incubated at 25 °C for 30 min. Two 2-ml aliquots of F medium (0.368 g KCI/liter, 18.4 g $CaCl_2.2H_2O$/liter, 0.8 g NaCl/liter, $Na_2HPO_4.2H_2O$/liter sterilized by filtration) were added at a 5-min interval. Cell groups were washed once with CPW solution and twice with N6 liquid medium, and then transferred to N6 medium with 2 ppm 2,4-D.

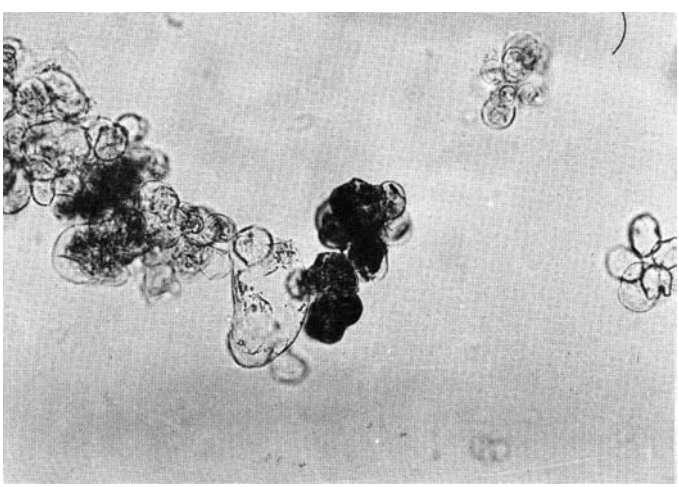
X-Gluc was used as the substrate for the assay of ß-glucuronidase (GUS) activity in treated cells (Jefferson et al 1987). Five milligrams of X-Gluc was dissolved in 50 µl dimethyl formamide and added to 50 mM $NaPO_4$ (pH 7.0) to 5 ml before assay. At 1 wk after gene transfer, aggregates from 0.5 ml of the treated cell groups were incubated with 150 µl of X-Gluc solution at room temperature for 3-4 d. The efficiency

Table 1. Effect of digestion time on amount of protoplast released.

Digestion time (min)	Protoplast released (cells × 10^5/ml)	Percent
60	0.212	7.7
120	0.275	10
180	0.625	22.7
240	2.42	88
420	2.75	100



1. Partially digested small cell groups in fluorescent brighter staining.



2. In situ GUS assay 1 wk after gene transfer of rice cells mediated by PEG with plasmid pBI121,

of gene transfer was determined by counting the number of blue cells. The best results were obtained with 120-min digestion, in which 10% of the protoplasts were released (Table 1). Fluorescent brighter staining showed that little cellulose remained on the surface of the enzyme-treated cells (Fig. 1). Only 12.9% of the total cells from the 120-min digest were found to become blue after the assay of GUS activity. Many more blue cells were produced by the use of a linear plasmid than by the use of a circular plasmid (Fig. 2).

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Introduction of exogenous DNA directly into rice

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Xing Guandang, and Zhang Yan

Since 1978, we have been studying techniques for introducing exogenous DNA directly into rice. Our objectives are to avoid the difficulties encountered in distant sexual hybridization, and to look for simpler gene transformation methods that do not involve protoplasts. The foreign DNA of donor rice plants that carries specific marker genes can be introduced into rice embryo sacs after self-pollination by the microinjection and pollen-tube pathway methods. The cells of fertilized eggs at this stage have not formed cell walls, so they can take up DNA. Using this method, genes controlling disease resistance and other traits from different sources have been successfully transformed into rice. Seedlings of the second generation were obtained after introducing p^{RR} plasmids with the *Kan*^{R} gene (PNEO105). Some offspring with high kanamycin tolerance were obtained; NPTII activity was detected; and Southern blot analysis was positive. The procedure for introducing exogenous DNA into plants after pollination is simple, and it is not necessary to prepare protoplasts to use in cell culture, or to regenerate plants. In principle, the procedure can be applied to any monocot or dicot cultivar (Duan and Chen 1985).

In 1987, we used this method for transferring DNA into rice to give the plants resistance to bacterial blight (BB). Using 9 BB-susceptible varieties as recipients, and total DNA from 2 resistant varieties as donors, 13 combinations were made involving 2,469 flowering glumes from 135 panicles. The seeds (671) were harvested in 1987 and sown the next spring. The 233 surviving seedlings constituted the first generation (D_1) of potentially transgenic plants. To test for resistance, all plants were inoculated with BB (3 races) by cutting their leaves before heading. After 2 wk, BB-resistant leaves were scored and classified on a 1-9 scale by percentage of infected area. One offspring from 856403 that had received foreign DNA from the donor rice Wase Aikoku 3 was highly resistant to BB. The degree of resistance was classified as 2, which was much higher than that for all other recipients (>7). The infected area was 5% of all leaves in the plant, the same as in the donor plants. Moreover, changes in ripening date, plant height, and panicle traits were found. The seeds from the resistant plants were sown in the spring of 1989 and became the D_2. All plants were BB resistant and had other characteristics similar to those in the D_1. Disease resistance was thus hereditary, and no segregation was seen in the offspring.

After harvest, the D_1 transgenic plant was root-propagated and moved into a greenhouse in the winter of 1988. The plant was moved out of the greenhouse the following spring and inoculated with BB. Not only was the plant still resistant, but its panicles now showed purple awns, one of the marker traits of the donor plant. Apparently, a DNA fragment of the donor that codes for purple awn introduced this trait into the recipient plants. Moreover, a comparison of the sodium dodecyl sulfate gel pattern of leaf proteins after two-dimensional electrophoresis showed that the pattern in D_2 transgenic plants was similar to that of the donor plants.

In summary, BB resistance can be transferred into recipient plants by the pollen-tube pathway method. This trait is transmitted to the offsprlng.

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Isolation of rice phytochrome genes from Nongken 58 and Nongken 58s

B. Wang, H.G. Zheng, Q.F. Xu, J.Z. Wang, D.D. Li,
S.T. Li, and Z.T. Zhang

Photoperiod-sensitive genetic male sterile rice Nongken 58s was derived from Nongken 58 through spontaneous mutation. Nongken 58s displays sterility when exposed to long days and appears fertile when exposed to short days (Shi and Deng 1986). Previous research suggests that sterility is determined by one or a group of regulatory gene(s), and the regulator is the length of illumination. Through a classical assay, red light induction and far-red light reversal, we know that phytochrome is involved in the expression of photoperiod-sensitive genetic male sterility in Nongken 58s (Table 1).

Kay et al (1989) have cloned the phytochrome gene from rice. To know the differences in DNA level and their relation to the expression of light-induced photoperiod-sensitive genetic male sterility, we are trying to isolate and compare the phytochrome genes from Nongken 58 and Nongken 58s. The genomic libraries of Nongken 58 and Nongken 58s have been constructed using phage EMBL4 as the vector. The recombinant bacteriophage numbers are 8.6×10^5 and 2×10^6, respectively, which are both up to the desired library size. By using 3-kb oat phytochrome complementary DNA (cDNA) as a probe (Hershey et al 1985), we isolated 2 clones (from Nongken 58 and Nongken 58s, respectively) containing homologous sequences to the oat phytochrome cDNA probe. In spot-blot hybridization, hybridization is proportional to the recombinant bacteriophage DNA. Subcloning and a comparative study of the phytochrome genes are being done in our laboratory.

The application of photoperiod-sensitive genetic male sterile rice Nongken 58s to hybrid rice production has simplified seed production by using a two-line instead of a three-line system. But little is known about the molecular mechanism. Phytochrome is involved in the morphogenesis of photoperiod-sensitive genetic male sterility induced by light. The comparison of the two phytochrome genes will help us get a better picture of events at the molecular level.

Table 1. Assay for involvement of phytochrome in expression of photoperiod-sensitive genetic male sterility in Nongken 58s rice.

Treatment[a]	Morphogenesis
Short day	Fertile
Long day	Male sterile
Short day + red light	Male sterile
Short day + red light + far-red light	Fertile

[a]Long day > 14 h, short day <10 h, red light: λ_{max}= 666 nm, far-red light: λ_{max}= 730 nm.

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Restriction fragment length polymorphism for characterizing monosomic alien addition lines of *Oryza sativa* having a single chromosome of *O. brachyantha*

O. Panaud, G. Magpantay, E. Galinato, D. Mahapatra, L.A. Sitch, and G.S. Khush

Wild species of the genus *Oryza* are an important source of useful genes for genetic improvement of rice. Among the various wild species, *O. brachyantha* (2n = 24, genome FF) is resistant to yellow stem borer and whorl maggot. From the BC_3 progeny (*O. sativa/O. brachyantha//O. sativa*), 6 aneuploid plants showing 25 chromosomes were isolated and characterized by restriction fragment length polymorphism (RFLP) analysis. Single-copy DNA probes of the rice genomic library (RG probes) (provided by S.D Tanksley) were labeled with nonradioactive digoxigenin-dUTP (Boehringer-Manheim, Genius kit). The DNAs from the two species were digested with five restriction enzymes (*Eco*RI, *Eco*RV, *Bam*HI, *Hin*dIII, and *Dra*I) and hybridized with a subset of RG probes containing at least one marker per chromosome. RFLP analysls showed that the six addition lines belonged to three groups. Three lines had chromosome 7 of *O. brachyantha,* showing the bands of both *O. brachyantha* and *O. sativa* with the RG probe of chromosome 7, and the *O. sativa* pattern with the RG probes of the 11 other chromosomes. We designated these three lines MAAL-7. Two lines had chromosome 11 of *O. brachyantha* (MAAL-11), and one had chromosome 6 (MAAL-6). These findings demonstrate the usefulness of the RFLP technique for characterizing monosomic alien addition lines of rice. In addition, this analysis will be used to monitor introgression of *O. brachyantha* genetic material in the 24 chromosome derivatives. A similar analysis is being used to identify monosomic alien addition lines of other *Oryza* species.

Notes

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Restriction fragment length polymorphism analysis of size of chromosomal segments retained around bacterial blight resistance genes in near-isogenic lines of rice

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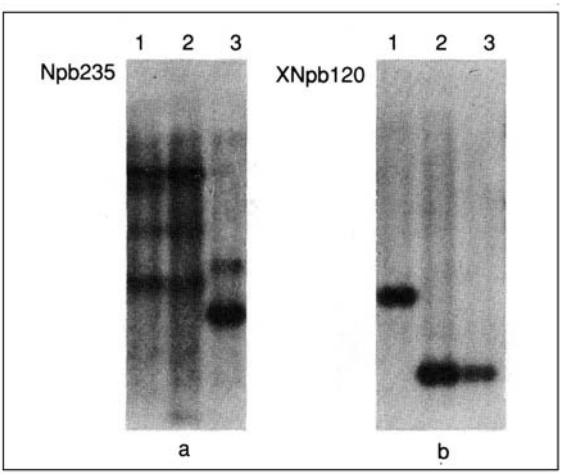
Several genes for resistance to bacterial blight were introduced into rice cultivars by repeated backcrossing, and near-isogenic lines were established (Ogawa et al 1988). We measured the sizes of the introgressed DNA segments in some of the near-isogenic lines by using a high-density map of restriction fragment length polymorphism (RFLP) markers (Kishimoto et al 1989, Yano et al 1990).

The genotypes at each RFLP marker locus flanking the resistance genes in the near-isogenic lines were determined by hybridization of labeled RFLP clones with restriction enzyme-digested genomic DNA extracted from the recurrent parent IR24, from isogenic lines, and from donor parents. Because of the sequence divergence between IR24 and the donor parents, RFLP could be identified for the markers (Fig. 1).

Line IR-BB1 (BC_4F_6; IR24*5/Kogyoku) contains the flanking segment of *Xa-1* on chromosome 4 from the resistant parent Kogyoku. RFLP marker Npb235 showed the genotype of Kogyoku in IR-BB1. Physiological marker *Ph* was also analyzed, and this locus in IR-BBI was of the Kogyoku type. Therefore, the size of the flanking segment of *Xa-1* was estimated to be approximately 7.2% based on the maps of Sakaguchi (1967) and Yano et al (1990) (Fig. 2a). The line also possessed the segment introgressed from Kogyoku in another region. To determine the genetic distance between *Xa-1* and nearby RFLP markers, an F_2 population and F_3 lines segregating for *Xa-1* resistance were analyzed. RFLP loci of XNpb102 and XNpb120 were linked to *Xa-1* with recombination values of 6.7 and 21.5%, respectively (Fig. 2a).

RFLP analysis of IR-BB3 (BC_4F_8; IR24*5/Chugoku 45) showed a crossover event between XNpb78 and XNpb186 on chromosome 11 in the process of developing this near-isogenic line (Fig. 2b). Since the segment containing the loci of XNpb78, XNpb181, and XNpb186 was from the resistant donor Chugoku 45 with *Xa-3, Xa-3* was inferred to be near the three RFLP markers in the linkage map.



1. Southern blot analysis of size of introgressed segments from resistant donor parents in near-isogenic lines for bacterial blight resistance genes. a) RFLP marker Npb235 exhibits hybridizing bands identical to those of donor parent Kogyoku and near-isogenic line IR-BB1, indicating segment at locus of Npb235 is introgressed from donor. b) XNpb120 exhibits hybridizing bands identical to those of recurrent parent IR24 and IR-BB1, showing segment at locus of XNpb120 of IR-BB1 is from IR24. Lane 1 = Kogyoku with *Xa-l,* 2 = IR-BB1 with *Xa-1,* 3 = IR24 without *Xa-1.*

In addition, RFLP markers Npb181 and Npb186 showed genotypes of the resistant donor IR20 in IR-BB4 (BC_4F_8; IR24*5/IR20), indicating that at least the regions between both markers were introgressed from IR20 in IR-BB4.

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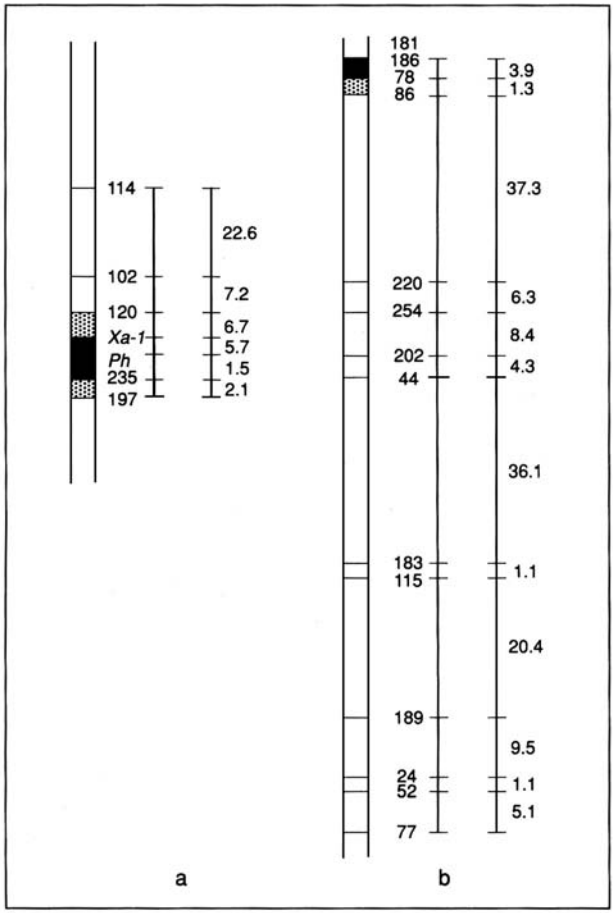

2. Ideograms showing genotypic constitution of chromosomes 4 and 11 relevant to respective genes *Xa-I* and *Xa-3* for bacterial blight resistance in near-isogenic lines. a = IR-BB1 with *Xa-1,* b = IR-BB3 with *Xa-3.* Empty intervals indicate segments derived exclusively from IR24. Blackened intervals indicate segments derived from donor parent. Shaded intervals indicate crossover event. RFLP linkage maps of chromosomes 4 and 11 (Yano et al 1990) are shown on right side of each ideogram.

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Accumulation signals of rice endosperm proteins

N. Mitsukawa and K. Tanaka

To Improve the digestibility and nutritional quality of endosperm proteins, it is important to understand the mechanism of the intracellular localization of individual polypeptides into their specific sites.

We constructed complementary DNA (cDNA) libraries for the messenger RNAs appearing in developing rice seeds. Several cDNA clones encoding glutelins (Masumura et al 1989a), 10-kDa prolamin (Masumura et al 1989b), 13-kDa prolamins (Kim and Okita 1989, Masumura et al 1990), and rice allergen proteins were isolated and analyzed. The amino acid sequences of the precursor polypeptides were deduced from these cDNA sequences.

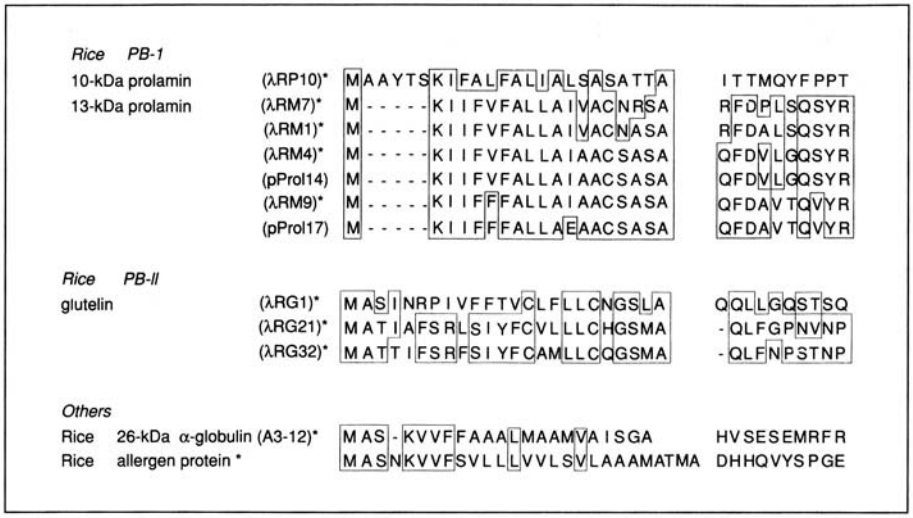
The 13-kDa prolamin polypeptides vary in molecular size and electrophoretic nature, and are classified into 3 groups by amino acid sequence:

- Class I: larger molecular size, containing cysteine residues
- Class II: smaller molecular size, containing no cysteine residues
- Class Ill: smaller molecular size, containing cysteine residues

Labeling with cysteine-specific fluorescence dye indicated that some 13-kDa prolamin polypeptides have no cysteine residues. This was supported by the amino acid sequences deduced from cDNA sequences. Although the structure and amino acid composition of 13-kDa prolamin polypeptides are different, their signal sequences are highly conserved.

The distribution of 26-kDa a-globulin was investigated. This storage protein was localized from the rough endoplasmic reticulum fraction to the protein body II (PB-II) fraction by sucrose density gradient centrifugation. Corresponding to the difference in the distribution of this polypeptide, the signal sequence of 26-kDa a-globulin showed no homology with those of rice glutelins specifically accumulating in PB-ll (Fig. 1).

These results strongly suggest that the intracellular localization of rice endosperm polypeptides determines their accumulation sites mainly by the aminoterminal structures including signal sequences of the precursor polypeptides.



1. Structures of signal sequences of rice endosperm proteins. Homologous amino acids are boxed. * = clones isolated in authors' laboratory.

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Assessment of protoclonal variation in seed progeny of transgenic plants regenerated from rice protoplasts

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Fertile transgenic rice plants were regenerated by somatic embryogenesis of callus derived from protoplasts isolated from suspension cells of rice (*Oryza sativa* L. var. Taipei 309) electroporated with pCaMVNEO carrying the neomycin phosphotransferase II (*npt*II) gene under the control of the CaMV 35S promoter (Zhang et al 1989). One protoplast-derived R_0 transgenic plant developed 7 tillers and produced 59 seeds. Sixteen green plants were obtained when these seeds were germinated on agar-solidified, hormone-free Murashige and Skoog's (1962) medium containing 20 µg kanamycim sulfate/ml. Subsequently, these plants were established in the glasshouse. The phenotypic characteristics of the R_1 transgenic plants were compared with the phenotypic variation already reported in nontransformed R_1 seed progeny of plants regenerated from protoplasts of the same rice cultivar (Abdullah et al 1989) in an attempt to identify any positive or negative shifts associated with transformation.

At maturity, the R_1 transgenic plants varied in height from 63 to 99 cm. They produced more tillers but flowered later than nontransformed R_1 plants and nontransformed seed-derived plants. Flag leaf length-width ratio in the R_1 transgenic plants was similar to that of nontransformed seed-derived plants. Panicles of R_1 transgenic plants were shorter, with fewer primary branches and spikelets than those of nontransformed R_1 plants and nontransformed seed-derived plants.

Molecular analysis of R_1 transgenic plants, involving dot blotting and Southern hybridization, confirmed the presence of the *npt* II gene in all R_1 transgenic plants and of neomycin phosphotransferase activity in the leaves of 7 of the 16 plants. Five of the R_1 transgenic plants were fertile and produced morphologically normal florets with viable pollen. However, seed production was limited; the 5 plants produced 79 seeds. Sterile plants failed to develop panicles or had abnormal panicles.

Details on the characteristics of the R_1 transgenic plants will be published elsewhere. Transgenic R_2 rice plants are also being characterized in the glasshouse at the University of Nottingham and in a field trial in collaboration with W. Schuh (Pennsylvania State University, USA) through support from the Rockefeller Foundation.

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Tissue culture and somatic hybridization of indica and japonica rices

I.H. Slamet

A potential technique for plant improvement is protoplast fusion (Toriyama and Hinata 1988). The establishment of an efficient protoplast system from which plants can be regenerated is a prerequisite for somatic hybridization (Abdullah et al 1986). Against this background, experiments were undertaken to investigate the optimum callusing medium for indica and japonica rice varieties, the effect of nurse cells on protoplast culture, and the attempted production of somatic hybrids between indicas and japonicas.

The tissue culture responses of indica type I (Glaszmann 1987) rice varieties IR54, IR43, IR36, and IR28 were compared with those of japonica varieties T309, Fujisaka 5, and Minehikari. In general, mature seed scutellum cultured on agarose-solidified Linsmaier and Skoog (LS) medium supplemented with 2.5 mg 2,4-dichlorophenoxyacetic acid (2,4-D)/liter showed the highest percentage of embryogenic callus formation. Of the indica varietles tested, IR54 showed the highest level of embryogenic callus formation on all media assessed. Response was optimal with filter-sterilized LS medium containing 2.5 mg 2,4-D/liter and 30 g maltose/liter (Table 1).

Japonica explants produced greater amounts of embryogenic callus, which was more friable than that obtained from indica explants. Production of phenolic compounds was encountered during indica cell suspension initiation. Japonica callus was less prone to browning and thus more suitable for suspension culture.

The effect of nurse cells on protoplast culture was thoroughly investigated as a model system for low-density culture of heterokaryons. Three nurse culture techniques were used. Specifically a membrane filter (0.8 µm cellulase acetate)(Lee et al 1989), a millicell unit, and mixed nurse cells (Kyosuka et al 1987) were compared with protoplast culture without the use of feeder cells (Fig. 1).

In contrast to the literature, where cell suspension protoplasts have been universally used for both parents in rice fusion programs, the work presented here uses the combination of leaf-base protoplasts (Finch et al 1990) from indica rice IR54 and cell suspension protoplasts from japonica rice T309. Protoplasts were fused at a ratio of 2:1 (IR54:T309) and aligned in an alternating current field (148 V/cm, 1 MHz), In general, two direct current pulses of 898 V/cm with durations of 500 and 100 µs (with a pulse delay of 1 s) gave the highest number of heterokaryons.

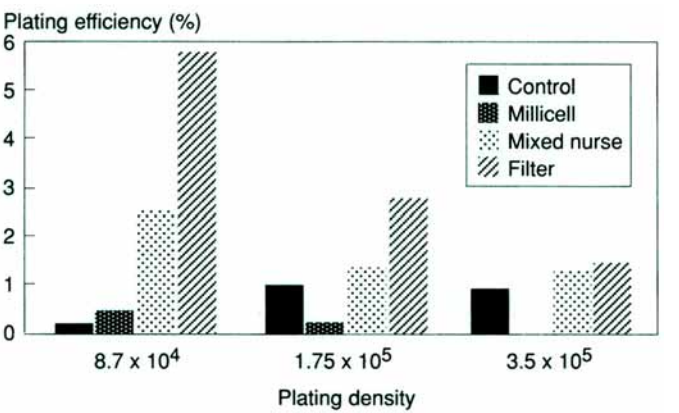
Initially, biochemical selection of fusion products was attempted. Iodoacetamide-treated T309 cell suspension protoplasts were fused with culture-recalcitrant IR54 leaf-base protoplasts. However, iodoacetamide proved to be inhibitory for heterokaryon division. Subsequently fusion products were mass cultured on agarose-solidified (1.2% wt/vol Seaplaque) Kao's protoplast regeneration medium. Heterokaryon division was identified visually by the presence of chloroplasts in a colorless background.

Isozyme analysis of putative somatic hybrid callus showed the combination of the two parental bands. Putative somatic hybrid plants are in the glasshouse and will shortly be characterized using isozyme and restriction fragment length polymorphism analyses.

Table 1. Effect of carbohydrate source on production of embryogenic callus in IR54.[a]

	Autoclaved		Filter-sterilized	
Supplement (at 25 g/liter)	Mature seed scutella (%) producing callus	Embryogenic callus (%)	Mature seed scutella (%) producing callus	Embryogenic callus (%)
Cellobiose	90.0 ± 6.6	14.3± 5.3	100	15.8± 3.2
Maltose	72.5 ± 1.6	9.6± 5.3	100	15.9± 2.4
Mellibiose	85.0 ± 9.0	15.2± 3.2	100	15.1± 3.5
Sucrose	73.0 ±11.7	7.7± 3.0	60.0 ± 7.0	14.3± 2.7
Galactose	35.0 ± 2.4	5.4± 6.5	90.0 ± 3.0	10.9± 4.3

[a] Linsmaier and Skoog medium. Each value is the mean of 3 experiments ± standard error. 50 seeds were used for each treatment.



1. Nurse culture of rice protoplasts (28 d after plating).

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Somatic hybridization studies between rice and *Porteresia coarctata*

R.P. Finch

The development of varieties capable of growth on marginal land may increase rice production. Wild rices are a natural source of genes controlling stress resistance traits, but many are sexually incompatible with cultivated rice. Protoplast fusion between rice and *Porteresia coarctata,* a highly salt-tolerant, sexually isolated relative, has been investigated in a study of somatic hybridization between these two species (Finch et al 1990).

Since cell cultures of the recalcitrant *P. coarctata* have not yet been established, protoplasts were isolated from chopped leaves of young, emerged rhizomes. The tissue was preplasmolyzed in CPW11SA medium (Finch et al 1990) and incubated in enzyme solution (CPW11SA with 1% wt/vol Cellulase RS and 0.1% wt/vol Pectolyase Y23; 4 h). The protoplasts were sieved (30 µm), pelleted (80 g, 5 min), and washed twice in electrofusion solutlon (EF11; 11% wt/vol mannitol, 0.5 mM $CaCl_2$). Rice protoplasts were isolated from embryogenic cell suspension cultures of the japonica variety Taipei 309 as previously described (Abdullah et al 1986, Finch et al 1991). The protoplasts were mixed (1:1, 2×10^5/ml in EF11) and fused using a newly developed electrofusion apparatus (B. Jones, pers. comm.). Fusion frequencies were determined by flow cytometry based on the dual fluorescence of heterokaryons with light excitation at 457 nm (red autofluorescence of *P. coarctata* mesophyll protoplasts, green fluorescence of rice cell suspension-derived protoplasts prestained with fluorescein diacetate). The treated protoplasts were cultured en masse in a modified Kao's protoplast regeneration medium (Abdullah et al 1986) at 8×10^5/ml in the dark at 28 °C.

Protoplast yields of $30 \pm 0.13 \times 10^6$/g fresh weight were routinely obtained. These protoplasts did not divide in culture. Optimal fusion parameters were AC: 80 V/cm, 1 MHz, 10 s, increased to 120 V/cm, 2 s; DC: 4 pulses (40, 10, 10, 10 µs, respectively), 300 V/cm, interpulse duration 1 s. Heterokaryon viability was 75-85%. Heterokaryons were readily identified as a discrete, quantifiable population of dual fluorescent particles using the flow cytometer. Heterokaryon formation frequency averaged 4.2%. Cultured heterokaryons, which were visually identifiable, began to expand and divide within 2-3 d, and distinct cytoplasmic strands were visible Within 8-9 d, some of the heterokaryons had undergone further division to form identifiable microscopic colonies, since they still contained chloroplasts from the orignal heterokaryon.

The isolation of large numbers of stable *P. coarctata* mesophyll protoplasts permitted a study of their fusion with regenerable protoplasts of rice. By subjecting protoplast mixtures to a high-voltage AC field but relatively low-voltage, multiple DC pulses, it was possible to obtain adequate fuslon frequencies with high heterokaryon viability. When the heterokaryons were cultured without selection (in the presence of dividing rice protoplasts, which possibly acted as a nurse culture), they divided to form putative hybrid microcolonies. Plants have recently been regenerated from callus derived from mass-cultured protoplast fusion mixtures, and these are currently being analyzed for hybridity. Because it was possible to identify and accurately quantify populations of heterokaryons using the flow cytometer, it is feasible to use fluorescence-activated cell sorting for this system as a large-scale heterokaryon isolation technique.

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Stress tolerance in rice cell lines

K.-I. Mori and T. Kinoshita

Somaclonal variation combined with in vitro selection is promising in breeding rice for disease resistance and stress tolerance. However, selection at the cellular level does not always contribute to a heritable trait in the regenerated plant. Therefore, we compared genetic tolerance for salt and herbicides at both the plant and cellular levels.

For salt tolerance, successive screening of calli resulted in tolerant cell lines that survived even when treated with 3% NaCl. However, it is uncertain whether the increased tolerance was due to genetic change or to metabolic adaptation of the cultured cells. For herbicide tolerance, we used seed calli induced from five cultivars and two mutants showing sensitivity to propanil and bentazon at the plant level. Callus growth was inhibited differentially at high concentrations of propanil and bentazon. Calli from a propanil-sensitive mutant showed strong growth inhibition only in the treatment with propanil, while calli from a bentazon-sensitive mutant were prominently inhibited by treatments with both bentazon and propanil. Thus, the gene responsible for herbicide sensitivity may have exerted pleiotropic action only at the cellular level.

Tolerance for benthiocarb and molinate at the plant level was correlated with tolerance at the cellular level except in one cultivar (Norin 8). After repeated screening with both herbicides, two plants (R_1 generation) were regenerated from tolerant cell lines. By using R_2 plants propagated from R_1 plants, tolerance for benthiocarb and molinate was compared with that of the control (no selection) at both the plant and cellular levels. Somaclonal selection for both herbicides was successful, because a significant increase in tolerance was confirmed at both the plant and cellular levels in the R_2 (Table 1).

In somaclonal selection of herbicides and salt, callus clones via primary selection showed significantly increased tolerance in secondary screening for tolerance. Therefore, repeated selection for tolerance was effective in raising the degree of tolerance in cell lines. In regeneration from tolerant cell lines, the somaclonal selection of herbicides contributed to a significant increase of tolerance at the plant level, possibly through a genetic change (mutation, etc.). Further investigations are needed to elucidate the genetic mechanism of tolerance in successful cases of somaclonal selection.

Table 1. Survival rate of callus produced from R_2 seeds and inhibition of plant height in R_2 in herbicide tolerance test.

Callus [a]	Herbicide	Cellular level		Plant level	
		Concentration (ppm)	Survival (%)	Concentration (ppm)	Relative plant height (%)
N-61	None		100		100
	Benthiocarb	100	100	0.25	63
		200	94	0.50	33
		400	86		
	Molinate	50	98	0.50	48
		100	96	1.00	18
		200	80		
NB-200	None		100		100
	Benthiocarb	100	100	0.25	84
		200	100	0.50	65
		400	100		
NM-100	None		98		100
	Molinate	50	96	0.50	49
		100	96	1.00	29
		200	98		

[a] NB-200: R_2 plant or calli produced regenerant after somaclonal selection in 200 ppm benthiocarb in N-61. NM-100: R_2 plant or calli produced regenerant after somaclonal selection in 100 ppm molinate in N-61.

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Novel proteins involved in dedifferentiation and redifferentiation in rice *Oryza sativa* L.

A. Tanaka and S. Tano

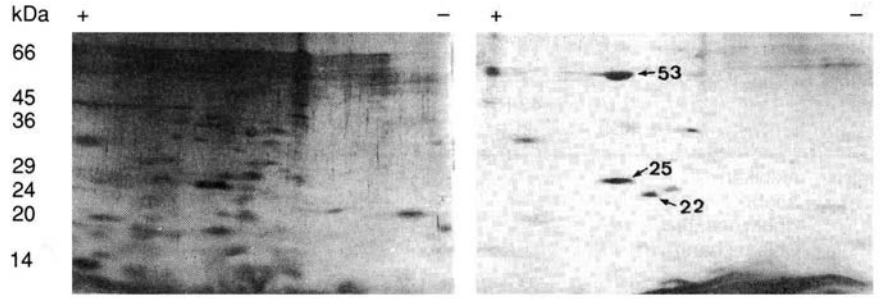
The mechanisms of dedifferentiation and redifferentiation in rice are not clear in many respects. Most information concerning the molecular aspects of somatic embryogenesis in plants has been obtained with carrot tissue on cell cultures (Sung and Okimoto 1983). Similar molecular markers have been identified in embryogenic callus culture of monocotyledonous plants (Chen and Luthe 1987, Hahne et al 1988). In each system, however, the relationship between dedifferentiation and redifferentiation has not been established. The present study was conducted to clarify the behavior of proteins in the early stages of dedifferentiation and redifferentiation.

One culture line was used to compare germination from embryos on Gamborg's basal medium (Gamborg et al 1968) with induction of callus from embryos on the medium containing 3 mg 2,4-dichlorophenoxyacetic acid (2,4-D)/liter, and another to compare mature callus formation with regenerating callus on medium containing 5 mg 6-benzylaminopurine (BAP)/liter. In qualitative studies, total soluble or alcohol-soluble protein extracts were analyzed by two-dimensional polyacrylamide gel electrophoresis.

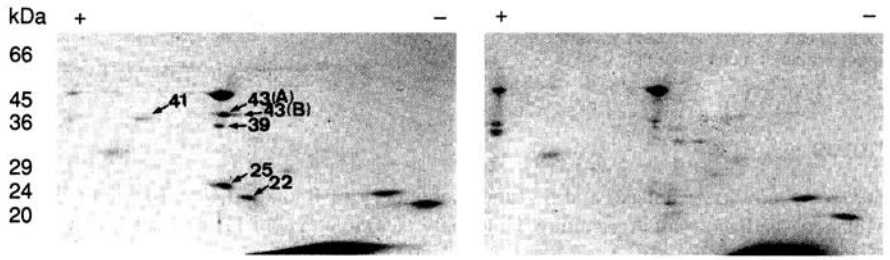
Total cellular protein extracts from germinating embryos and callus-forming embryos indicated qualitative differences in the 2 tissues after 5 d of Incubation, but large differences were not found at earlier stages. In redifferentiation from callus, differences between mature callus and regenerating callus on medium with BAP were very slight.

On the other hand, the alcohol-soluble fraction showed distinct differences in the two tissues in each line. The major proteins in the callus-forming embryo extracts were 53, 25, and 22 kDa. They were prevalent in callus-forming embryos and increased after incubation (Fig. 1). For further incubation on medium with 2, 4-D about 3 wk after induced callus matured, proteins of 43, 43, 41, and 39 kDa were newly synthesized (Fig. 2). These spots were probably specific proteins for mature callus.

Meanwhile, when callus was transferred to medium with BAP, the callus-specific proteins decreased and were not produced as early as the transfer (Fig. 2). Two proteins of 25 and 22 kDa, which were found in callus-forming embryos, also decreased. Furthermore, these six proteins commonly existed in mature callus of six rice varieties belonging to three subspecies—japonica, Indica, japonica/indica hybrids—and they were mostly reduced in regenerating callus on the medium with BAP (Table 1).



1. Silver-stained 2-dimensional polyacrylamide gel electrophoresis of alcohol-soluble proteins. Submerged embryos of japonica variety Akihikari were incubated for 5 d on basal medium (left) or on medium with 3 mg 2,4-D/liter (right), where callus was induced from embryo.



2. Coomassie Brilliant Blue R-stained 2-dimensional polyacrylamide gel electrophoresis of alcohol-soluble proteins. Mature calli of Akihikari were subcultured on medium with 3 mg 2,4-D/liter (left) or kept on medium with 5 mg BAP/liter for 67 h (right), where calli regenerated.

The proteins of 25 and 22 kDa are probably required for callus induction, and the proteins of 43, 43, 41, and 39 kDa are specific for callus growth. These proteins may play a regulatory role in opposite ways for dedifferentiation and redifferentiation.

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Table 1. Comparison of changed protein spots among rice varieties.

Medium	Variety [a]	Presence of proteins [b]					
		43 (A) kDa	43 (B) kDa	41 kDa	39 kDa	25 kDa	22 kDa
Control	Akihikari	+	+	+	+	+	+
	Asahi	+	+	+	+	+	+
	Chukyoasahi	+	+	+	+	+	+
	Chiem chanh	+	+	+	+	+	+
	Milyang 20	+	+	+	+	+	+
	Milyang 21	+	+	+	+	+	+
BAP	Akihikari	–	–	–	–	–	–
	Asahi	–	–	–	+	+	–
	Chukyoasahi	+	–	–	+	+	–
	Chiem chanh	–	–	+	–	+	–
	Milyang 20	–	–	+	+	+	–
	Milyang 21	–	–	–	+	–	–

[a] Mature calli (control were kept on medium with 5 mg BAP/liter for 2 d. [b] + = detectable, (–) = undetectable or much lower than control.

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Cytohistological observations on plantlet regeneration from rice calli

A.B. Mendoza, Y. Futsuhara, and Y. Takeoka

The role of genetic background on callus formation and plant regeneration from seed callus has been extensively studied (Abe and Futsuhara 1986). The origin and ontogeny of somatic embryos from seed callus have been shown to be single cells from the basal scutellar epithelium (Jones and Rost 1989). However, the role of the callus induction medium in conditioning regeneration by somatic embryogenesis in different rice varieties has not been reported. This study presents efficient regeneration of green plants by somatic embryogenesis in rice seed-derived callus.

Twenty seeds from indica and japonica varieties were induced to form callus on different callus induction media and then transferred to regeneration medium composed of Murashige and Skoog's (MS) salts supplemented with 1 mg naphthaleneacetic acid (NAA)/liter, 2 mg kinetin/liter, 2 mg 6-benzyladenine (6-BA)/liter, and 3% sucrose. adjusted to pH 5.8 and gelled with 0.6% agar.

The ability to regenerate plants was promoted by the amounts and kinds of growth regulators added to the callus induction medium. MS media components for positive results were as follows: MSD = 2 mg 2.4-dichlorophenoxyacetic acid (2.4-D)/liter; MSD.5 = same as the preceding plus 0.5 mg kinetin/liter; MS2 = 0.5 mg NAA/liter plus 0.2 mg each of 2,4-D and kinetin/liter: MS6 = 0.2 mg each of 2,4-D and NAA/liter plus 0.5 mg 6-BA/liter; MS7 = same auxins as MS6 plus 0.5 mg kinetin/liter; MS8 = same auxins as MS6 plus 2 mg 6-BA/liter. Regeneration data for all varieties studied are in Table 1. A high number of regenerated plantlets in indica types was associated with early onset of regeneration at 1 wk after transfer to regeneration medium. while japonicas like Nipponbare and Murasaki-ine regenerated 1 mo later. Sakaikaneko, a japonica, regenerated plants from callus 2 wk after culture on the callus lnduction medium and continued to form new plants when transferred to regeneration medium.

To study the regeneration process, calli were induced for 1 mo in those media in which better regeneration was obtained. Calli were transferred and kept on regeneration media for 4-7 d and fixed in formalin:acetic acid. 70% ethanol (5%:5%:90%) solution. Cytohistological observations by light microscopy of callus paraffin sections at 5 µ revealed that compact calli in the indica varieties were associated with abundant vascular bundles in the callus. Sakaikaneko had soft and friable callus, but vascular bundles were also observed. In Nipponbare and Murasaki-ine, highly meristematic celis were seen inside the callus bounded by parenchyma cells, although many also

Table 1. Effect of callus induction medium on plant regeneration.

Variety	Regenerated plants (no.) on given medium[a]					
	MSD	MSD.5	MS2	MS6	MS7	MS8
Nipponbare	3(2)	20(7)	0	0	0	0
Sakaikaneko	0	0	17(4)	0	0	0
Murasaki-ine	1(1)*	2(2)*	0	0	0	0
UPLRi-3	0	0	104(14)	0	5 (1)*	0
UPLRi-4	0	0	21(3)	0	0	0
UPLRi-5	9(4)	49(5)	0	0	0	0
IR8	0	0	0	3(2)	1(1)*	187 (10)

[a] Numbers in parentheses are numbers of calli regenerating plants. * = only albino plants regenerated.

occurred on the surfaces of parenchyma cells. Embryogenic cells in all the varieties appeared on the periphery of the callus as elongated and tubular, with dense cytoplasm and sometimes vacuolated with a highly visible nucleus. They appeared in clusters, and in these regions could be found different stages of somatic embryo formation. Secondary shoot formation of Sakaikaneko, UPLRi-3, and IR8 appeared common among germinating somatic embryos. In IR8, formation of adventitious leaf and shoot in the scutellar area was observed among germinating embryos. Thus, multiple shoots arise from somatic embryos.

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Production of aneuhaploids (2n=13) in rice *Oryza sativa* L. by anther culture of trisomic plants

Wang Zixuan and N. lwata

To obtain aneuhaploids (2n=13) and tetrasomics (2n=26) of rice *Oryza sativa* L., anther culture was carried out in a trisomic series of japonica variety Nipponbare (Iwata and Omura 1984), except for one type having no normal anther. About 125,500 anthers from 11 types of trisomics were plated on N6 inducing medium with 2 mg 2,4-dichlorophenoxyacetic acid/liter, and 3,734 regenerated plants were obtained. There were significant variations in both callus induction rate and plant regeneration rate among the types of trisomics. Morphological and cytological studies were done on the plants. In each population of plants derived from the 10 types of trisomics, a small number of plantlets showed unique morphological features compared with most normal plants.

The chromosomes of plants with unique morphological feaures were counted in root tip cells. Forty-three plants with 2n=13 chromosomes were detected in the plants derived from 9 trosomic types—A (pale), B (awned), C (small grain), D (erectoides), E(spreading), G (pseudo-normal), H (large grain), L (short panicle), and O (grassy)—and O (grassy)— and 51 aneuploid plants with 2n=26 chromosomes were detected in the plants derived from 9 trisomic types— A, B, C, D, E, F (rolled leaf), H, L, and O (Table 1). Other aneuploids such as monosomics and 2n=39 plants were also obtained.

Cytological studies of the aneuploids with 2n=13 and 2n=26 derived from trisomic types C, E, and H suggest that the extra chromosomes of those aneuploids originated from their donor trisomic plants. Both aneuhaploids and tetrasomics of rice could be obtained by culturing the anthers of trisomics, and those aneuploids could serve as useful materials in breeding and genetic studies.

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Table 1. Chromosome number of plants derived from each trisomic type by anther culture.

Trisomic type	Plants obtained (no.)	Plants (no.) with 2n=									
		12	13	23	24	25	26	36	39	48	73
A	317	7	11				14		1		
B	236	4	8		3		11				
C	616	68	7 (1[a])	1	16	1	8	2	2	2	
D	470	57	4		17	1	5		1		
E	384	54	3		6		2			2	
F	125	23			6	3	6		1	1	
G	351	64	2[a]		10	1				1	
H	205	7	6	1	4	1	3				1
L	228	44	1[a]		14	4	1	1		1	
M	560	91		1	13					1	
0	242	40	1		18	1	1	1		1	
Total	3734	459	43	3	107	12	51	4	5	9	1

[a]Extra chromosome is a fragment.

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Assessment of gametic selection in indica/upland japonica rice anther cultures by isozyme markers

E. Guiderdoni

Wide integration of anther culture-derived plants in rice genetics requires testing the existence of in vitro gametic selection. To know whether androgenetic microspores represent a random gametic array, we compared segregations and recombinations of 10 and 12 isozyme marker genes in the anther culture derivatives—nonmorphogenic microspore-derived calli (NMC) and anther-culture plants (ACP)—and the F_2 progenies of indica/japonica hybrids IR64/Azucena and IR64/IRAT216.

Significant departures from the expected 1:2:1 ratio were detected in 2 of the 10 segregations monitored in the F_2 progenies of IR64/Azucena and in 6 of the 12 segregations from IR64/IRAT216 (Table 1). These deviations were due mostly to dissimilar frequencies of the parental alleles. Segregational bias is a common feature of indica/japonica progenies that has been explained by several genetic models involving the action of sterility genes.

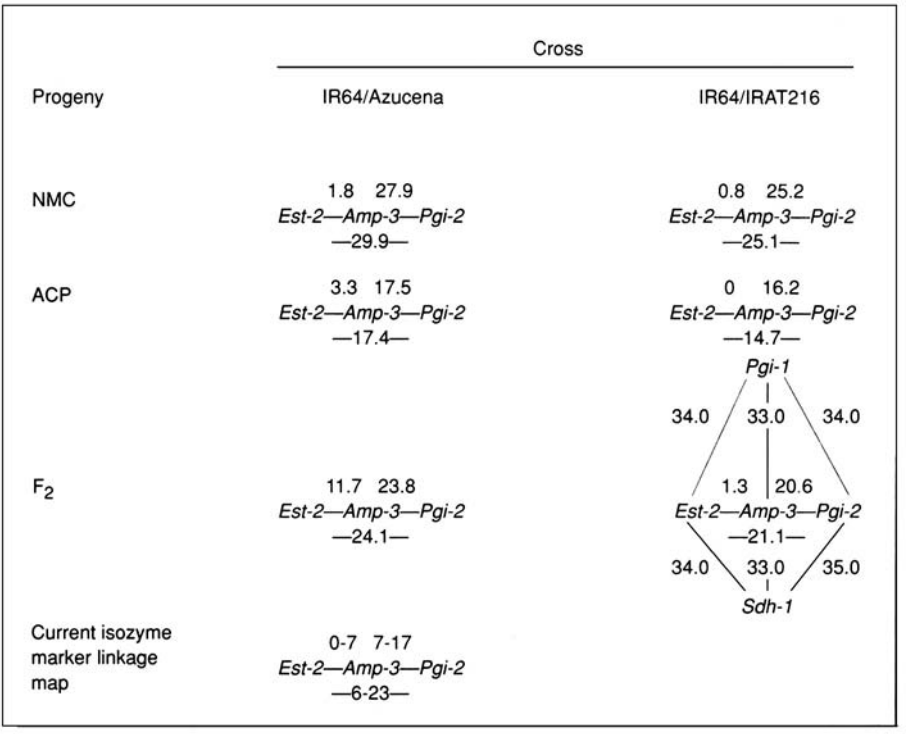
The alleles in excess in the F_2s were consistently overrepresented in the NMC populations. These alleles were also generally overabundant in the corresponding ACP populations. In these cases, there is no difference among the allelic frequencies calculated from the F_2, NMC, and ACP data at the deviated loci. Conversely, the segregational skewings at 5 isozyme loci found in the F_2 and NMC progenies segregated 1:1 in the ACP population of IR64/IRAT216. Allelic frequencies at these loci calculated from ACP data differed significantly from those estimated from F_2 and NMC data, suggesting that selection of the microspores occurred at two stages of gametogenesis: before the mid-uninucleated stage and shortly before first pollen mitosis. The latter could be due to the action of sterility genes in selecting the late uninucleated microspore pool, which is known to produce mostly nonregenerating calli when cultured.

Two additional deviations were found specific to anther culture derivatives in IR64/Azucena, and five in IR64/IRAT216. However, this selection related to the mechanisms of androgenesis appeared neutral with regard to indica and japonica differentiation, since the pooled allelic frequencies did not deviate from a 1:1 ratio.

Chi-square values for independence were insignificant for all the combinations of pairs of loci except for *Est-2—Amp-3,Amp-3–Pgi-2,* and *Est-2—Pgi-2* in the NMC, ACP, and F_2 progenies of IR64/Azucena and IR64/IRAT216 (Fig. 1). The linkage estimates calculated from anther culture-derived materials were consistent with those of the F_2s and with current knowledge of the isozyme locus linkage map.

Table 1. Segregations of heterozygous isozyme markers among nonmorphogenic microspore-derived calli (NMC), anther culture-derived plants (ACP), and F_2 progenies generated from IR64/Azucena and IR64/IRAT216. Segregation of isozyme phenotypes follows the order JJ:JI:II for F_2 progeny and J:I for NMC and ACP populations (J and I are alleles contributed by japonica and indica parent, respectively). Segregations given in boldface and in italics deviate significantly (at least 5%) from expected 1:2:1 (F_2 progeny) or 1:1 (AC derivatives) ratio due to overrepresentation of J and I parental allele, respectively. Asterisk (*) stands for significance at 5% level of chi-square values for panmictic segregation, indicating nonrandom assortment of parental alleles.

Cross	Progeny	Isozyme locus											
		lcd-1	*Amp-3*	*Est-2*	*Pgi-2*	*cat-1*	*Pgi-1*	*Sdh-1*	*Acp-1*	*Est-9*	*Amp-2*	*Pgd-1*	*Mal-1*
IR64/ Azucena	NMC		**285:199**	**300:211**	266:230	*184:288*	*232:279*	273:243	190:162	**269:168**	*112:207*	–	*174:238*
	ACP		**233:172**	**222:174**	214:191	*168:225*	*162:243*	178:207	88:106	195:210	*143:259*	–	*151:210*
	F_2		90:145:96	78:126:92	101:157:106*	*46:37:77**	95:86:101	67:122:72	58:97:61	79:140:67	47:146:67	–	*52:100:109**
IR64/ IRAT216	NMC	**313:177**	**294:189**	**292:186**	**287:191**	*182:279*	264:244	239:245	**240:160**	**298:171**	*143:249*	243:246	242:216
	ACP	142:129	141:130	141:130	151:120	123:148	124:147	137:134	43:33	**166:105**	*112:155*	124:147	129:128
	F_2	**133:64:34***	**83:98:51***	**83:96:53***	**88:84:50***	*46:90:95*	62:80:90*	54:31:49*	35:52:44	**96:76:60***	*74:71:87**	4:121:44*	54:40:49*



1. Representation of linkages between isozyme loci borne by chromosome 6 deduced from NMC, ACP, and F, data in IR64/Azucena and IR64/IRAT216. Recombination rates in anther culture derivatives and F_2s were calculated from percentage of recombinant associations and through the maximum likelihood method, respectively.

Cosegregation analyses also identified loose pseudolinkages that were specific to the F_2 of IR64/IRAT216: *Est-2, Amp-3,* and *Pgi-2*—all borne by chromosome 6—were found linked with both *Sdh-1* (chromosome 12) and *Pgi-1* (chromosome 3) Such pseudolinkages between independent genes are a common feature of progenies of indica/japonica crosses and have been tentatively explained by linkage of marker genes with duplicate gametic genes whose recombination produces disadvantaged gametes. The fact that pseudolinkages and aberrant segregations at loci borne by chromosome 12 found in the F_2 of IR64/IRAT216 were not observed in its ACP population must be stressed. This may suggest that the androgenetic pathway permits us to bypass problems related to the hybrid sterility breakdown existing in the gametogenesis of distant hybrids.

Given the average neutrality of gametic selection with regard to the indica and japonica differentiation, the absence of pseudolinkage, and the consistency of the linkage estimates found in ACPs, their use as molecular mapping and gene tagging populations can be further investigated with confidence.

Notes

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Salt tolerance in rice callus cultures

P.B. Kavi Kishor and G.M. Reddy

Salt accumulation affects about 400 million ha of land throughout the world. Crop plants selected for increased tolerance for saline environments should permit use of those soils. In vitro techniques were exploited to isolate salt-tolerant rice callus and to probe the physiological mechanism of salt tolerance. Callus cultures of rice selected for NaCl stress were found to accumulate considerable amounts of free proline, compared with unselected callus. Salt-selected callus grown for 10 passages (25 d each) on NaCI-free medium accumulated proline on re-exposure to salt, as did cells grown continuously on NaCI, suggesting the appropriateness of the selection procedure. On replacing NaCl (100 mmol) with 100 mmol KCI, the fresh and dry weights as well as the free proline content of salt-selected callus declined compared with that attained on 100 mmol NaCl medium. Omission of Ca^{2+} from the growth medium inhibited the growth of salt-selected callus in the presence of NaCl but had little effect on the growth of unselected callus. Ca^{2+} at a concentration of 9 mmol and 10 mg gibberellic acid/liter alleviated stress up to 200-300 mmol NaCl and KCl—otherwise an inhibitory concentration.

Replacing NaCl with KCl (100 and 200 mmol) inhibited the growth of salt-selected and unselected calli, but 10 mmol of proline had an ameliorating effect. Abscisic acid (ABA) suppressed the growth of both salt-selected and unselected calli in the absence of salt stress. ABA suppressed the growth of callus selected and grown in 100 and 200 mmol NaCl or when it was replaced by equimolar concentrations of KCI. The growth of 100 mmol NaCI-selected callus was inhibited when it was transferred to medium containing 200 mmol NaCI, but in the presence of ABA, growth was stimulated. ABA increased the growth of unselected callus subjected to NaCI, KCI, Na_2SO_4, and K_2SO_4. ABA accelerated the adaptation of cells exposed to salt, but not to water deficit imposed by nonionic solutes. Further studies are needed to learn the mechanism whereby ABA enhances the adaptation to or tolerance of callus for salts. The adaptation or nonadaptation of cells may be due to the ionic and nonionic nature of the osmotica used in the medium. Preliminary observations with rice callus cultures indicate that specific proteins are induced by both NaCl and Na_2SO_4 stress, and these proteins are now being characterized.

Notes

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Regeneration of plants from cultured anthers of salt-tolerant CSR-1 rice

N.R. Yadav, D.R. Sharma, and J.B. Chowdhury

Breeding for new varieties will remain a challenge because land area with high salinity is increasing; the new races of insects, pests, and pathogens are emerging; and world population is rapidly growing. Plant tissue culture techniques can identify desirable genes and incorporate them into high-yielding cultivars. Anther culture can shorten breeding time and generate new variability. Gametoclonal variation can provide a good means for selecting desirable types (Schaeffer 1983). Successful anther culture in rice has been reported by many workers (Chaleff and Stolarz 1981, Niizeki and Oono 1968). However, anther response seems to be highly genotype-specific. CSR-1 is a salinity-tolerant rice variety with medium yield and grain characteristics. Anther culture was undertaken in this cultivar to select variants endowed with salinity tolerance and improved grain characteristics.

Anthers at the uninucleated stage from cold-pretreated panicles (10 °C for 10 d) were cultured on modified N6 medium with 2 mg 2,4-dichlorophenoxyacetic acid/liter, 0.1 mg zeatin/liter, 0.06 mg picloram/liter, and 6% sucrose, and incubated in the dark at 27± 2 °C. Anther culture and regeneration response are summarized in Table 1. Of 2408 anthers cultured, 615 showed callus induction.

For regeneration, androgenic calli were transferred to Murashige and Skoog's basal medium supplemented with 0.3 mg naphthalene acetic acid/liter and 2.0 mg kinetin/liter. Green plant regeneration was very low (6.4%). The green plants were transferred to pots and raised to maturity. Gametoclonal variation was recorded for agronomic traits such as height, tiller number, panicle length, and 1000-grain weight. Subsequent evaluation of these in vitro plants for two generations confirmed their homozygous nature.

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Table 1. Anther culture and regeneration response in CSR-1.

Character		Results
Anther response (%)		25.5
Time to callus induction (d)		19-22
Calli (no.) per anther	Range 1-1 8, mean	5.2
Embryogenic pollen (%)		49.8
Regeneration (%)		80.2
Green plant regeneration (%)		6.4
Shoots (no.) per callus		4.0
Time to visible shoot formation (d)		15-22

Notes

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Chromosome studies and multiplication of embryonic cells derived from anther culture of indica rice

Wu Jiadao, Huang Zhongxiang, Liu Zuling, Zheng Leya, Yan Jianbo, and Chen Yan

Anther culture breeding is rapid, but the lower frequency of green plantlets regenerated limits its usefulness in rice culture, especially for indica varieties. We discovered the embryonic cell mass during anther culture of Indica rice and have cloned rice pollen in subculture, making it practicable to increase selection. Calli were derived from anther culture of 110 indica crosses cultured on N6 basic medium supplemented with 2 mg 2,4-dichlorophenoxyacetic acid/liter, 100 mg glutamine/liter, 500 mg lactalbumin hydrolysate/liter, and 50 mg sugar/liter. The embryonic cell mass of the pollen was discovered in the cultures of three crosses with 871015, 871025, and 871109 while the calli were differentiating. By repeatedly cutting the embryonic cell mass at regular intervals, a granular tissue (3-5 mm) was separated from the mass. The tissue was cultured on differentiating medium for 7-10 d, when it became an embryonic cell mass. More than 10,000 plantlets were obtained within 2 mo (Fig. 1) and transplanted in soil, where they grew continuously (Davidonis and Hamilton 1983). One hundred ninety-one plants were seeded both artificially and naturally.

Among 532 cells inspected from pollen calli of 871109 in subculture for 16 mo, n£12 made up 16.9%, and n>12, 83.1% (Table 1). Among the cells with n>12, 54.9% had 18-20 chromosomes; the number of chromosomes had obviously increased after subculture. These chromosomes could still be divided into 12 homologous groups, just as could the haploid pollen cells. But chromosome ploidy among the homologous groups was different. Sometimes, a homologous group could be lost in the cell, and it became n<12 (Chu and Zhang 1985; Wu 1987, 1989).

We think that the embryonic cells discovered will efficiently raise the frequency of regenerated green plantlets. To obtain the embryonic cells, it is important to choose material with the proper genotype that can produce hard but loose calli (easy to separate to form a granular tissue). The results of the study on chromosome variation of the clone in subculture agree with those of other researchers.

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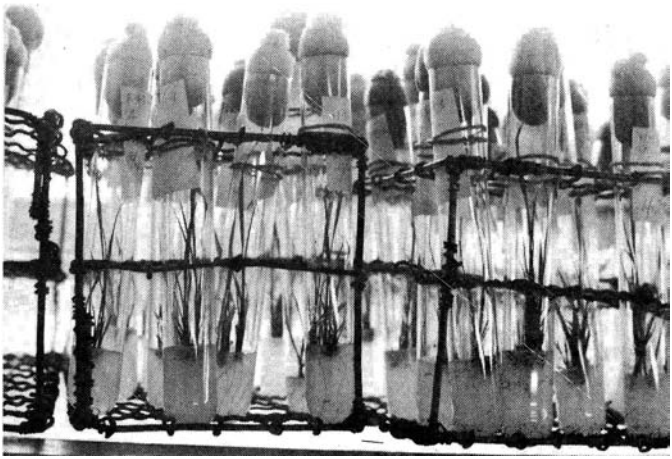

1. Growing large plantlets.

Table 1. Change of chromosome number,.

Chromosomes (no.)	Cells (no.)	Percent of cells inspected
<12	40	7.5
12	50	9.4
13-17	108	20.3
18-20	292	54.9
>20	42	7.9

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Current linkage maps in rice

T. Kinoshita

The linkage data presented in Table 1 include the latest information, mostly from Rice genetics newsletter (RGN), Volumes 1-6.

Two hundred eighty-six genes were assigned to 12 linkage groups, and 25 genes for economic characters such as semidwarfness, panicle and grain characteristics, heading habit, male sterility, disease and insect resistance, and stress tolerance were positioned on the chromosomes together with 123 markers.

The use of restriction fragment length polymorphism and other biochemical markers has proceeded rapidly. Integration of conventional and molecular linkage maps is important for rice genetics and breeding. Further studies on expression and regulation of rice genes must be done using modern tools to complement conventional genetics.

For information on the name of gene, gene stocks, and references, please refer to RGN 1:4-77 (1984) or RGN 4:11-37 (1987).

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Table 1. Marker genes assigned to linkage groups.

Gene	Locus
Group 1	
d-4	0
I-PI-4	9
wx	22
dp-1	24
ms-1	27
Pgd-2	29
v-3	30
ga-1	36
C	44
S-5	48
Amp-3	52
Est-2	53
bl-3	54
alk	55
st-1	64
Pgi-2	66
Se-1	66
Pi-z	68
S-6	73
d-9	75
rcn-1	76
gf	79
chl-4	81
bl-2	82
fs-1	94
Cl	95
Pi-i	99
ms-9	99
ur-1	122
Unlocated genes	
al-1	*mp-2*
al-9(t)	*Pox-5*
bc-4	*S-1*
Cat-1	*s-a-1*
chl-7(t)	
d-21	*s-c-1*
dw-1	*s-d-1*
Enp-1	*S-A-1*
fc-2(t)	*S-B-1*
drp-6(t)	*spl-3*
ga-4	*Stv-1*
ga-5	*Un-a*
HI-a	*v-1*
I-PI-2	*zn*
Group II	
d-2	0
d-3	25
PI	61
Prp-b	61
Pi(t)	66
flo-3(t)	82
lg	92
ga-6	96
st-4	97
d-42	102
Ph=Bh-c	113
Xa-1	119
Xa-12	121
Xa-2	123
nal-4	124
d-31	131
Pr	137
Ps-2	141
rcn-2	156
drp-5(t)	160
d-11	160
Ik-i	170
Unlocated genes	
al-5	*P*
al-7	*Pin-1*
An-1	*rk-1*
aul	*rl-2*
Bph-1	*s-c-2*
bph-2	*s-e-2*
drp-1	*sc-1*
drp-8(t)	*ssk*
ga-10(t)	*Wh*
nal-1	*ylm*
nal-5	*z-5*
Group III	
eg	0
ga-8	0
spl-6	8
lax(lx)	13
chl-5	22
rl-1	26
d-10	28
ga-7	39
sd-1	43
A	50
Rd	51
shr-1	52
Shp-1	53
rl-4	70
Pn	70
Unlocated genes	
al-4	*ga-9*
al-8	*I-Ps-b*
chl-6	*lgt*
d-18	*mp-1*
d-26(t)	*Prp-a*
d-54	*sh-2*
d-55	*ts-a*
fs-2	*v-6*
Group IV	
d-6	0
g-1	6
spl-5	29
Rc	42
v-11(t)	43
z-6	45
rfs	56
Unlocated genes	
d-7	*m-Ef-1*
esp-1	*ms-8*
Est-9	*rl-6(t)*
ge	*se-2*
lp-1	*Un-b*
Group VI+IX	
gh-1	0
nl-2	6
d-1	28
st-2	46
al-3	48
spl-8	48
al-6(t)	53
v-10(t)	54
ops	66
bgl	79
ri	82
spl-7	97
nl-1	107
al-2	108

continued

Table continued.

Gene	Locus
Unlocated genes	
An-2	*I-PI-1*
bd-1	*ms-14*
er(o)	*Shp-3*
eul	*xa-5*
gl-1	*ylb*
Glh-6	
Group *VII*	
Dn-1	0
drp-2	14
dp-2	14
Unlocated genes	
Bp	*Pi-ta*
d-57	*sl*
ms-10	
Group V (include in Group VII)	
gm	0
l-Bf	39
lam (t)	39
Group Vlll	
Pi-k	0
d-27	28
z-2	33
drp-7 (t)	50
la	55
v-4	66
Pgd-1	71
d-28	81
sp	89
Adh-1	90
Pi-a	91
sh	94
v-9 (t)	104
z-1	118
D-53	127
Unlocated genes	
esp-2	*Pi-is-1*
M-Pi-z	*S-3*
nal-2	*Xa-3*
Pgd-1	*Xa-4*
Pi-f	*Xa-10*
Pi-se-1	
Group X	
d-29	0
tri	12
bc-3	25
bl-1	34
Chl-10	54
d-5	59
gh-3	69
d-32	107
d-30	125
gh-2	159
spl-2	178
Unlocated genes	
ms-17	
Pi-b	
Group XI+XII	
Chl-1	0
Nal-6 (t)	4
chl-3	19
fc-1	40
v-1 (t)	58
bc-1	76
ms-7	76
chl-2	106
v-2	106
al-10	110
d-56	125
Ihs-1	131.5
dl	132
Hg	132
drp-4	139
spl-3	149
drp-3	149
z-3	149
d-52	149
Unlocated genes	
An-3	*Mi*
bl-4	*Psi-1*
d-14	*rl-5*
d-20	*s-e-1*
ga-2	*Shp-4*
ga-3	*5t-3*
Gdh-1	*v-5*
Lk-f	*v-7*
fgl group	
pgl	0
Rf-1	12
fgl	12.5
Ef-1	28
Unlocated genes	
Bph-3	*Glh-3*
bph-4	*rk-2*
d-20	*ygl*
du-1	
d-33 group	
Acp-1	0
pox-2	24
d-33	32<
Sdh-1	37
Unlocated genes	
du-4	*rl-3*
nal-3	*spl-1*
sug group	
sug	0
v-8	49
Unlocated genes	
Amp-2	*Stv-b*
An-4 (t)	*ur-2*
d-51	*z-4*
shr-2	

Notes

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Characterization of rice chromosomes by imaging methods

K. Fukui and K. Iijima

Samples of somatic chromosomes of the haploid japonica rice cultivar Koshihikari (2n=12) were prepared by enzymatic maceration and air-drying. They were stained with Wright solution, and the good chromosome samples at the prometaphase stage were selected and photographed. Photographs of 30 rice chromosomal spreads were subjected to image analysis to obtain numerical and image data.

Besides the conventional parameters of total chromosome length and arm ratio, the condensation pattern (Fukui and Mukai 1988) or the density distribution along the midrib of each chromatid was digitally measured by using the chromosome image analyzing system (Fukui 1986, 1988; Kamisugi and Fukui 1990). The characteristics of each rice chromosome extracted by visual inspection were also summarized into the essential key characters. One hundred eighteen key characters were obtained and checked for agreement with 360 chromosomes one by one, and the value of the fitness probability of each key character was calculated.

Using the numerical parameters, the condensation pattern, key characters, a discrimination chart, and a flow chart to identify each rice chromosome were developed. All the rice chromosomes were objectively identified by using the chart. The standard karyotype of the somatic rice chromosomes at prometaphase was established based on the data in the 30 rice chromosomal plates. Figure 1 shows the idiogram of the rice chromosomes. The chromosome numbers were based on the order of their relative length. Table 1 shows the average arm ratio and the relative length of the rice chromosomes with standard deviation.

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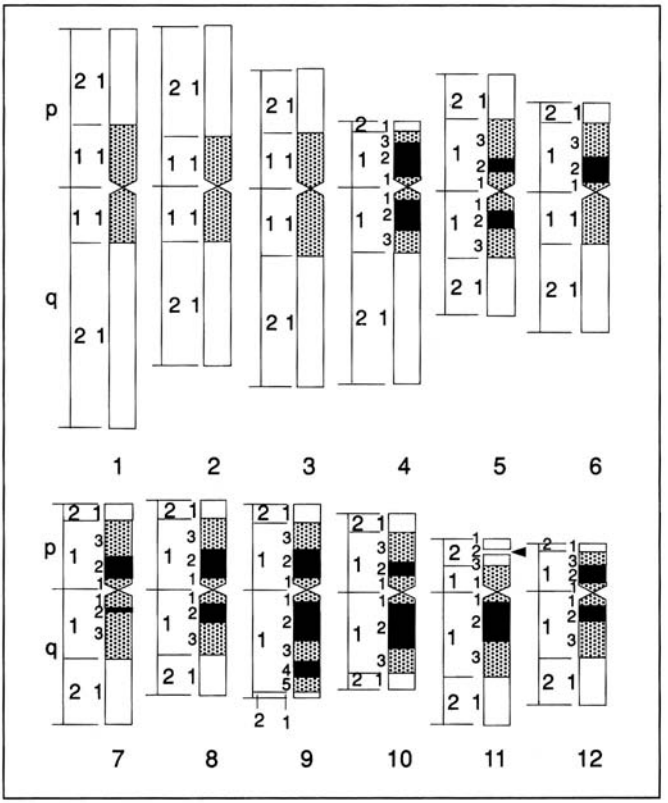

1. Quantitative ideogram of somatic rice chromosomes. Shaded and black regions correspond to condensed regions of prometaphase rice chromosomes that show characteristic uneven staining during prometaphase. Black regions show most condensed part among condensed regions. p, q, and ◄ indicate short arm, long arm, and nucleolar organizing region, respectively.

Table 1. Numerical data on somatic chromosomes of rice.[a]

Chromosome no.	Relative length (%)	Arm ratio
1	13.6 ± 1.1	1.58 ± 0.23
2	11.7 ± 1.2	1.10 ± 0.25
3	10.9 ± 0.9	1.72 ± 0.21
4	9.1 ± 0.6	2.93 ± 0.29
5	8.3 ± 0.6	1.12 ± 0.09
6	7.9 ± 0.6	1.68 ± 0.21
7	7.6 ± 0.5	1.61 ± 0.31
8	6.6 ± 0.4	1.24 ± 0.13
9	6.6 ± 0.5	1.26 ± 0.11
10	6.1 ± 0.4	1.26 ± 0.12
11	5.8 ± 0.6	3.90 ± 1.11
12	5.8 ± 0.4	2.00 ± 0.37

[a] Average data of 30 prometaphase plates with total chromosome length ranging between 39.7 and 54.4 µm. ± indicates standard deviation. Length of the satellite was excluded from calculation.

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Chromosome constitution of diploid-like plants derived from gamma-irradiated tetraploids

H. Fukuoka, Y. Kageyama, K. Yamamoto, and G. Takeda

There is widespread acceptance of the hypothesis that structural chromosome rearrangement plays an important role in initiating speciation. Induction of chromosome rearrangement may then be an effective method to extend the range of phenotypic variation. Whereas chromosome aberration generally involves reduced variability and fertility, tetraploids, being genetically redundant, are expected to have greater capacity than do diploids to maintain and hand down the rearranged chromosomes. We repeatedly irradiated artificial autotetraploid plants of japonica rice cultivars Nipponbare and Fukunishiki with gamma-rays through several generations. Eventually an almost normal diploid phenotype was observed at the rate of 1 or 2 individuals per generation. These plants were designated as diploid-like plants and were maintained as panicle lines. The irradiation procedure and the mutant characters observed in these derived lines were reported previously (Yamamoto et al 1988, 1989). All of the examined lines had the normal diploid chromosome number of 2n=24.

To detect chromosome rearrangement, restriction fragment length polymorphism (RFLP) of the nuclear DNA was examined. Nuclear DNA was extracted from leaf tissue of the original cultivars: 25 mutant lines derived from Nipponbare and 14 lines derived from Fukunishiki. These lines belong to eight mutant line groups derived from Nipponbare (Tr##) or one mutant line group derived from Fukunishiki (FTr86). Each DNA sample was digested with restriction endonuclease Xbal, EcoRI, or EcoRV in separate reactions, electrophoresed, and Southern blotted. To utilize as probe fragments, nuclear DNA extracted from Nipponbare was digested with BamHI or Pst and ligated into plasmid pHSG399 (Takara Shuzo Co., Ltd.). Random genomic clones were given numbers as NB## for BamHI clones and NP## for Pst clones. Forty-three probes and 3 enzymes, i.e., 129 probe-enzyme combinations, revealed 29 cases of RFLP in at least 1 mutant line. The results are summarized in Table 1. Eighteen of 39 mutant lines examined exhibited at least one RFLP. They belonged to four of nine mutant line groups. Clone NB36 was the only high-copy-number fragment that showed RFLP. Southern hybridization analysis of NB36 probed with a rice ribosomal DNA (rDNA) clone indicated that NB36 was a clone of the ribosomal RNA (rRNA) coding region. Hence, the RFLP revealed with NB36 indicated that repeated units of rRNA gene had changed in length during seven or eight generations. The mechanisms involved in the conversion of molecular species will be investigated. We are presently attempting to determine the kind of change that occurred in the rDNA region of the mutant lines. High resolution analysis of karyotypes is required

Table 1. Mutant lines that exhibited RFLP compared with the original cultivars.[a]

Probe	Mutant line group and number																	
	Tr86a			Tr87a							Tr881-CM		FTr86					
	W17	R3	R22	16	31	46	47	54	79	120	15		123	133	404	438	519	601	641
NB36	X1	X1	X1	X2	X2	X2		X2	X2	X2				X3	X3	X3	X3	X3	X3
	EI	EI	EI	E2	E2	E2		E2	E2	E2				E3	E3	E3	E3	E3	E3
	V1	V1	V1	V2	V2	F2		V2	V2	V2				V3	V3	V3	V3	V3	V3
NB122					E														
NB123				E	E		E	E	E	E									
NP11				X	X	X		X	X	X									
NP25		x1		X2	X2	X2	X2	X2	X2	X2									
				E	E	E		E	E	E									
				V															
NP48				E	E	E	E			E									
NP55				X		X	X	X											
				E	E	E	E	E		E									
				V		V	V	V											
NP65					X					X			X	X			X	X	
										E			E	E			E	E	E
					V				V	V			V	V			V	V	
NP68											X								
											E								
											V								
NP71					X		X	X	X	X									
NP72				X	X	X	X	X	X	X									
				E	E	E	E	E	E	E									

[a]Enzymes detecting polymorphism are represented as follows. X = *Xbal,* E = *Eco*RI, V = *Eco*RV. Numerals indicate size class.

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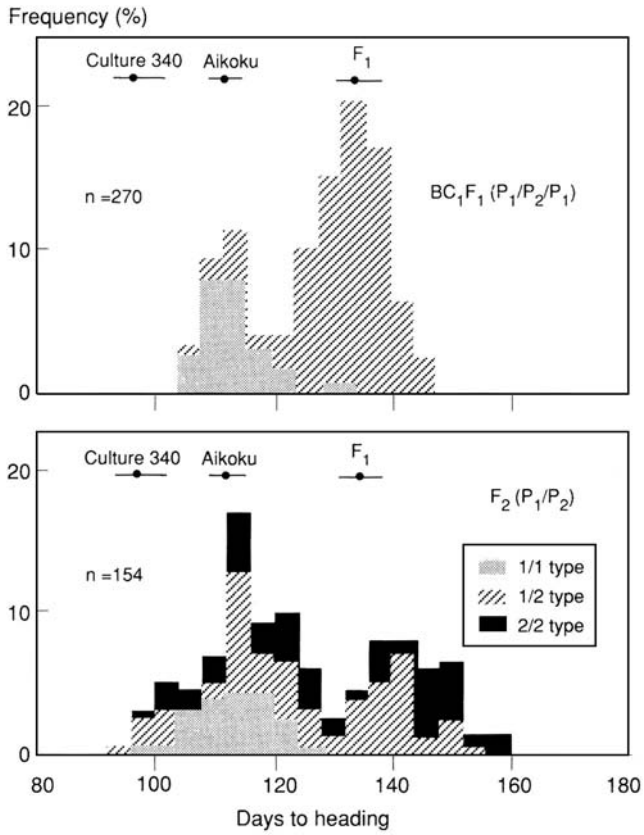
Genetic analysis of heading time by using linkage relationship with isozyme gene *Pgi-2* in rice

I. Imuta, F. Kikuchi, I. Watanabe, and M. Yusa

Heading times of hybrids between the early variety Culture 340 from India and Japanese varieties were much longer than those of the parents under long-day conditions in Tsukuba (36° N), Japan. In daylength experiments, Culture 340 had a short vegetative period and photoperiod insensitivity. The F_1 hybrids of Culture 340 with Japanese varieties showed a similar short vegetative period but fairly strong photoperiod sensitivity, which was not expected from the photoperiod responses of the parents (Watanabe et al 1990).

The present study was undertaken to investigate whether the photoperiod sensitivity of the hybrids is the result of the gene effect of the *Se-1* locus of linkage group I, which is known to control heading time in cultivated rice. The genetic analysls was carried out on a cross between the Japanese photoperiod-insensitive variety Aikoku and Culture 340 by utilizing segregation of genotypes for phosphoglucose Isomerase isozyme locus *Pgi-2,* which is closely linked with the *Se-1* locus Aikoku and Culture 340 have the 1/1 and 2/2 alleles for *Pgi-2,* respectively. The heading times of the BC_1F_1 (Aikoku/ Culture 340//Aikoku) and F_2 populations were measured under field conditions, and the plants were also tested for the *Pgi-2* genotype. In the BC_1F_1. the variation of heading time showed a clear-cut bimodal distribution with two peaks corresponding to the heading times of Aikoku and the F_1 (Fig. 1). The early heading group consisted of plants with mostly the 1/1 genotype (Aikoku type) for *Pgi-2.* while the late group consisted of plants with mostly the 1/2 genotype (F_1 type). The association between heading time and *Pgi-2* indicates that the alleles of the *Se-1* locus control heading time in this cross, and that Culture 340 has a photoperiod-sensitive gene at the *Se-1* locus. The heading time of the F_2 population also showed a wide bimodal distribution (Fig. 1). This population was partitioned by three genotypes for *Pgi-2* 1/1 1/2, and 2/2. Plants with the 1/1 genotype were exclusively early, like those of the BC_1F_1, but plants with the 1/2 and 2/2 genotypes appeared in the early- as well as late-heading groups. This phenomenon is explained by the complementary action of a recessive inhibitor (*i-Se-1*) for photoperiod-sensitive gene *Se-1* (Chang et al 1969) Under this assumption, the homozygote genotype for *i-Se-1* will segregate in the F_2, which may result in early heading, irrespective of the presence of the photoperiod-sensitive *Se-1* gene.

Thus, the early variety Culture 340 carries a gene for photoperiod sensitivity with large effects at the *Se-1* locus, and a recessive inhibitor for sensitivity as well, causing early heading even under long-day conditions.



1. Segregation for heading time and *Pgi*-2 genotypes in the BC_1F_1 and F_2 populations of a cross between Aikoku (P_1) and Culture 340 (P_2).

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Effects of dwarfing genes on cell characteristics in rice internodes

O. Kamijima

The mechanism by which dwarfing genes shorten the three upper internodes of rice was investigated by studying the effects of dwarfing genes on cell number and length. Normal variety Shiokari and 19 near-isogenic dwarf lines, which were obtained by more than 4 backcrosses with Shiokari (Kinoshita and Shinbashi 1982), were used. Parenchyma cells in a longitudinal cell line were measured in each internode using methods similar to those described by Kamijima (1981).

In general, dwarf lines whose internodes were shorter than those of Shiokari had fewer cells than Shiokari, and cell number was highly and positively correlated with internode length within each internode (r = 0.946-0.967). However, dwarf lines did not always have shorter cells, even when they had shorter internodes than Shiokari. Correlation coefficients between cell length and internode length were statistically significant but relatively low (r = 0.491-0.782). As shown in Table 1, the 19 dwarf lines could be categorized into 8 groups based on the cell characteristics that cause the shortening of each internode.

The dwarf line that has dwarfing gene *d-12* showed the same abnormality in its cell morphology as did dwarf line Fukei No. 71 (Kitano and Futsuhara 1982). This is further evidence that Fukei No. 71 has the *d-12* gene, as Murai et al (1990) reported.

This information on cell characteristics will be useful for revealing the physiological and biochemical mechanisms of gene action that cause dwarfness in rice.

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Table 1. Classification of 19 dwarf lines based on cause of internode shortening for each of the 3 upper internodes.

Cause of internode shortening[a] in			Line[b]
Internode I	Internode II	Internode III	
1	1	1	*d-1, d-12*[c], *d-30, d-a*
1	1	2	*d-27, d-47 (sd-1), d-b*
1	2	1	*d-3, d-4, d-5*
1	2	2	*d-2, d-10, d-14, d-17, d-18*[h], *d-18*[k], *d-35*
+	2	2	*d-6*
2	2	+	*d-11*
2	2	2	*d-13*
1	3	3	*d-7*

[a] 1 = fewer cells, 2 = fewer cells and shorter cell length, 3 = shorter cell length, + = no shortening of internode length. [b] Name of line is indicated by symbol of dwarf gene involved. [c] Abnormal cell morphology.

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Effects of dwarf genes on rice root elongation

H. Kitano and Y. Futsuhara

The effects of dwarf genes on root elongatlon at the seedlmg stage were examined in 18 near-isogenlc dwarf lines that were bred at the Plant Breedmg Institute of Hokkaido University using Shiokarl as the recurrent parent (Kinoshlta and Shinbashi 1982). Seedlings of the lines and Shlokari were grown for 10d after germlnatlon by soil culture in the greenhouse and by hydroponics in a growth cablnet at 28 °C. Thereafter, data on shoot, seminal root, and nodal root lengths and on number of roots were recorded. In addition, average nodal root length and total root length were calculated from the mean values of the measurements of root characters. Table 1 shows the results of the soil culture experiment. Except In a few lines, shoot and/or root growth inhibition by each dwarf gene showed a slmilar tendency in the two cultural condltions. The lines were classified into four categories: Group A having a prominent decrease in root length and shoot length *(d-1, d-10, d-11, d-13, d-27),* Group B having a greater decrease In shoot length than in root length *(d-18^k, d-35, d-b),* Group C having a greater decrease In root length than in shoot length *(d-3, 4, 5, d-7, d-14, d-17,* and Group D composed of resldual lines *(d-2, d-6, d-12, d-30, d-47, d-a).* Although it is confrrmed that *d-7* and *d-27* significantly decrease radicle length in the mature embryonic stage (Kamglma and Watanabe 1985), in this experiment reduclble effects by these dwarf genes could not be detected on seminal root elongatlon after germmatlon. On the other hand, excised root tips of the isogenic lines were cut at 1 cm from the top of the seminal root of seedlings at 6 d after germination and cultured on White's medlum for 21 d at 26 °C in the dark. The root growth responses of the 18 lines were compared wlth that of Shiokari. The results of this experiment were conslderably different from those gwen above, suggesting that dwarf gene action on root growth is affected by shoot growth.

These results reveal that the expression of dwarf genes on root elongation varies remarkably.

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Table 1. Top and root characters of 18 near-isogenic lines and their recurrent parent Shiokari raised by soil culture.

Line	Gene	Donor parent	Generation	Shoot length (cm)	Seminal root length (cm)	Total nodal root length (cm)	Nodal roots (no.)	Average nodal root length (cm)	Total root length (cm)
Shiokarl				17.4	19.7	31.0	4.4	7.1	50.7
Daikoku dwarf	*d-1*	H-86	BC_8F_7	8.0	19.6	19.5	3.8	5.2	39.1
Ebisu dwarf	*d-2*	H-85	BC_7F_7	15.1	24.1	32.3	4.9	6.6	56.4
Bunketsu-waito dwarf	*d-3, 4, 5*	H-2	BC_4F_7	16.8	20.4	13.1	3.9	3.3	33.5
Ebisumochi dwarf	*d-6*	H-126	BC_6F_7	17.3	20.8	36.6	4.1	8.9	57.4
Heiei-daikoku dwarf	*d-7*	N-7	BC_8F_7	15.9	200	200	4.9	4.1	40.0
Toyohikari-bunwai dwarf	*d-10*	N-70	BC_8F_7	14.5	15.3	6.8	3.0	1.9	22.1
Norin-28 dwarf	*d-11*	N-58	BC_6F_7	9.0	16.0	12.8	2.6	4.2	28.8
Yukara dwarf	*d-12*	N-62	BC_5F_7	17.6	24.7	27.6	4.8	5.6	52.3
Short-grained dwarf	*d-13*	M-51	BC_5F_7	11.0	17.7	13.8	4.2	3.2	31.5
Kamikawa-bunwai dwarf	*d-14*	H-147	BC_7F_7	17.2	19.7	8.6	2.8	3.0	28.3
Slender dwarf	*d-17t*	1-71	BC_5F_7	16.4	15.1	3.1	1.7	1.3	18.2
Kotake-tamanshiki dwarf	*d-18*[k]	F1-26	BC_8F_7	12.1	26.0	32.0	5.9	5.6	58.0
Bunketsuto dwarf	*d-27*	F1-86	BC_6F_7	15.1	19.3	4.7	2.0	2.2	24.0
Waisei-shirazasa dwarf	*d-30*	F1-3	BC_5F_7	18.7	20.4	34.2	5.0	6.9	54.6
Tanginbozu dwarf	*d-35t*	N-77	BC_7F_7	9.5	20.4	32.6	5.6	5.8	53.0
Dee-geo-woo-gen dwarf	*d-47t*	I-120	BC_4F_7	15.4	20.7	32.2	4.8	7.0	52.9
[Unnamed]	*d-a*	M-290	BC_4F7	17.9	21.9	39.1	5.7	6.8	61.0
Hiroba dwarf	*d-b*	N-100	BC_4F7	14.6	21.4	28.4	4.8	5.0	49.8

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Genetic aspects of organ differentiation during rice embryogenesis

Y. Nagato, H. Kitano, O. Kamijima, S. Kikuchi, and H. Satoh

In plant developmental biology, the regulatory mechanism of gene expression during organ differentiation is of primary concern. It can be effectively investigated if suitable developmental mutants are available. To date, we have used N-methyl-N-nitrosourea treatment to isolate 28 single-gene recessive mutants (*odm-1* to *odm*-28) affecting organ differentiation during rice embryogenesis (Nagato et al 1989).

Embryos of the 28 mutants were sectioned at various developmental stages, phenotypically characterized, and classified into four groups.

- Arrested at the early globular stage: *odm-7, -13,* and *-17.* In these three mutants, small globular embryos of 100-200 cells were formed but then degenerated, resulting in the embryoless feature in mature seeds.
- Undifferentiated: *odm-7, -72, -20, -27, -23,* and *-28.* Although these mature embryos were larger than the maximum size of the normal globular embryo, they appeared globular and showed no sign of organ differentiation. They grew very slowly and were viable throughout embryogenesis.
- Abnormal organ differentiation: Some embryos were arrested at the early organ differentiation stage (*odm-2, -8,* and *-10*); they differentiated the coleoptile and, in some cases, both shoot and root primordia. In others, abnormality was detected in the position of the shoot (*odm-4*) or of both shoot and root (*odm-14*); these wild-type genes must be involved in determining organ position. A third type lacked a shoot (*odm-22*); the root was normally formed and grew after germination. Therefore, this wild-type gene participates in the determination of the shoot. In a fourth type. both shoot and root were differentiated, but the morphology was unusual (*odm-5, -6, -19, -24,* and *-25*); they were considered mutants on the elaboration of organ morphology. The fifth type was unique in that the mutation was concerned with size regulation (*odm-16);* not only embryo size but also sizes of shoot and root were reduced.
- Wide phenotypicvariation: *odm-3, -9, –11, -15, -18, -26,* and *-27*. Unexpectedly, many mutants showed variable phenotypes in spite of single-gene mutations. Each mutant showed three or four discrete phenotypes. Each of these wild-type genes regulates the expression of several other developmental genes.

The phenotypic diversity of these 28 mutants indicates that a large number of genes and their successive stage- and organ-specific expressions are required for normal embryonic development.

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Genetic identification of mutant genes for reduced culm number

I. Takamure

The first report of a reduced-culm-number mutant in rice was made by Futsuhara and Yamaguchi (1963). A similar mutant, N-133, was reported to be controlled by a single recessive gene, *rcn-1,* belonging to the first linkage group (Takamure and Kinoshita 1985). The mutant gene *rcn-1* is temperature sensitive, and gene expression is restricted only under low temperature. The author studied three new mutants—N-174, N-176, and N-175—which showed similar culm number. N-174 was induced by ethylmethane sulfonate treatment of cultivar Shiokari. N-176 and N-175 were induced by gamma-ray irradiation of two doubled haploid lines, AC-15 and AC-34, produced by anther culture from F_1 hybrids H-59/H-120 and A-5/H-69, respectively.

Character expression of N-174, N-176, and N-175 was compared with that of the original strains under both field (low temperature) and plastic house (high temperature) conditions. N-174 showed the reduced-culm-number type and normal plant height under both conditions, and the decrease in culm number was not as striking as in N-133. N-176 and N-175 had one or two panicles with short culms under field conditions, and growth was nearly normal in the plastic house. N-176 and N-175 were sensitive to low temperatures.

Genetic analyses and allelism tests were done under field conditions. Recessive genes *rcn-2, rcn-3,* and *rcn-4,* which have independent relationships to *rcn-1,* were responsible for the reduced culm number of N-174, N-176, and N-175, respectively. *rcn-2* was linked to the marker genes belonging to the second linkage group such as *Ph* (phenol staining) and *Pr* (purple hull) in the order *Ph - Pr - rcn-2.* In addition, *rcn-1* was epistatic to *rcn-2, rcn-3,* and *rcn-4,* showing that the temperature sensitivity of *rcn-1* is higher than that of *rcn-3* or *rcn-4.* The genetic Interactions between *rcn-2* and dwarf tillering genes (*d-10, d-14, d-17,* and *d-27*) were additive with regard to culm number, and a similar relationship was recognized for both culm number and culm length between *rcn-3* or *rcn-4* and the dwarf tillering genes Genes *rcn-2, rcn-3,* and *rcn-4* can be effectively used as markers in various genetic and physiological studies.

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Grain-shattering gene linked with semidwarfing gene *sd-1*

S. Oba and F. Kikuchi

Genetic studies on grain shattering have been undertaken by many researchers, but only the *sh* gene has been mapped—in the 8th linkage group (Kinoshita 1984). This paper discusses the inheritance and character expression of a new grain-shattering gene linked to the semidwarfing gene *sd-1.*

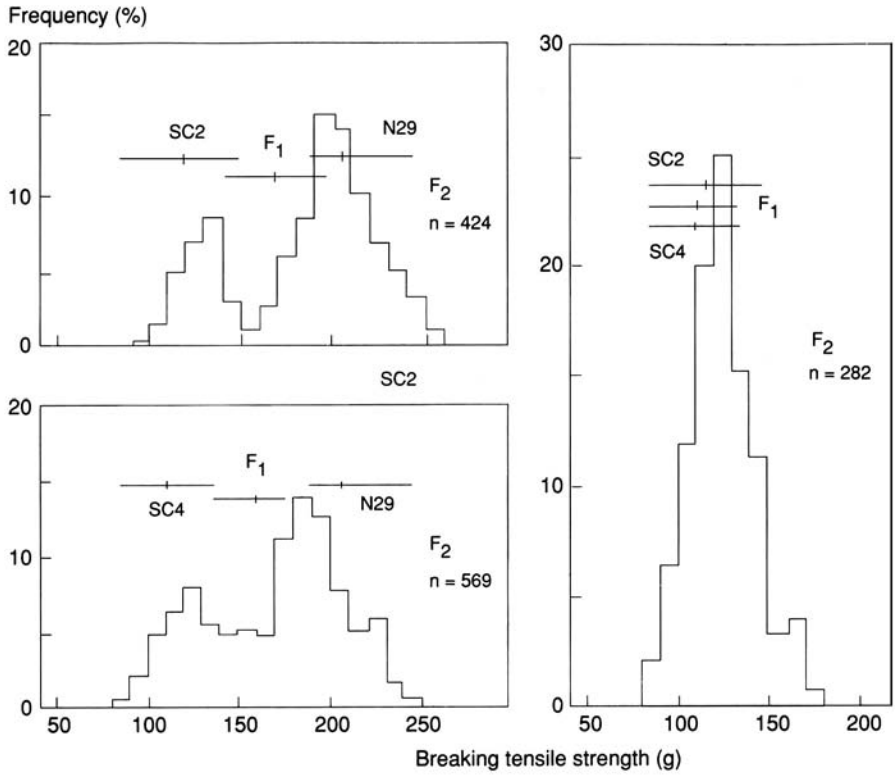
Japanese tall variety Norin 29 and near-isogenic semidwarf lines SC2 and SC4 were used. SC2 and SC4 were developed by using Norin 29 as the recurrent parent, and semidwarf varieties Taichung (Native 1) from China and Shiramui from Japan, respectively, as donor parents (Kikuchi 1986). Norin 29 is nonshattering, while SC2 and SC4 are susceptible to shattering. Grain shattering was evaluated with an apparatus for measuring the breaking tensile strength at the base of the sterile lemmas.

Crosses among SC2, SC4, and Norin 29 were made to investigate the inheritance of grain shattering. Figure 1 shows the variation in breaking tensile strength among the three hybrid populations. The values of the F_1s of SC2/Norin 29 and SC4/Norin 29 were similar to the average values of the parents, indicating that the nonshattering habit of Norin 29 is incompletely dominant over the shattering habit of SC2 and SC4. The segregation patterns of breaking tensile strength in the F_2s of SC2/Norin 29 and SC4/Norin 29 fit a 3 nonshattering : 1 shattering segregation ratio. These results show that the two lines carry a single recessive gene controlling shattering habit. The F_1 and F_2 of SC2/SC4 showed the same breaking tensile strength as that of both parents, indicating that SC2 and SC4 have the same grain-shattering gene.

A close association between semidwarfness and breaking tensile strength was observed in the F_2s of SC2/Norin 29 and SC4/Norin 29: the combined recombination value between the two characteristics was 10.6±4.1%. A linkage relationship between the recessive shattering gene and the semidwarfing gene *sd-1* was also found in Chinese semidwarf variety Ai-Jio-Nan-Te, with a recombination value of 13.7±4.7% (Oba et al 1990). These results suggest that the gram-shattering gene linked with *sd-1* may be present in many semidwarf varieties.

The base of the sterile lemmas was observed under a microscope. Norin 29 did not develop an abscission layer, while SC2 and SC4 showed Its early formation, indicating that the shattering gene controls the formation of the abscission layer.

The new grain-shattering gene was registered as *sh-2.*



1. Frequency distribution of degree of grain shattering in F_2 populations of SC2, SC4, and Norin 29 (N29). Solid horizontal lines show the range of parents and F_1s.

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Semidwarfing genes influencing morphological traits and physiological ability of rice roots

M. Ichii, Shui Shan Li, and H. Hasegawa

An experiment was conducted to determine the effect of semidwarfing genes on the morphological traits and N uptake of rice roots. Norin 29 (Sd_1Sd_1) and the near-isogenic line SC4 (sd_1sd_1), which was developed by transferring the semidwarf trait from Shiranui into Norin 29 through backcrossing, were grown in liquid culture from the seedling stage to maturity.

Plant height after transplanting and shoot weight after flowering were significantly higher in Norin 29 than in SC4 (Fig. 1). A significant difference was not detected in root length and root weight. Root cells were longer in Norin 29 than in SC4 after the active tillering stage (Fig. 2). The uptake rate (mg plant wt/g per h) of NH_4^+-N was significantly higher in Norin 29 than in SC4 until panicle initiation, but it soon became nearly equal in the two lines (Table 1). Uptake rate is an index of the absorbing ability of the genotype. The uptake rate of NO_3^-–N was significantly higher in Norin 29 than in SC4 except at 3 wk before flowering. These facts suggest that the operating time of semidwarfing genes varies with the character, and that semidwarfing genes act to decrease the metabolic rate. In developing superhigh-yielding rice cultivars, therefore, it is important to improve genetic productivity by investigating the characteristics of semidwarfing genes.

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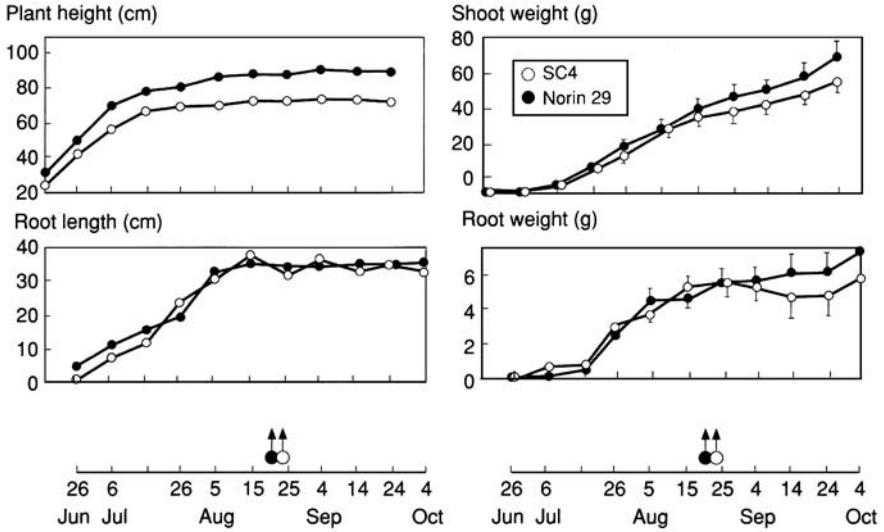
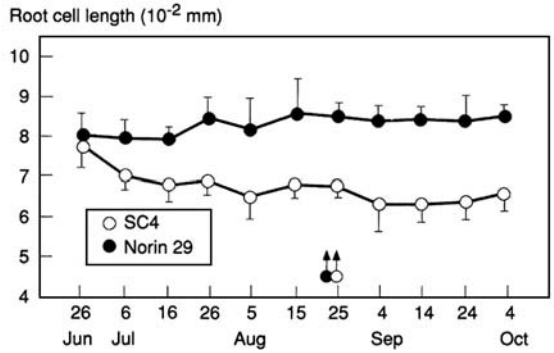


1. Changes in plant height, root length, shoot weight, and root weight in the near-isogenic line SC4 and recurrent parent Norin 29. Arrows show heading dates.



2. Changes in root cell length in the near-isogenic line SC4 and recurrent parent Norin 29. Arrows show heading dates.

Table 1. NH_4^+-N and NO_3^-–N uptake rate at different stages in near-isogenic line SC4 and recurrent parent Norin 29.

Nutrient	Line	Genotype	Uptake (x 10^{-2}mg plant wt/g per h) on[a]										
			26 Jun	7 Jul	16 Jul	26 Jul	5 Aug	15 Aug	25 Aug	4 Sep	14 Sep	24 Sep	4 Oct
NH_4^+-N	Norin 29	Sd_1Sd_1	78.7 b	37.8 b	21.0 b	8.40 b	4.50 a	2.90 a	3.30 a	2.10 a	1.00 a	1.20 a	0.90 a
	SC4	sd_1sd_1	48.1 a	23.2 a	18.3 a	7.70 a	4.80 a	3.00 a	3.70 a	1.80 a	1.10 a	1.30 a	0.90 a
NO_3^--N	Norin 29	Sd_1Sd_1	27.9 b	16.0 b	94.0 b	9.80 b	6.30 a	2.10 a	3.30 b	0.90 b	0.40 a	0.40 b	0.30 b
	SC4	sd_1sd_1	25.8 a	11.0 a	7.20 a	7.80 a	5.70 a	1.90 a	2.70 a	0.50 a	0.30 a	0.20 a	0.10 a

[a] Within a nutrient, means in a column followed by a common letter are not significantly different at the 5% level by *t*-test.

Eating quality of rice cultivars in reference to chemical components

A. Matsuzaki, T. Takano, and G. Takeda

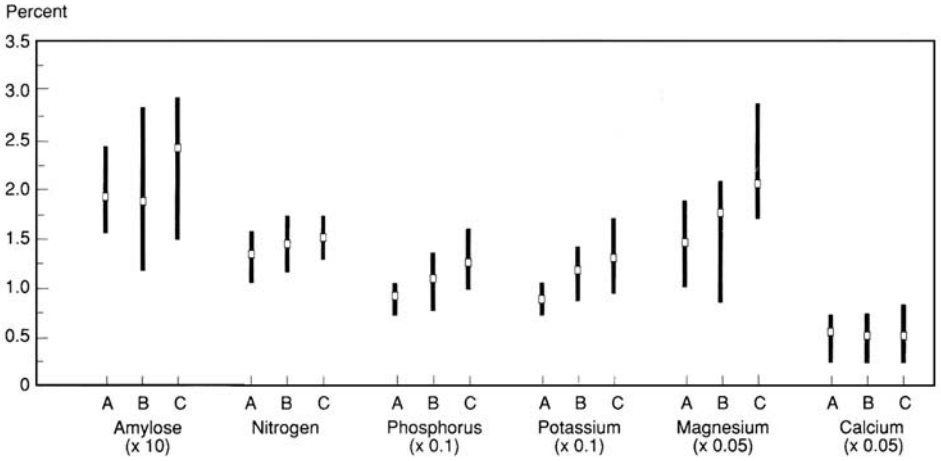
Many studies support the hypothesis that rice eating quality is influenced mainly by N content in the grains (Matsuzaki et al 1973). Horino et al (1983) reported that the balance of Mg and K also plays an important role in eating quality. Recently, Inatsu (1988) reported that eating quality is controlled by both N and amylose content.

To clarify the characteristics responsible for eating quality, three cultivars grown in a ricefield with abundant $CaCO_3$ and superphosphate were topdressed with $(NH_4)_2SO_4$ at booting and full heading. Eating quality was evaluated by sensory tests carried out at the National Food Research Institute, Japan. A ^{15}N application test with ^{32}P and ^{45}Ca was also carried out to confirm N accumulation in the rice grains, and 66 cultivars including japonica, indica, and javanica types were grown in 1985-88 to clarify the interrelationship of cultivars and cultural conditions and their effect on eating quality.

N topdressing at booting and heading decreased eating quality, due mainly to increased N content in the grains, whereas the presence of abundant Ca and P restricted N accumulation. Ca and/or P thus probably play an important role in improving eating quality when too much N is applied at heading. In general, each cultivar has a significant correlation between N content in the grains and eating quality; however, the high-quality rice cultivar Koshihikari showed a weak correlation between protein content and eating quality when evaluated by the sensory test.

The chemical components of 66 cultivars were analyzed by the autoanalyzer and atomic absorption spectrophotometer. The cultivars were classified into 3 groups: A = 29 Japanese cultivars, B = 19 javanicas and japonicas introduced from other countries, and C = 18 indicas. The average amylose content was 19.4% in group A cultivars, 18.9% in group B, and 24.3% in group C (Fig. 1). Comparatively high N content was observed in groups B and C. P and K contents showed nearly the same levels, and higher values were observed in groups B and C. The contents of Mg and Ca were about 1/3 and 1/10 of the P content, respectively, although the Mg contents of groups B and C were also higher than that of group A.

The coefficient of variation among cultivars for each component was also high in groups B and C. From these results, we hope to discover valuable characteristics for improving eating quality in existing cultivars.



1. Variation in components among rice grains. A = 29 Japanese cultivars, B = 19 javanicas and japonicas, C = 18 indicas.

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Genetic analysis of grain size in rice

T. Kato

In rice breeding programs in Japan, improvement of sink size has so far been attained through increasing panicle number per plant and/or grain number per panicle. Recently, some breeders have set about increasing grain size to improve sink size. This paper evaluates the genetic properties of rice grain size through two kinds of quantitative study: diallel analysis and estimation of narrow-sense heritability for this character.

Five rice cultivars whose grain length ranged from 7.1 to 11.2 mm were intercrossed as half-diallel crosses. The data for grain length and width of these five cultivars and their F_2 progenies were examined using the variance analysis of Jones (1965). The mean square for the differences between mean parental effects was significant at the 1% level for both grain length and grain width. The mean square for mean dominance effect among parents and that for dominance effect ascribed to specific parents were also significant for grain length and grain width, respectively. Since the data were regarded as satisfying the assumptions underlying diallel analysis, the genetic parameters defined by Jinks (1956) were estimated. For both grain length and width, additive gene effect (D) was considerably larger than dominance effect (H_1 and H_2). The average degree of dominance, $\sqrt{H_1/D}$,, was 0.49 for gram length and 0.06 for grain width, indicating that the alleles for grain length showed a partial dominance effect. The dominance alleles for gram length were estimated to cause shorter grains from the fact that there existed a positive and significant correlation (r = 0.99) between $W_r + V_r$ and the mean of the common parent, where W_r and V_r, respectively, mean array covariance and array variance as defined by Hayman (1954).

To estimate the narrow-sense heritability for grain length and grain width, parent-offspring correlations were calculated from the F_2 and F_3 data in 10 crosses. The heritability estimate averaged over the 10 F_2 populations was 0.80 for grain length and 0.56 for grain width. As for part of the 10 crosses, the narrow-sense heritability was also estimated in the F_3 and F_4 by the same method. The estimates for grain length and width did not fluctuate drastically with generation advancement from the F_2 to the F_4. The narrow-sense heritability for grain length on an F_2 plant basis was also estimated from selection response—the ratio of selection response in the F_3 to selection differential in the F_2. The average heritability estimate was 0.68 by this method.

These analyses reveal the predominance of the additve gene effect for rice grain length and width, causing high narrow-sense heritability for both traits. This result strongly suggests that selection for grain size, a polygenic character, could be done effectively even in early segregating generations. Grain size of rice shows genetically determined relationships to some physiological and morphological characters like grain-filling rate (Kato 1989), cold tolerance at the young microspore stage (Koike et al 1990), and occurrence of white-belly kernels (Takeda and Saito 1983). These characters, for which direct selection is rather difficult, could be manipulated indirectly through selection for grain size.

Grain length and grain width are probably controlled by different genetic systems. Although a positive phenotypic correlation between grain length and width is often found in rice, genetic improvement might be achieved separately for these traits.

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Core collections: providing access to wild *Oryza* germplasm

D.A. Vaughan

Core collections are representative subsamples consisting of 5-10% of a germplasm collection (Brown 1989a, b). Core collections of wild *Oryza* species are useful because

- they provide breadth of genetic diversity without duplication;
- they provide a set of materials for cost-effective initial evaluation; further evaluation of germplasm of particular species or of species from a particular region showing promise can be undertaken and related accessions tested; and
- determining several core collections that are more or less equally representative of the entire collection prevents oversupply of a few accessions that could exhaust seed stocks.

The core collections do not substitute for complete screening of germplasm when looking for rare traits, such as some virus resistances. However, a core collection is an appropriate place to begin a search.

Core collections are more easily designated for germplasm collections that are relatively small (a few thousand); contain germplasm with many distinctive features, which permits accurate assignment to different germplasm groups in the collection; and have comprehensive passport information primarily and characterization/evaluation data secondarily.

The International Rice Germplasm Center (IRGC) has about 2,000 accessions of wild rice. This collection is growing because of an intensive Asia-wide campaign to save these endangered genetic resources.

Initial steps in developing the core collection for wild rice were

- surveying the literature and major herbaria (Kew, Paris, Calcutta, Singapore, Bogor) for information on wild *Oryza* taxa to determine the basis for grouping the germplasm into species, subspecies, or races; and designating the germplasm groups for the core collection (Vaughan 1989);
- determining the geographic range of each species or Intraspecies group;
- identifying or reidentifying accessions and assigning accessions of the conserved germplasm to each species or intraspecies group;
- determining the number of accessions to be assigned to each species or intraspecies group; for this, proportional sampling was estimated on the basis of geographic range and known variability within each species; and

Table 1. Genomic and continental representation[a] of wild *Oryza* species in core collections.

Genomic			Continental		
Designation	Species (no.)	%	Region	Species (no.)	%
AA	6	61.0	Asia	10	60.2
BB	1	1.2	Africa	5	26.3
CC	3	14.4	Latin America	4	9.9
CCBB	2	5.6	Australia	2	4.3
CCDD	3	7.2			
EE	1	1.6			
FF	1	2.4			
Unknown	4	7.2			

[a] Sum of percentages is greater than 100.0 because of rounding.

- selecting accessions to be assigned to one of five different but approximately equal core collections (Table 1).

Twenty-six groups were designated on the basts of past chromosome, ecological, and taxonomic studies to represent the major distinctive forms in the genus *Oryza.* Several of these groups are not generally considered species.

A proportion or weight was assigned to each group on the basis of known geographic range and diversity within the group. Representation of some groups in the germplasm collection is inadequate, and extra conservation efforts for these species are necessary; these species include *O. alta, O. granulata, O. ridleyi,* and *O. rufipogon*

Theconserved germplasm that could readily be placed into 1 of the 26 predetermined groups was assigned to 1 of 5 more or less equally representative core collections.

Five core subsets, each representing 5-10% of the total collection of wild *Oryza* species conserved in the IRGC, were designated These core subsets are not static but are dynamic; as more information is gathered or more collections are made, they will be updated. This will provide scientists easy and efficient access to the range of diversity present in this exotic germplasm.

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Monosomic alien addition lines of *Oryza australiensis* and alien gene transfer

K.K. Jena, D.S. Multani, and G.S. Khush

Oryza australiensis (EE genome), a diploid wild species, is an important source of resistance to brown planthopper (BPH) and of tolerance for drought. Crosses were made between an induced autotetraploid of elite line IR31917-45-3-2 of *O. sativa* (2n=48=AAAA) and an accession of *O. australiensis.* Hybrids were produced following embryo rescue. Average crossability between the parents was 0.26%. The F_1 hybrids (AAE) showed model chromosome configuration 12II + 12I and were completely male sterile. The chromosome number of BC_1 plants *(O. sativa/O. australiensis//O. sativa)* varied from 28 to 31, These plants were male sterile. Only two BC_2 plants with 28 chromosomes set seed upon backcrossing. Eighteen BC_2 plants having 24-28 chromosomes were obtained and were grown to maturity. Monosomic alien addition lines corresponding to 8 of the 12 possible types of the haploid chromosome complement of *O. australiensis* were isolated among BC_2F_2 progenies. The transmission rates of alien chromosomes in selfed progenies varied from 8.4 to 37.6%. Pollen mother cells with chromosome configuration 11II + 1III ranged from 7.5 to 24.0%. Disomic BC_2 progenies and alien addition lines were examined for the presence of *O. australiensis* traits, Some traits, such as awns, shorter growth duration, purple stigma and apiculus, BPH resistance, and stress tolerance were identified in these progenies, indicating introgression of genes from *O. australiensis* to cultivated rice.

Notes

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Reexamination of genetic control of the reproductive barrier between *Oryza longistaminata* and *O. sativa,* and relationship to rhizome expression

A. Ghesquiere

Oryza longistaminata is a perennial wild rice isolated from all other rice species by a strong reproductive barrier. According to Chu and Oka (1970), D1 and D2 genes are present in O. *longistaminata and O. sativa,* respectively, and induce differential levels of albumen deterioration according to their dosage in the triploid endosperm. D1D1D2 induces early deterioration with no visible seeds; D2D2D1 produces late deterioration, resulting in shrunken kernels.

Rare F_1 hybrids obtained at ORSTOM in the Ivory Coast were used in an extensive backcross and testcross program. The testcrosses of the backcross offspring indicated clearly that Chu and Oka's model is rigorous only for F_1 crosses. In the F_1 embryo sacs, the differential deteriorations provoked by D1 and D2 were not as strong as expected and suggested modifications of gene effects. Assuming that in the F_1 the D1 gene becomes inactive and is no longer complemented by D2, tentative genotypes can be inferred to account for the experimental data (Table 1).

This new hypothesis found strong support in the observations of the rhizomes, which showed nearly unilateral inheritance in their backcross offspring (Table 1). Contrasting with the nearly complete restoration of rhizome expression in the backcrosses on *O. longistaminata,* backcrosses on *O. sativa* and their subsequent selfed progenies indicated that the rhizomatous habit was definitely lost (Causse 1991); under these conditions, since our model predicts the eviction (D2) or the inactivation (D1) of a lethal gene in the backcross process, rhizome expression could be attributed to the presence of an active D1 gene.

Electrophoretic observations on seven isozyme loci segregating in strictly controlled backcross offspring displayed a few patterns that could be interpreted as inactivation or reactivation events of various electromorphs (Ghesquiere 1988). Thus, genetic modifications and rearrangements evoking "transposable element activities" were suggested and could be associated with the confrontation between D1 and D2. Interspecific hybridization between *O. longistaminata* and *O. sativa* seems to evidence a "mutagenic-like" effect, which possibly indicates genomic reorganization. This phenomenon needs further investigation at the molecular level.

Table 1. Rhizome expression in interspecific progenies and presumed relationship with lethal genes controlling the reproductive barrier.

Progeny[a]	Plants (no.)	Rhizomatous plants (%)	Presumed genotype for reproductive barrier[b]	
			D1/d1	D2/d2
F1	4	0	d1d	D2d2
BCL	350	96	D1/(d1-d)	d2d2
Selfed BCL	300	80	D1/(D1-d1-d)[c]	d2d2
S/BCL	2	0	d1/(d1-d)	D2d2
BCS	3000	0	d1/(d1-d)	D2/(D2-d2)
Selfed BCS	1000	0	d1/(d1-d)-dd	D2/(D2-d2)-d2d2
BCS/L	32	100	D1/(d1-d)	d2d2

[a]Line of *O. sativa* or strain of *O. longistaminata* (L) used as tester for the reproductive barrier, BCL = backcross on *O. longistaminata,* BCS = backcross on *O. sativa.* [b]d = presumed inactive form of D1. [c]Presumed genotypes for rhizomatous plants only.

Assuming that the presence of rhizomes is a typical attribute of a perennial form, the coupled heredity of rhizome expression with the reproductive barrier reinforces the genetic isolation between *O. longistaminata* and other A genome species in Africa, since this original system separates the introgressed forms into annual and perennial clusters.

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Relatedness of annual and perennial strains of *Oryza rufipogon* investigated with nuclear DNA sequences

P. Barbier and A. Ishihama

The Asian wild rice species *Oryza rufipogon* is distributed in tropical Asia from West India to South China and Indonesia. This species is differentiated into annual and perennial populations. Geographical variation has also been found in molecular markers such as isozymes, chloroplastic DNA, and ribosomal DNA spacers. It is difficult, however, to relate the annual-perennial differentiation to the variation in molecular markers (Barbier 1989, Dally 1988, Second 1985). In particular, present molecular data do not provide a clue as to whether the differentiation into annuals and perennials occurred once in the remote past, or whether it could have occurred repeatedly and independently in several geographic regions. We attempted to infer a phylogeny for annual and perennial strains from various geographic regions using the variation in some nuclear DNA sequences. Here we describe a summary of nucleotide sequence data for two kinds of genes: 1) three introns within the phytochrome gene (introns 2, 3, and 4); and 2) members of a gene family encoding a seed storage protein, 10-kDa prolamin. The origin and ecotype of the wild rice strains analyzed (eight strains of *O. rufipogon* and one of *O. longistaminata* used as a reference outgroup) are given in Table 1. Data were obtained by direct sequencing of the relevant genes amplified by polymerase chain reaction (PCR). Forty amplification cycles were carried out from 1 µg total DNA. Annealing with synthetic primers was allowed for 2 min at 55 °C for the phytochrome introns and at 65 °C for the prolamin genes. Aliquots of purified PCR products were then submitted to dideoxy termination reactions. Further details are given elsewhere (Barbier and Ishihama 1990, Barbier et al 1991). In case of a multigene family such as the prolamin genes, all copies are supposed to be amplified by the PCR and sequenced in the mixture. Thus heterogeneity among copies, if any, can also be detected.

The numbers of base substitutions between pairs of strains summed over the three phytochrome introns (total 602 bp) are given in Table 2. Besides base substitutions, short insertions were also found in strains NE88 and W2025, and large deletions in strain W1444 (about 60 bp in intron 3). Within *O. rufipogon,* strains originating from geographic regions as remote as Thailand, Indonesia, and India differed at most by 2 or 3 substitutions, i.e., 3.3-5.0 x 10^{-3} substitutions/site. This pattern of variation yields a star phylogeny (i.e., nonsignificant differences between pairs of strains). Yet, a trend

for clustering of annuals and perennials from the same region seems to exist (Thailand: NE4, CP20, W180, and NE88; India: W106 and W108). Essentially the same trend was observed using Southern hybridization analysis. This could mean that annuals and perennials differentiated within the same geographic regions. In any case, the pattern of variation observed argues against the possibility of the spread of one single annual ecotype. On the other hand, all *O. rufipogon* strains differed from *O. longistaminata* by 22-24 substitutions, i.e., 36-40 x 10^{-3} substitutions/site. Assuming a neutral substitution rate for introns of 3 x 10^{-9}/site per yr would date back the *O. longistaminata-O. rufipogon* divergence to 4-6 million years ago and the divergence between strains of *O. rufipogon* to 800,000 years ago at most.

The prolamin gene sequences of strains W2025, W106, W108, W180, NE4, and CP20 were identical. Within *O. rufipogon,* one base substitution was found in W2008 (amino acid #38, third position) and two in NE88 (#84, third position; #177, second position), whereas four substitutions and the "insertion" of two additional codons were found in *O. longistaminata* (Barbier and Ishihama 1990). No conspicuous heterogeneity

Table 1. Wild rice strains used.

Code	Ecotype	Origin
W106	Annual	India (Orissa)
W2008	Annual	West India
NE4	Annual	Thailand (Northeast)
W108	Perennial	India (Orissa)
W180	Perennial	Thailand (North)
CP20	Perennial	Thailand (Central Plain)
NE88	Perennial	Thailand (Northeast)
W2025	Perennial	Indonesia (Kalimantan)
W1444	Perennial	Africa

Table 2. Variation in phytochrome genes among wild rice strains.

	Total point mutations (no.) between pairs of strains								
	W1444	W2025	W2008	W108	W106	NE4	NE88	CP20	W180
W1444		25	23	26	26	25	24	23	25
W2025	31		2	3	3	2	1	2	2
W2008	28	3		3	3	2	1	0	2
W108	31	4	3		2	3	2	3	3
W106	31	4	3	2		3	2	3	3
NE4	30	3	2	3	3		1	2	2
NE88	30	3	2	3	3	2		1	1
CP20	28	3	0	3	3	2	2		2
W180	30	3	2	3	3	2	2	2	
	W1444	W2025	W2008	W108	W106	NE4	NE88	CP20	W180
	Total point mutations, insertions, and deletions (no.) between pairs of strains								

among prolamin gene copies could be detected. The variant sequences were further estimated to have spread to at least 80% of the total number of copies. The evolutionary implications are different from those of one substitution in a single-copy gene, as the time from the occurrence of mutation to its spread to most gene copies must be considered. However, variant strains W2008 and NE88 belong neither to the same ecotype nor to the same geographic region.

Different regions of the same genome may have different evolutionary histories The construction of a phylogeny of strains belonging to the same species may be complicated by recombination events. Hence, a certain amount of contiguous sequence data is preferable to infer such a phylogeny. For a substitution rate similar to the above, about 2500 bp of such a contiguous sequence (noncoding sequence) are necessary to obtain a reliable phylogeny of wild rice strains.

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Increasing cold and drought tolerance in rice with a new phytohormone analogue

A.A. Flores-Nimedez, K. Dörffling, and B.S. Vergara

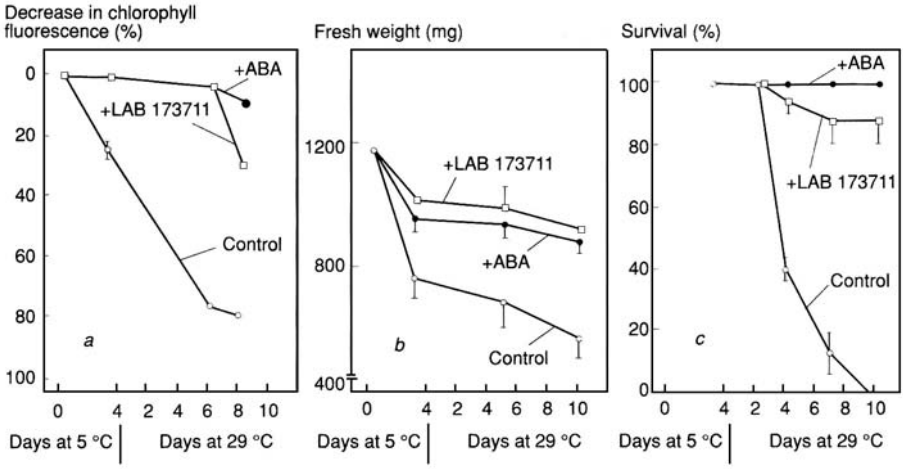
Two factors that limit the growth and yield of rice are chillig and drought stress. Because of low temperature, seeding or transplanting is usually delayed to avoid chlorosis or death of seedlings. Rice seedlings are protected from cold injury by growing them under protective structures, which are very expensive. During the first 3 wk after transplanting in the field, leaves wilt and turn yellow. Transplanting stress causes root damage, imbalances between shoot and root, and water stress. An abscisic acid (ABA) analogue under laboratory conditions is very effective in preventing leaf-rolling and death of seedlings during low-temperature (Flores et al 1988a) and drought stress. This is the first time a plant growth regulator with such a dramatic effect has been found. The use of the ABA analogue to prevent cold damage and transplanting shock might be more economical in the future.

This research aimed to

- characterize the role of the ABA analogue in chilling damage at the seedling stage and its interaction with transplanting stresses, and
- determine the effect of the ABA analogue on grain yield under stressed and nonstressed conditions.

Twelve-, 27-, and 36-d-old Samgangbyeo and IR36 were sprayed with 10^{-3} ABA and 10^{-4} mol LAB 173711 (BASF)/liter in the greenhouse (Grossman and Jung 1984). After 24 h, the seedlings were transferred to a water tank with a root temperature of 11 °C for 4 and 10 d and 5 °C for 2 d. Leaf-rolling, water potential, electrolyte leakage, chlorophyll fluorescence, and survival were measured. During chilling at 11 °C for 4 d, leaf water potential significantly decreased and leaf-rolling increased. These were, however, minimized by the application of LAB 173711. Fresh weight and survival were higher in plants treated with ABA and the ABA analogue (Fig. 1). The plants with LAB 173711 treatment exhibited less yellowing and lower decrease in chlorophyll fluorescence than did the control. The new ABA analogue is effective in maintaining not only water balance but also membrane stability. Application of LAB 173711 decreased electrolyte leakage. Spikelet sterility was likewise increased by chilling stress. Application of LAB 173711 before chilling at 5 °C and 11 °C minimized sterility.

The analogue is also effective in reducing transplanting shock. Twelve-day-old IR20 seedlings were pulled, and the soil was removed. Roots were dipped in 10^{-4} mol



1. Changes in chlorophyll fluorescence, leaf fresh weight. and survival at initial temperature of 5 °C followed by 29 °C for recovery in ABA- and LAB -treated 27-d-old IR36. Data are means ± standard error of a) 10 measurements, (b) and c) 5 parallel experiments each with 10 plants.

LAB 173711/liter. After 24 h, the seedlings were transferred to trays and exposed to direct sunlight for 3 h prior to transplanting in the field. Survival was higher in seedlings treated with LAB 173711 prior to drought stress and transplanting (Fig. 2). The percentage of filled spikelets was higher when monitored during harvest.

The protective mechanism of the new ABA analogue LAB 173711 seems to involve the following processes:

- *stomatal closure,* which reduces water loss during chilling and drought stress, thus increasing leaf water potential and fresh weight, and reducing leaf-rolling (Flores and Dörffling 1990);
- *stability of the membrane* by a decrease in electrolyte leakage (Flores et al 1988b);
- *prevention of chlorosis* by an increase in chlorophyll fluorescence;
- *stability of the root system;* and
- *decrease in spikelet sterility.*

The use of LAB 173711 in increasing cold and drought tolerance in rice is now being monitored in the field.

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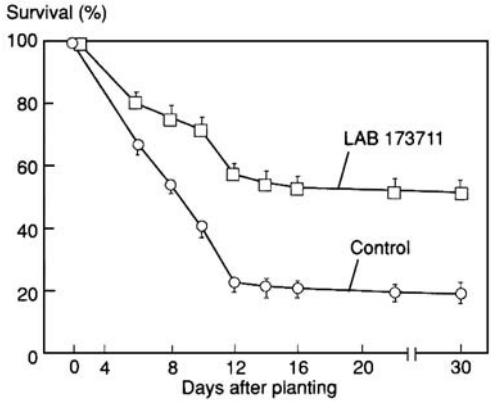

2. Changes in survival percentage of drought-stressed IR20 seedlings pretreated with LAB 173711. Data are means ± standard error of 16 measurements.

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Genetic control of chilling injury in rice seedlings detected by low-temperature treatment

T. Nagamine and M. Nakagahra

We have established an optimum method for evaluating chilling injury at the seedling stage and reported wide genetic variation and geographic distribution in the character among indigenous rice varieties collected from various countries (Nagamine and Nakagahra 1989). Tolerant varieties were distributed mainly at high latitudes. Genetic control of chilling injury was analyzed at 5 °C for 4 d in a growth cabinet. F_1 plants of reciprocal crosses between tolerant and sensitive varieties were always tolerant, and the F_2 populations showed a monogenic segregation ratio (3 tolerant: 1 sensitive; Table 1). Based on the phenotypes of F_3 lines derived from selfed F_2 plants, the F_2 genotypes were confirmed as 1 tolerant homozygote: 2 heterozygotes: 1 sensitive homozygote (Table 2). Accordingly, tolerance for chilling injury at the seedling stage is controlled by a single dominant gene. Low-temperature chlorosis is another response expressed under a temperature treatment of 15-20 °C during the seedling stage. No linkage was found between chilling injury and low-temperature chlorosis, indicating that the characters are controlled by different loci (Table 3). Thus, at least two loci control tolerance for low-temperature stress during the seedling stage.

Esterase isozyme *Est-3* was used to distinguish indica and japonica varietal groups (Nakagahra 1978). The relationships between chilling injury and allele difference in *Est-3* were studied in 1,548 indigenous varieties. Most varieties having allele $Est\text{-}3^2$ were sensitive to low temperature, while varieties having $Est\text{-}3^1$ were mainly tolerant (Table 4). A clear correspondence existed between the genotype of *Est-3* and the response to low-temperature stress.

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Table 1. Genetic analysis of F_1 rice plants and F_2 populations of crosses between tolerant and sensitive parents for chilling injury in seedlings.[a]

Cross (P_1/P_2)	Phenotype			Segregation in F_2 population			c^2 (3:1)
	P_1	P_2	F_1	T	S	Total	
Dular/In Sitt	S	T	T	443	149	592	0.009
Guizhao 2/Dourado Precoce	S	T	T	221	66	287	0.615
Guizhao 2/R.M.	S	T	T	373	114	487	0.657
IR30/North Rose	S	T	T	225	84	309	0.787
Nepal 8/Shirakawa	S	T	T	232	70	302	0.535
Lizihong/IR30	T	S	NE	205	69	274	0.009
North Rose/Dee-geo-woo-gen	T	S	T	235	76	311	0.052
Shirakawa/Pusur	T	S	T	235	76	311	0.052
Wzbeuskij 2/Guizhao 2	T	S	T	445	135	580	0.920

[a]T = tolerant, S = sensitive, NE = not examined. P_1 = female parent, P_2 = male parent.

Table 2. Segregation of 3 rice genotypes by chilling injury expected in F_3 seedlings.

Cross (P_1/P_2)		F_3 lines (no.) tested				c^2 (1:2:1)
		Tolerant homozygote	Segregating heterozygote	Sensitive homozygote	Total	
Lizihong/Guizhao 2	Observed	74	144	55	273	3.469
	Expected	68	137	68		
Lizihong/IRBO	Observed	69	126	57	252	1.142
	Expected	63	126	63		
North Rose/IR30	Observed	74	164	74	312	0.820
	Expected	78	156	78		

Table 3. Segregation of tolerance genes for chilling injury and low-temperature chlorosis in F_2 hybrid population of rice seedlings.[a]

Cross (P_1/P_2)	Genotype of			Segregation (no.) in F_2 hybrid population					c^2 (3:1) for chilling injury	c^2 (3:1) for chlorosis	c^2 for Independency
	P_1	P_2		+/+	+/c	s/+	s/c	Total			
Dular/In Sitt	s/c	+/+	Observed	167	53	46	28	294	0.005	1.020	5.625*
			Expected	162.6	54.2	54.2	18.1				
Shirakawa/ Dular	+/+	s/c	Observed	162	60	43	20	285	1.274	1.432	0.425
			Expected	160.3	53.4	53.4	17.8				
Shirakawa/ Pusur	+/+	s/c	Observed	166	69	55	21	311	0.053	2.573	0.103
			Expected	174.9	58.3	58.3	19.4				

[a]s =chilling injury, c = low-temperature chlorosis, P_1 = female parent, P_2 = male parent. * = significant at the 5% level.

Table 4. Relationship between genotypes of *Est-3* and degree of chilling injury at seedling stage in indigenous rice.

Genotype	Seedlings (no.) with given degree of chilling injury						
	0	1	2	3	4	5	6
Est-3[1]	51	137	159	71	15	12	14
Est-3[2]	2	14	58	163	189	217	446
Total	53	151	217	234	204	229	460

Notes

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Simple, rapid procedure for analyzing polypeptide pattern of thylakoid membrane in rice

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Photosynthetic proteins in the chloroplast have very complicated pathways. Proteins in the thylakoid membrane act in H^+ transfer during reduction and phosphorylation in the light reaction. Many studies of protein synthesis in the thylakoid membrane show that genetic information about proteins is coded somewhere in the chloroplast genome and/or nuclear genome.

We analyzed the polypeptide pattern of the protein on the thylakoid membrane to determine the genetic markers of the nuclear and chloroplast genes by a simple and rapid procedure instead of using the conventional density gradient ultracentrifugation method. We tried to detect differences in the polypeptide pattern of the protein in M_1 plants obtained from embryo-irradiated (^{60}Co gamma ray) Nipponbare rice.

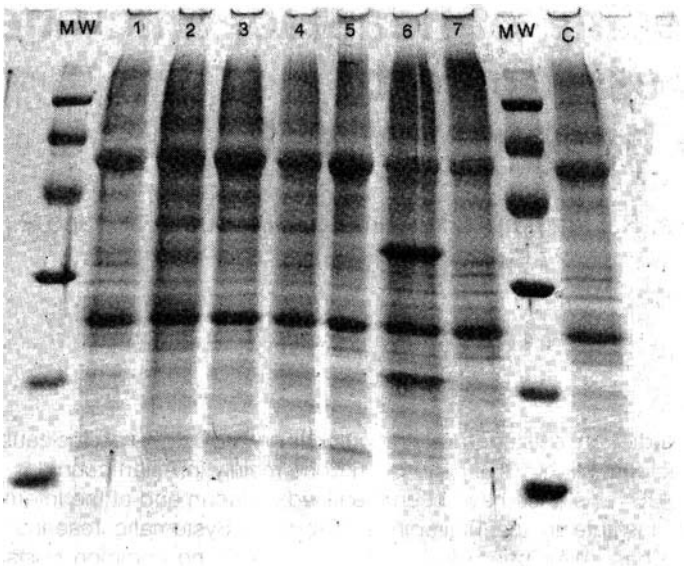
About 0.1 g (fresh weight) of rice leaf blades was crushed and ground in liquid N_2 by hand, applying the principle of the Potter-Elvehjem type tissue grinder. After grinding, 0.1 ml of Tris-HCI buffer (pH 6.8) was added, glass beadswere included, and the mixture was homogenized in a vibrator at 4 °C for 20 min. The liquid mixture was centrifuged at 500 g for 5 min, and the green supernatant was decanted and centrifuged again at 15,000 g for 20 min. The green pellet obtained was resuspended with buffer. This green solution was used for purification of thylakoid by floating centrifugation. Purified thylakoid was solubilized in the buffer containing 5% sodium dodecyl sulfate (SDS) by heating at 95 °C for 5 min. This solution was used for SDS-polyacrylamide gel electrophoresis as developed by Doucet (1988).

By this simple and rapid procedure, *Oryza latifolia* and three varieties of cultivated rice were compared. All but *O. latifolia* showed very similar polypeptide patterns.

One hundred five of 4,962 M_1 plants obtained from embryo irradiation differed from the control in the polypeptide pattern of thylakoid protein. Differences in the polypeptide band pattern occurred mainly near the 22- and/or 38-kDa protein. A remarkably different pattern was observed in two plants (Fig. 1), both of which died before seed ripening. Therefore, a progeny test could not be performed.

Our simple procedure is very useful for analyzing the polypeptide pattern of the thylakoid membrane. Many materials can be rapidly analyzed without ultracentrifugation.

It is not known if the 105 M_1 plants with differing band patterns from the control were mutants for the chloroplast and/or nuclear genome. A progeny test needs to be conducted to determine the induction of gene mutations concerning differences in polypeptide pattern.



1. Polypeptide patterns of some M_1 plants and control. MW = molecular weight markers (upper to lower, 94, 67, 43, 30, 20.1, 14.4 kDa), C = control (Nipponbare), 1-7 = M_1 plants. No. 6 has a remarkably different band pattern.

Notes

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Breeding of near-isogenic lines for resistance to rice bacterial blight

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Genetic studies on resistance to *Xanthomonas oryzae* pv. *oryzae,* the causal organism of rice bacterial blight (BB), have been done mainly in Asian countries. To date, 12 genes for BB resistance have been identified in Japan and at the International Rice Research Institute in the Philippines (Table 1). Systematic research on varietal resistance has never been done because there is no common basis for genetic research (Ogawa and Khush 1989). Each country has used different BB races or a different set of differentials.

International differentials—a set of near-isogenic lines (NILS)—can be the general basis of systematic evaluation of resistance. These lines can be the major tool for identifying genes and BB races, and their subsequent distribution. Moreover, these can be valuable materials for basic research and as donors in resistance breeding programs.

To develop NlLs with a monogenic basis, the 11 donor parents were backcrossed 4 times to 3 recurrent parents, namely IR24 (indica), Toyonishiki (japonica), and Milyang 23 (indica/japonica derivative). Among the 12 genes confirmed, 4—*Xa-1, Xa-2, Xa-11,* and *Xa-12*—were susceptible to all Philippine races. Thus, isogenic lines carrying these four genes were developed at the Tropical Agriculture Research Center, Japan, using Japanese BB races (Table 2). For other genes—*Xa-3, Xa-4, xa-5, Xa-7, xa-8, Xa-10, xa-13,* and *Xa-14*—isogenic lines were developed at IRRI using Philippine races.

The NlLs were named in three series based on their genetic background. For *Xa-1,* lines IR-BB 1, IR-BB 101, and IR-BB 201 have the genetic background of IR24, Toyonishiki, and Milyang 23, respectively. The last two digits in each name of the NlLs indicate the specific major gene involved.

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Table 1. Near-isogenic lines for BB resistance developed at IRRI and the Tropical Agriculture Research Center, 1990.

Gene	Donor	Lines obtained with given recurrent parent [a]		
		IR24	Toyonishiki	Milyang 23
Xa-1	Kogyoku	IR-BB 1 (t)	IR-BB 101 (t)	IR-BB 201 (t)
Xa-2	Tetep	IR-BB 2	IR-BB 102	IR-BB 202
Xa-3	Chugoku 45	IR-BB 3	IR-BB 103	IR-BB 203
Xa-4	IR20	IR-BB 4	IR-BB 104	IR-BB 204
xa-5	1Rl545-339	IR-BB 5	IR-BB 105	IR-BB 205
Xa-7	DV85	IR-BB 7	IR-BB 107	IR-BB 207
xa-8	PI 231 129	IR-BB 8	IR-BB 108	IR-BB 208
Xa-10	Cas 209	IR-BB 10	IR-BB 110	IR-BB 210
Xa-11	IR8	IR-BB 11	IR-BB 111	IR-BB 211
Xa-12	Kogyoku	IR-BB 12	IR-BB 112	IR-BB 212
xa-13	BJ1	IR-BB 13	BC_4F_3 [b]	BC_4F_3 [b]
Xa-14	TN1	IR-BB 14	IR-BB 114	IR-BB 214

[a](t) = tentative designation. [b]Latest generation.

Table 2. Reaction of near-isogenic lines to 6 Philippine BB races.[a]

Line	Reaction to					
	PXO 61 Race 1	PXO 86 Race 2	PXO 79 Race 3	PXO 71 Race 4	PXO 112 Race 5	PXO 99 Race 6
IR-BB 1 (t)	S	S	S	S	S	S
IR-BB 2	S	S	S	S	S	S
IR-BB 3	R	R	R	R	R	S
IR-BB 4	R	S	S	MR	R	S
IR-BB 5	R	R	R	MS	R	S
IR-BB 7	R	R	R	MR	R	S
IR-BB a	R	R	R	MR	R	R
IR-BB 10	S	R	S	S	R	S
IR-BB 11	S	S	S	S	S	S
IR-BB 12	S	S	S	S	S	S
IR-BB 13	S	S	S	S	S	MR
IR-BB 14	S	S	S	S	R	S

[a]S = susceptible, R = resistant, MR = moderately resistant, MS = moderately susceptible.

Notes

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Variation in resistance to bacterial blight between and within wild rice populations

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Comparison of host-pathogen interactions in a natural ecosystem and in an agroecosystem can provide perspectives on the evolutionary dynamics of plant-pathogen systems. The present study dealt with inter- and intrapopulational variation in response to bacterial blight (BB) in the wild rice *Oryza rufipogon.*

Forty-two strains of wild rice (each representing a natural population) collected from various regions of Asia were examined for response to infection by five races of *Xanthomonas oryzae* pv. *oryzae* isolated in Japan. Multivariate analysis of the data showed variation in both general resistance and race-specific resistance. The pattern of variation, however, did not show a distinct relationship to geographical distribution, nor to ecotypic differentiation (perennial vs annual type). Some Chinese strains showed race-specific responses.

To assess the intrapopulational variation in response to the pathogen, three wild rice populations from Thailand (one annual and two perennial types) were studied together with two land race populations from China. In each population, 30-40 lines derived from randomly chosen mother plants growing in their natural habitats were studied by the injection method as well as by clip inoculation, using three Japanese pathogen races. Wild rices as well as land races were found to preserve a large amount of intrapopulational genetic variability in response to the pathogen races. In the host-pathogen associations used in the present study, wild rices were generally resistant, although land races were susceptible. Table 1 shows an example of frequency distribution of response to infection by BB race T7133. The intrapopulational variation in BB resistance was not related to the genetic diversity estimated from isozyme variation.

Responses to three pathogen races were compared among lines in each population. In most cases, intensities of infection (lesion lengths on leaves) by different races were positively correlated, indicating that race-nonspecific resistance, possibly governed by polygenes, was mainly responsible for the intensity of infection. In addition, the existence of resistance governed by a major gene was suggested by the discontinuity in the response to race T7133 in NE4 (Table 1). This was confirmed by the injection method. Furthermore, the genetic structures of two populations, NE4 and CP20, each consisting of 30 lines, were compared with regard to BB response. Analysis of variance indicated that between-line variation (heterogeneity in the field)

Table 1. Intrapopulational variation found in 3 wild and 2 cultivated rice populations in response to infection by BB pathogen race T7133.

Population[a]	Plants (no.) showing given lesion length (cm) on leaves												Mean (cm)	Standard deviation (cm)	H[b]
	0	2	4	6	8	10	12	14	16	18	20	22			
Wild rice (Thailand)															
NE4	7	3	4	1	4	6	2		1				5.5	4.01	0.193
CP20	8	16	5										2.0	1.17	0.360
NE88	7	19	4	6	5	2	1			1			4.1	3.57	0.390
Land race (China)															
Ch 54					1			4	6	4	11	10	19.8	4.00	0.074
Ch 55			1	2	1	9	11	8	3				11.9	3.40	0.095
Control															
Norin 8				(5	2)[c]		2	3	2	1			14.4	1.78	
Java 4	6	3											0.8	0.62	
WA3	3	4		1									1.8	1.89	

[a] NE4 =annual, CP20 and NE88 = perennial, Ch 54 = lowland, Ch 55 = upland. [b] H =average genetic diversity estimated from isozyme variation. Cited from Barbier (1989) for wild rices and from Morishima (1989) for land races. [c] Tested in the experimental field; others were in a greenhouse.

was larger in the annual population NE4, while within-line variation (heterozygosity in the field) was larger in the perennial population CP20. This could be explained by the difference in their breeding systems.

To understand the complex pattern of host-pathogen interactions, more of the pathogen races, particularly those prevailing in the areas from which study populations are sampled, should be used.

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Citation information: International Rice Research lnstitute (1991) Rice genetics II. P.O. Box 933, Manila, Philippines.

Genetic analysis of rice blast resistance using doubled haploids, single seed descent lines, and isozyme marker genes

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Thanks to molecular techniques, the genetic map of rice chromosomes is becoming densely populated, and genes of interest can be located and further tagged, isolated, and cloned. Blast (BI) caused by *Pyricularia oryzae* is the most serious rice fungal disease. To identify genetic markers linked with BI resistance genes would facilitate breeding programs and improve basic knowledge of the action of such genes.

We initiated the use of recombinant doubled haploid (DH) and single seed descent (SSD) inbred lines derived from the cross IRAT177/Apura to locate the genes controlling BI resistance through cosegregation analysis with 12 isozyme markers.

lRAT177 and Apura parentals have contrasting alleles at 12 isozyme loci on at least 6 of the 12 chromosomes, namely chromosomes 1, 3, 7, 8, 11, and 12. IRAT177 and Apura plants were inoculated by syringe injection with suspensions containing 25,000 conidia/ml of 25 BI isolates collected from 11 countries. Symptom reactions were scored according to a 1-6 scale. Three BI strains (CD101 , BR14, and BR26)were found virulent on a single parent.

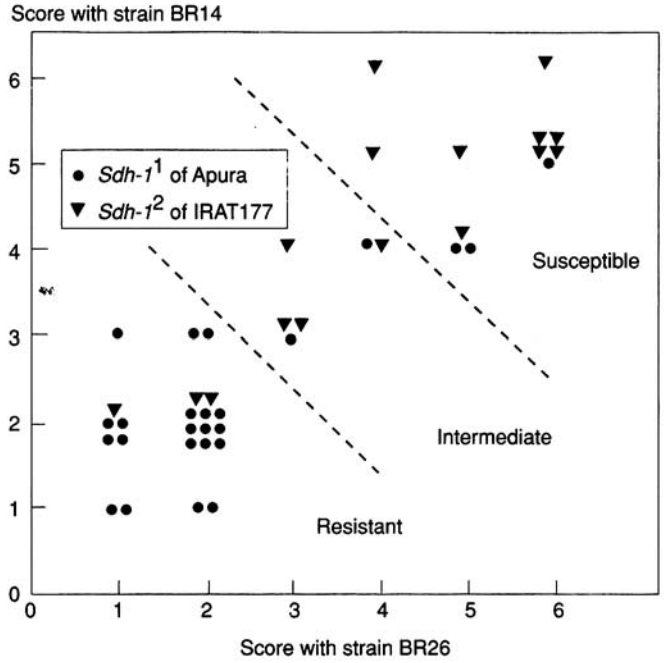
Forty-two DH lines and 89 F_7 SSD lines generated from lRAT177/Apura have been isozyme analyzed, then artificially inoculated with the 3 BI isolates.

The DH and SSD lines showed a clear bimodal distribution for BR14, BR26, and CD101, allowing them to be classified as resistant (R) or susceptible (S).

The segregations of R and S phenotypes were between 1:1 and 3:1, indicating either monogenic or digenic control of resistance. Moreover, reactions to the isolates were tightly correlated (Fig. 1), suggesting identical genetic control for resistance to both strains.

Cosegregation analysis of resistance gene(s) revealed linkage with the *Sdh-1* locus (chromosome 12), which also segregated slightly differently from 1:1 (Fig. 1, Table 1). The simplest model involves a single resistance gene located 15 cM from *Sdh-1* and 30 cM from *Pox-2.* Preliminary restriction fragment length polymorphism (RFLP) assay of the DH line population recently confirmed the location of the resistance gene on chromosome 12 (S.R. McCouch, IRRI, pers. comm.).

The 3:1 segregation of R and S phenotypes among DH lines suggests digenic



1. Correlation between reactions to blast isolates BR14 and BR26 and parental origin of allele at the *Sdh-1* locus in DH lines derived from IRAT177/Apura.

Table 1. Cosegregation of resistance and *Sdh-1* and *Pox-2* alleles among DH and SSD lines, and linkage estimates.

Progeny		*Sdh-1*		*Pox-2*	
		Apura	IRAT177	Apura	IRAT177
DH lines	Resistant	20	3	14	6
	Susceptible	3	9	3	8
		r = 17.1%		r = 29.0%	
SSD lines	Resistant	19	9	20	11
	Susceptible	2	19	10	12
		r = 14.4%		r = 32.8%	

[a]For DH lines, the r value is calculated from the percentage of recombinant associations. For SSD lines, the r value is obtained from the formula $a = 2r/(1 + 2r)$, where a is the apparent rate of recombinants.

control, whereas segregation among SSD lines was atypical and was between 1:1 and 3:1. Based on these values, resistance could be controlled by either one independent or two partially linked genes. No clear linkage was found through cosegregation analysis of resistance to this strain with isozyme markers.

This study illustrates the suitability of recombinant inbreds generated through anther culture or SSD to locate genes controlling BI resistance using marker genes. Ongoing assay of the two-line populations with RFLP markers (S.R. McCouch and S.D. Tanksley, pers. comm.) should sharpen the analysis considerably and possibly locate other BI resistance genes.

We surveyed other parents of hybrids for which large sets of DH or SSD lines are available for their reaction to 20 BI strains. Nineteen strains differentiated parentals for cross IR64/Azucena and for cross IR34583-22-1-2/Bulu Dalam. This implies wide scope for identifying and locating BI resistance genes with the help of biochemical and molecular genetic markers.

Notes

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Induced mutations for rice improvement in Malaysia

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High yield, good eating quality, early maturity and resistance to major pests and diseases are characters usually desired in a rice variety. The use of induced mutations for rice improvement at the Malaysian Agricultural Research and Development Institute (MARDI) started in 1972. However, a more concerted effort was begun in 1979 to look for blast (BI) resistance (Arasu et al 1983). Since then, mutation breeding work encompassing other objectives as well has been intensified. To date, 48 varieties and elite breeding lines have been treated with ethyl methane sulfonate (1.5-2.5%) and gamma radiation (15-40 kr). Mutant lines with short stature, early maturity, improved grain quality, increased BI and brown planthopper (BPH) resistances, and photoperiod insensitivity were obtained (Table 1). Mutant Muda 2 was released directly to farmers to replace the slightly taller Muda variety, which is susceptible to lodging. Another mutant from Muda, MR90, with moderate improvement in BPH resistance, is a promising line for rainfed areas. Mahsuri Mutant, which has good eating quality, is now being popularized as a high-quality rice (Hadzim et al 1988). A short-statured, early-maturing mutant of Mahsuri Mutant, MM98, has lower amylose content. An aromatic hill rice, Pongsu Seribu 2, had its height halved and its yield potential improved through the mutant PS1297 (Mohamad et al 1988). A short, early, and photoperiod-insensitive mutant has been recovered from the tall, traditional, good-eating variety Jarum Mas. Other mutants are being used in cross breeding. The International Atomic Energy Agency sponsored a 5-yr project on semidwarf rices with PUSPATI using the variety Manik beginning in 1984. Several promising mutants from the variety are being evaluated for their yield potential, and an allelic test for semidwarfism is under way. The glutinous mutant Manik 817 has shorter stature than the previously popular glutinous varieties PMI and Pulut Siding, and gives higher head rice recovery. Reduction in duration and plant height, disease and pest resistance, and improved eating quality are attainable objectives in mutation breeding without reducing grain yield, as experienced at MARDI.

Table 1. Grain yield and other important traits of mutants and parents.

Variety	1000-grain weight (g)	Plant height (cm)	Amylose content (%)	Resistance to or tolerance for	Eating quality	Yield (t/ha)	Grain length (mm)	Aroma	Maturity (d)
Mahsuri	16.6	102.5	26.9	None	Good	4.2	7.75	None	134-138
Mahsuri Mutant	19.0	115.0	25.3	Blast	Better	4.4	9.14	None	131-148
Mahsuri Mutant MM98	20.6	76.0	19.4	Blast	Good	4.7	7.72	None	117-122
Pongsu Seribu 2	20.8	128.5	23.9	Blast	Good	2.2	8.40	Present (strong)	140-148
PS1297	21.5	72.0	28.4	Blast	Intermediate	4.0	8.42	Present (less strong)	140-148
Muda (MR71)	25.8	113.0	28.7	Blast, tungro, BB	Intermediate	5.2	9.78	None	126-132
Muda 2	25.9	93.6	N.A.	Blast, BB	No change	5.2	9.70	None	125-140
MR90	26.7	116.6	N.A.	BPH, blast, BB	No change	4.6	9 86	None	136-145

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Mutagen-induced male sterility in rice

J.L. Minocha, J.S. Sidhu, and R.K. Gupta

To diversify the sources of cytoplasmic male sterility in rice *Oryza sativa* L., attempts were made to induce this character in the popular indica cultivar PR106 using chemical mutagens. Seeds were treated with different doses of ethidium bromide, ethyl methanesulfonate (EMS), and streptomycin (SM).

In the M_2, male sterile plants were detected in 11 progenies, 1 from the SM, and the remaining from the EMS treatments (Minocha and Gupta 1988). The male sterile plants were hand-pollinated or were left to open-pollinate in the field of PR106. A stable male sterile mutant was obtained after treatment with 0.4% EMS for 48 h at 10 °C. The breeding behavior of the mutant in the M_2-M_5 is given in Table 1

Some of the plant-to-row progenies were completely male sterile in the M_5 (Table 2).

PR106 had normal meiotic behavior and fertile pollen grains. The male sterile plants flowered 3-5 d later than PR106, had erect panicles, and remained green for a longer period. The male sterile plants had normal meiotic behavior but had 100% aborted pollen grains, nonstainable with both acetocarmine and I-KI solution. There was no seed on the selfed panicles, and various seed sets were obtained on crossing with PR106.

Thus the sterility of PR106A is cytoplasmic and is maintained by the parental variety. PR106 restores fertility when crossed with Chinese male sterile line V20A. Therefore, the male sterile line PR106A is a different source of male sterility and may help in diversifying the sources of male sterility for hybrid seed production in indica rices.

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Table 1. Breeding behavior of selected male sterile line PR106A.

Generation	Year	Plants (no.)		
		Sterile	Fertile	Total
M_2	1986	9	5	14
M_3	1987	14	6	20
M_4	1988	31	0	31
M_5	1989	464	22	486

Table 2. Breeding behavior of selected M_5 progenies.

Row no.	Sterile plants (no.)[a]
87	10
88	13
115	21
118	11
121	18
125	7
136	32

[a] No fertile plants

Notes

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Induction of inheritable dwarfism in rice by DNA demethylation

H. Sano, S. Youssefian, I. Kamada, and M. ltoh

A single exposure of germinated rice *Oryza sativa* seeds to either of the DNA demethylating agents 5-azacytidine (azaC) or 5-azadeoxycytidlne induced dwarf plants. The seedlings were most sensitive to the drug during the first 6 d after germination. The duration of drug treatment determined the number of dwarf samples but not the extent of dwarfing. The reduction in height was proportional to the azaC dose up to 1.5 mM; above that concentration no further effects were observed. Mature plants treated with azaC as seedlings exhibited normal characteristics, except that the height was reduced about 15% from that of untreated controls. The M_1 progeny, obtained by self-fertilization of an azaC-induced dwarf plant, segregated into dwarf (35%) and apparently tall (65%) types. The M_2 progenies, obtained by self-fertilization of dwarf M_1 plants, were also dwarf, while those from tall M_1 plants were all tall. Genomic DNA isolated from mature leaves of azaC-treated seeds showed about a 16% reduction in 5-methylcytosine (m^5C) content compared with DNA from untreated samples. A similar reduction in the m^5C content was also observed in M_1 and M_2 progenies. Thus, both undermethylation and dwarfism induced by azaC treatment are heritable. These results suggest that azaC-induced demethylation of genomic DNA causes an altered pattern of gene expression and consequently a reduction in plant stem length.

Notes

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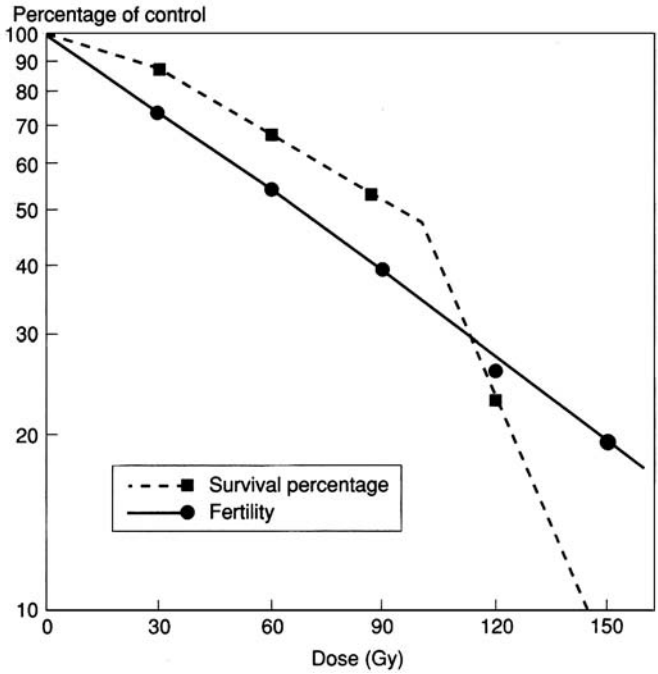
Mutagenic effects of accelerated argon ion irradiation in rice

M.T. Mei, Q.F. Zuo, Y.G. Lu, H. Deng, and T.C. Yang

Since research work with accelerated heavy ions has shown that high-linear energy transfer (LET) ion radiation can induce certain types of mutation more effectively than low-LET radiation in mammalian cells (Cox et al 1977), yeast, and maize (Mei et al 1987, Yang 1979), we initiated this study in 1987 to explore the potential use of heavy ion radiation in rice mutation breeding. Dry seeds of Bianpizhan, an indica variety, were irradiated with various doses of an ^{40}Ar ion beam (E=400 MeV/u; LET_{∞} = 117 keV/µm) generated by the BEVALAC accelerator at Lawrence Berkeley Laboratory. The field experiment was implemented at the South China Agricultural University. Injurious effects on the M_1 were observed. The dose-seedling survival curve as well as the dose-fertility curve (Fig. 1) showed that $D_{50\text{-}s}$ and $D_{50\text{-}f}$ were about 93.7 Gy and 67.0 Gy, respectively.

By using the plant-to-row method, more than 7,000 M_2 plants were investigated, and mutants with variations in quantitative or morphological characters were sought. Doses from 60 to 120 Gy were effective in inducing mutation in dry seeds, and the mutation frequency was rather high, especially for mutations involving semidwarfism, earliness, and grain shape (Table 1). Also, more than one mutated character was frequently found in a single plant. Thus, accelerated heavy ion radiation might be useful in rice mutation breeding. Further studies comparing the mutation effectiveness of various heavy ion and gamma ray treatments are being conducted at the South China Agricultural University.

Seedlings of 43 stable mutants in the M_5 and the original variety were tested for allozymic differences in 10 isozymes—SDH, EST, ICD, PGI, GOP, POX, ACP, AMP, PGD, and ME, which represent 17 loci—by starch gel electrophoresis (Second 1982, Wu et al 1988). Alteration of the electrophoretic banding pattern of one or more enzymes was observed in 37 mutants. Such alterations were found for only three enzymes—SDH-1, PGD-1, and PGI-2—among the 17 surveyed and were related to mutations in apiculus color, grain shape, and plant height, respectively. Thus, for some mutated characters, a linkage between mutated loci and isozyme loci might exist.



1. Dose-response curves of M_1 injury observed on the 30th day after seed germination (average of 50 single plants for each treatment).

Table 1. Mutation spectra and mutation frequency[a] in the M_2 generation.

Dose (Gy)	Mutation frequency											
	Semi-dwarfism (1)	Early maturity (2)	Grain shape (3)	Apiculus color (4)	Sheath color (5)	Total (I)[b]	Panicle length[c] (6)	Effective panicles per plant[c] (7)	Spikelets per panicle[c] (8)	Grains Per panicle[c] (9)	1000-grain weight[c] (10)	Total (II)[b]
30	1.67 (3120)	1.15 (3120)	1.19 (3120)	1.51 (3120)	1.57 (3120)	2.02 (3120)	0 (150)	0 (150)	0 (150)	0.63 (150)	2.00 (150)	2.00 (150)
60	4.16 (1470)	3.13 (1470)	2.43 (1470)	2.93 (1470)	3.12 (1470)	4.56 (1470)	0 (150)	2.67 (150)	0 (150)	0 (150)	2.00 (150)	4.00 (150)
90	4.10 (1390)	5.90 (1390)	3.47 (1390)	3.04 (1390)	3.47 (1390)	6.83 (1390)	0.67 (150)	3.33 (150)	2.67 (150)	4.67 (150)	4.00 (150)	12.00 (150)
120	3.52 (630)	4.29 (630)	1.77 (630)	2.86 (630)	3.35 (630)	4.92 (630)	0 (150)	2.67 (150)	0 (150)	0 (150)	0.67 (150)	3.33 (150)
150	1.40 (140)	0 (140)	1.43 (140)	1.43 (140)	1.43 (140)	1.43 (140)	0 (42)	0 (42)	0 (42)	0 (42)	0 (42)	0 (42)

[a] Figures in parentheses represent number of M_2 plants from which frequency was calculated. [b] Calculated on the basis of number of plants with mutated characters (one or more)/total number of plants sought. For total (I), mutated characters of (1), (2), (3), (4), and (5) were sought; for total (II), mutated characters of (6), (7), (8), (9), and (10) were sought. [c] Only promising mutations were sought.

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Exploitation of useful semidwarfing genes by mutation

T. Tanisaka and H. Yamagata

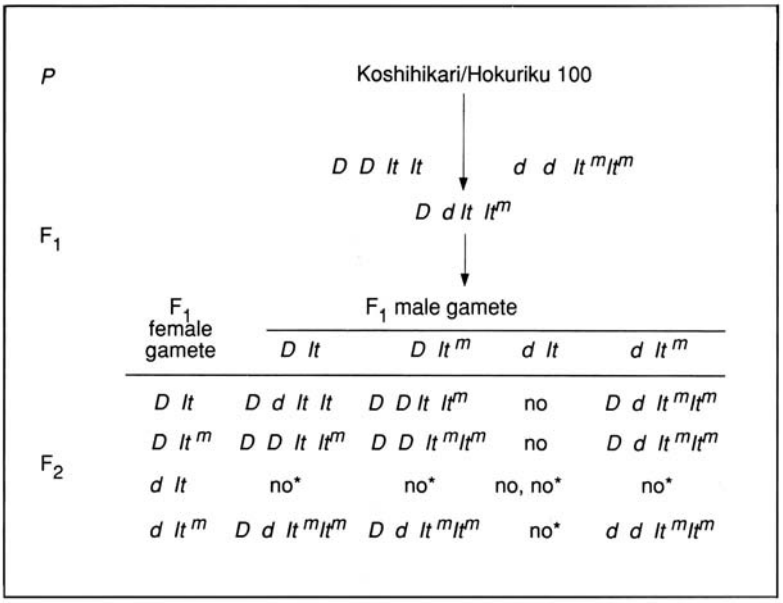
The existing semidwarfing gene resource base is much smaller than originally thought, and the semidwarf varieties under cultivation are controlled mostly by the same semidwarfing gene, *sd-1.* Such a situation suggests the urgent necessity of exploiting new semidwarfing genes to provide genetic diversity. This paper discusses the genes responsible for the semidwarfism of 24 mutants induced from 4 Japanese rice varieties.

All the mutants were crossed with their respective original varieties to learn the number and direction of dominance of mutated loci in the F_2 and F_3. Then, some of the mutants that were supposed to have a single semidwarfing mutant gene were crossed with the variety(-ies) and/or strain(s) having *sd-1* to test the allelism of the mutant genes with *sd-1.* In each cross, about 300 F_2 plants and 50-100 F_3 lines raised from randomly selected F_2 plants were analyzed. These materials were grown together with the parents and measured for culm length and heading date.

Each of 18 mutants proved to be controlled by a single recessive semidwarfing gene, 2 were governed each by 2 recessive semidwarfing genes, and the remaining 4 were due to the mutations induced in respective minor-gene systems.

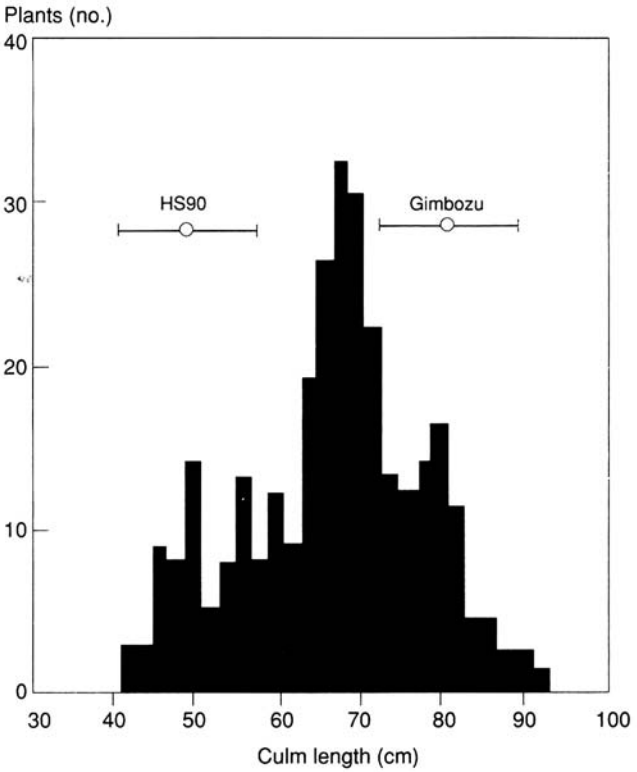
Among the 18 single recessive semidwarfing genes, *d-60* from Hokuriku 100 proved nonallelic to *sd-1,* suggesting the extreme importance of this gene in breeding for semidwarfism. To introduce *d-60* into some varieties or strains, however, a much greater number of offspring is needed than usual, because this gene cannot express itself when it coexists with the gametic lethal gene *It* (Fig. 1). Three genes from IM222, M686, and X82 were nonallelic not only to *sd-1* but also to each other, although their agronomic importance could not be evaluated in this study. Two genes from HS90 and IM265 were allelic to *sd-1* and are considered useful for hybrid rice seed production because they cause more remarkable reduction of culm length than *sd-1;* besides, their genotypic values in the heterozygous state were almost equal to those of the midparents, which came within the category of semidwarfism (Fig. 2).

Experimental results indicate that semidwarfism is induced by a single recessive gene mutation in many cases, and suggest that induced mutation successfully opens a way to create new semidwarfing gene sources. Besides, two facts suggest that the



1. Hypothesis for segregatim of semidwarfness and seed fertility in Koshihikari/Hokuriku 100. d and D = induced semidwarfing gene and its allele, respectively. Genes d-60 and 0-60 are here expressed as *d* and *D,* respectively, for convenience. *It*^{m} and *It* = induced nongametic lethal gene and its allele, which causes gametic lethality in coexistence with *d,* respectively. no and no* = no zygotes formed because of gametic sterility due to *d lt* in male and female gametes, respectively; in the latter case, F_1 plants and some of their F_2 progenies show partial seed sterility. A segregation ratio of 8:1 is expected in the F_2 for Koshihikari type *(DD, Dd)* and Hokuriku 100 type (*dd*).

sd-1 locus is easily mutated by mutagenic treatment: 1) two mutants controlled by semidwarfmg genes allelic to *sd-7* were detected; and 2) two excellent semidwarf mutant varieties, Reimei and Calrose 76, which have remarkably increased rice production in Tohoku District, Japan, and in California, USA, respectively, each have a semidwarfing gene at the *sd-1* locus.



2. Frequency distribution of culm length in the F_2 of Gimbozu/HS90.

Notes

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Combining ability and heterosis for yield and yield components related to reproductive-stage salinity and sodicity tolerance in rice *Oryza sativa* L.

B. Mishra

Genetic studies on reproductive-stage salinity and sodicity tolerance following line x tester analysis (Kempthorne 1957) Involving genetically divergent lines (15) and testers (4) having varying degrees of salt tolerance were conducted in normal (no stress), saline (ECe 10), and sodic (ESP 73, $pH_2$10) edaphic environments at the Central Soil Salinity Research Institute, Karnal. lndia. Analysis revealed significant general combining ability (GCA) for male and female parents in all environments as well as in the pooled data over the environments for plant height, total and fertile tillers per plant, grains per panicle, gram and straw yield per plant. and days to 50% flowering, while panicle weight and sterility percentage showed significant GCA in all environments individually but were nonsignificant in pooled data. Mean squares due to GCA as well as specific combining ability (SCA) were significant for all (the characters in each environment, indicating the importance of both additive and nonadditive gene effects in controlling these traits. The high GCA/SCA ratio for all characters showed a preponderance of additive gene action in the stressed environments. Parents highly tolerant of salinity or sodicity also had high GCA effects, and F_1s of two tolerant parents exhibited better tolerance. Parents CSR1, CSR2, CSR3, and CR-22-2685 were found promising for future breeding programs to exploit salt-affected soils. Significant heterosis and heterobeltiosis were observed for yield and yield components. The heterotic effect for grain yield was maximum in crosses involving the most tolerant parents.

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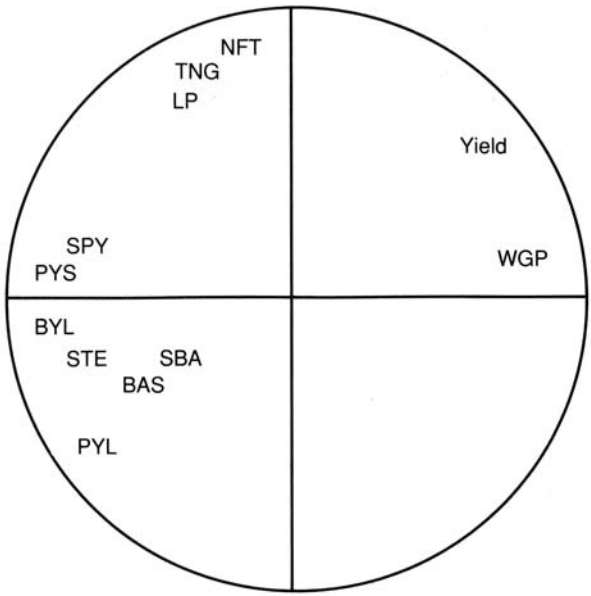
Genetics of quantitative traits in rice in mid-altitude swamps of Burundi

J.P. Tilquin, J.P. Chapeaux, and J.F. Detry

Rice breeding for swampland is very complicated. The combination of purely soil, temperature, relative humidity, and topographic factors makes each swamp a special case. To find a set of stable varieties, a multilocational trial was done with 24 preselected varieties (Malagasy or hybrid families from crosses involving IRRI lines bred for cold tolerance), 5 sites, and 4 replications. Twenty-two variables were measured during growth or at harvest. Analysis of variance showed high interaction of variety with site (G x E). The Finlay-Wilkinson and Eberhart-Russel stability models were applied to yield and to the severity of infection with *Pseudomonas fuscovaginae* and *Pyricularia oryzae,* which, singly or together, greatly affect yield in the region. Two major areas are present: one colder and one warmer. The Malagasy varieties escaped *Pseudomonas* infection in the colder area, while the hybrids were resistant to *Pyricularia* in the warmer area. Principal component analysis (Fig. 1) showed the more sensitive variables (less heritable) linked with the major axes and the interactions between them by projection on the main planes. *Pseudomonas* severity was correlated negatively with panicle length, number of grains per panicle, and 1000-grain weight. Therefore, the well-known dwarfism of the stem, which leads to poor panicle exsertion and concomitant sterility, is only one aspect of the dwarfism induced by *Pseudomonas.*

The following year, another trial was done with 16 preselected varieties at 18 sites. The greatest variation was induced by soil (fertility) and not by climatological conditions. Principal component analysis of the variables of a stable variety (Kirundo 9) tolerant of *Pyricularia* and *Pseudomonas* showed on the main axis all the constraints (sterility due to low temperature or nutritional deficiencies, to *Pseudomonas* or *Pyricularia*) negatively correlated with 1000-grain weight measured on disease-free panicles (axis 2). Tillering ability and panicle length are almost independent of constraints, explaining 65% of the variability. For a known variety, the 1000-grain weight is the best factor for predicting yield.



1. Circle of correlations. WGP = weight of 1000 grains, LP = length of panicle, TNG = total number of grains per panicle, STE = sterility of disease-free panicles, NFT = number of fertile tillers per m^2, SBA = seventy of bacteriose, BAS = bacterial sterility, BYL = bacterial yield loss, SPY = severity of pyriculariose, PYS = sterility due to pyriculariose, PYL = pyriculariose yield loss.

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Genetic studies on intercalary elongation

Yi-Shin Chen and Chia-Yi Aes

As a step in studying the genetics of intercalary elongation, the first internode lengths of two varieties with different elongation and of their progenies were investigated, and the number of genes controlling the characteristic was estimated.

Tainung 70 from Taiwan and Koshihikari from Japan—and their reciprocal F_1 and F_2 segregating populations (550 plants)—were grown in the field in the first season of 1987 and the results analyzed by the maximum likelihood method. Every sample was composed of three tillers sampled from a single-plant hill.

The range of F_2 segregation in the first internode was 14.3-45.7 cm. Koshihikari was 32.8 cm, and Tainung 70 was longer by only 0.4 cm.

Varietal (additive) and environmental variations were calculated (Table 1). First internode length appeared to be controlled by three recessive genes. The dominant effect (H) was greater than the additive effect (D) under the genetic model with no limitation to H. If we assume that H = D or—D, the results were different. This may be explained by the large variance in the model due to environment.

We confirmed that the intercalary pattern is an indicator for identifying varieties. The length of the third internode seems more important than that of the first, and maternal effects were apparent.

Table 1. Estimates of genetic parameters in F_2 populaton of Koshihikari/ Tainung 70.

Method	Parameter[a]					
	N	RN = exp (en-eN)	U	D	H	5
H unlimited	1	0.9706	33.163	0.1056	–1.055	16.01
	2	0.9996	33.205	0.0986	–0.570	16.11
	3	1.0000	33.198	0.0799	–0.375	16.17
	4	0.9976	33.191	0.0645	–0.278	16.19
	5	0.9949	33.186	0.0535	–0.220	16.21
	6	0.9928	33.183	0.0454	–0.182	16.22
H limited	1	0.9223	32.791	0.2957	–0.296	16.22
	2	0.9778	32.835	0.1928	–0.193	16.23
	3	0.9928	32.849	0.1382	–0.138	16.24
	4	0.9977	32.855	0.1064	–0.106	16.25
	5	0.9994	32.857	0.0861	–0.086	16.25
	6	1.0000	32.858	0.0721	–0.072	16.26
Moment			33.018	42.722	–191.17	11.45

[a] RN = ratio of likelihood, U = population mean, D = additive effect, H = dominant effect, S = environment effect.

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Some quantitative characters of rice *Oryza sativa* L. studied by triple testcross analysis

Bui Chi Buu and Thai Thi Hanh

The ultimate aim of this study was to identify cultivars and crosses that can be profitably utilized in breeding programs for augmenting grain yield with special emphasis on semidwarf rices. The optimum requirement for efficient detection of epistasis is a modified triple testcross (Perera et al 1986).

The nature of gene action for grain yield in short-duration varieties (OM43, OM559, OM201, OM87-9, OM606, OM296) was studied in a 6 x 6 diallel cross. All varieties were short duration (95-105 d), semidwarf, and tolerant of brown planthopper (having the *bph-2* gene) and blast. Subsequently, the triple testcross analysis provided additional information regarding the epistatic components of variation and the effects of the order in which the parents were Involved in the crosses. Estimates of the components of variation from the product of three parents, for instance A/B//C, were made using the formula of Singh and Chaudhary (1985). There were 60 possible 3-way crosses.

Additive gene action and additive x additive epistasis were found among the parents for days to heading, grams per panicle, and 1000-grain weight. Dominance x dominance epistasis was also found to control panicles per plant, days to heading, grains per panicle, and 1000-grain weight (Table 1).

Unidirectional dominance and the absence of asymmetrical gene distribution of both positive and negative alleles in the parents were shown, except for 1000-grain weight and days to heading.

The slope of the regression line for grain yield differs from unity, Indicating epistasis. This shows the preponderance of additive x dominance and dominance genetic variations for grain yield and plant height. Improvement by simple selection would thus be difficult. The importance of indirect selection for yield through the use of component characters governed predominantly by genes with additive action and showing strong correlation with yield should be emphasized (Falconer 1981). Panicles per plant should be emphasized as an indicator for indirect selection for yield, because this character is suitable for the two suggestions through path analysls (Table 2).

Table 1. Components of variation and epistasis in 3-way hybrids involving 6 lines.[a]

Genetic variation	Yield	Plant height	Days to heading	Panicles per plant	Grains per plant	1000-grain weight
Additive (d)	ns	ns	**	ns	**	**
Dominance (h)	**	**	ns	ns	ns	ns
Additive x additive (i)	ns	ns	**	**	**	**
Additive x dominance (j)	**	**	ns	ns	ns	ns
Dominance x dominance (l)	ns	ns	**	**	**	**

[a]ns = nonsignificant, ** = significant at the 1% level.

Table 2. Path analysis of yield components in 3-way hybrids.[a]

Variable	Days to heading	Plant height	Grains per panicle	Panicles per plant	1000-grain weight	Total genotypic correlation with yield
Days to heading	*0.045*	-0.033	-0.001	-0.129	-0.021	-0.139 ns
Plant height	-0.007	*0.202*	0.095	0.270	0.014	0.573 **
Grains per panicle	-0.004	0.106	*0.180*	0.229	0.004	0.519 **
Panicles per plant	-0.009	0.083	0.062	*0.660*	-0.0003	0.796 **
1000-grain weight	-0.012	0.035	0.010	-0.003	*0.081*	0.111 ns

[a]Italicized figures are direct effects; others are Indirect effects. Residual effect = 0.513.

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Genetic analysis of wide compatibility varieties of rice *Oryza sativa* L.

Minghong Gu, Aiqing You, and Xuebiao Pan

Partial sterility is common in rice hybrid F_1s of japonica/indica crosses. However, some varieties with wide compatibility (WCV) produce fertile F_1s when crossed to either indicas or japonicas (Ikehashi et at 1984, 1985). With the great potential of using WCVs for solving the problem of sterility in indica/japonica hybrids, extensive screening of WCVs has been conducted in China.

To analyze the genetic behavior of the wide compatibility genes, eight WCVs of different origins were collected and tested against standard testers (three japonicas and three indicas). All WCVs were crossed in a diallel design. Both pollen and spikelet fertilities were investigated in the F_1, F_2, and TF_1(F_1 of the three-way cross).

Pollen and spikelet fertilities in the F_1s of WCVs and standard testers were normal except in 68-83/Natehao (indica tester). In the F_1s of diallel crosses of WCVs, pollen fertility in 23 of 28 possible crosses was >90%. Four crosses showed partial sterility ranging from 61.8 to 78.7%, most of which was related to WCV Ketan Nangka or Lunhui 422 crossed with Dular or Aus 373. Three crosses showed partial spikelet fertility; two were related to Lunhui 422 crossed with Dular or Aus 373 (Table 1). The fertility of the F_1s of crosses of 02428 or Lunhui 422 with Cpslo 17, Calotoc, or Ketan Nangka was normal. No significant segregation for fertility was found in the F_2, meaning that the gene or genes in 02428 and Lunhui 422 are allelic to that at the S_5 locus.

Sterility occurred in the F_1 of crosses among WCVs, indicating that a genetic difference in fertility existed even though hybrid fertility was normal when they were crossed to indica or japonica testers. WCV Dular and Aus 373 may possibly have another locus related to wide compatibility.

Obvious segregation for fertility was detected in the F_2s of crosses that showed partial sterility in the F_1 (Ketan Nangka and Lunhui 422 crossed to Dular or Aus 373). The ratio of fertile to partially sterile (taking 75% as the criterion) was close to 3.5:1, which met the theoretical segregation ratio for a gametophytic sterile model of two loci. On the other hand, in the F_2s of crosses in which no sterility was detected in the F_1 (Dular or Aus 373 crossed to Cpslo 17, 02428, or Calotoc), the frequency of fertile plants was much higher than in previous crosses. In the F_2 of other crosses, mild variations in fertility were also observed, which might be due to minor gene effects.

Table 1. Pollen and spikelet fertilities in F_1s of WCVs in diallel cross.

Cross	Pollen fertility (%)	Spikelet fertility (%)
Calotoc/Ketan Nangka	95.6	95.2
Calotoc/68-83	97.5	91.9
Calotoc/Lunhui 422	94.0	84.2
Galotoc/Cpslo 17	97.1	79.2
Galotoc/02428	96.4	90.8
Calotoc/Aus 373	92.4	83.0
Galotoc/Dular	93.7	96.1
Ketan Nangka/68-83	95.3	91.9
Ketan Nangka/Lunhui 422	94.9	87.3
Ketan Nangka/Cpslo 17	93.4	73.5
Ketan Nangka/02428	94.1	87.4
Ketan Nangka/Aus 373	67.8	78.1
Ketan Nangka/Dular	67.9	89.4
68-83/Lunhui 422	95.2	80.4
68-83/Cpslo 17	96.5	83.9
68-83/02428	97.1	90.9
68-83/Aus 373	78.7	80.2
68-83/Dular	90.9	92.2
Lunhui 422/Cpslo 17	93.5	81.6
Lunhui 422/02428	93.3	85.4
Lunhui 422/Aus 373	61.8	55.4
Lunhui 422/Dular	84.2	65.3
Cpslo 17/02428	93.9	85.3
Cpslo 17/Aus 373	93.8	94.9
Cpslo 17/Dular	92.3	92.6
02428/Aus 373	93.4	81.0
02428/Dular	94.2	91.5
Aus 373/Dular	96.1	96.4
Dular/02428	96.0	91.3
Dular/Cpslo 17	92.3	89.3
02428/Cpslo 17	94.7	80.8
Ketan Nangka/Calotoc	95.4	90.1

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An approach to the genetic pattern of compatibility in an *O. sativa* indica/japonica cross

Qi Zu-bai, Cai Ye-Tong, and Li Bao-jian

The hereditary basis of wide compatibility in rice was Investigated through the expression of fertility in the F_1s of 3-way crosses, the hybrids of F_1 to F_3, the backcrosses of F_1 to F_2 , and the hybrid offspring of the generations tested above with the parents of 4 wide compatibility varieties, 22 *O. sativa* indica varieties, and 7 *O. sativa* japonicas.

The genetic pattern of wide compatibility governed by nuclear genes in indica/ japonica crosses is probably dominated by a major gene along with the minor genes. The major gene, which is a dominant single gene, has remarkable influence on wide compatibility and is closely linked to the marker gene for apiculus color. Compared with the major gene, the minor genes exert only a slight effect and bear no relationship to the marker gene.

Notes

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Does hybrid sterility isolate indicas from japonicas?

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Hybrid sterility due to gametic selection has been regarded as one of the most plausible mechanisms of isolation between indica and japonica rices. The validity of this idea was examined, using the Monte Carlo computer simulation.

In the modeling, hybrid sterility is regulated by two independent loci X and Y, each carrying two alleles designated as X_1 and X_2, and Y_1 and Y_2, respectively. Two types of gametic selection were studied. In model I, gametic selection occurs by the genotypes of the gametes themselves; gametes carrying X_2Y_2 are assumed to be aborted, while gametes carrying other combinations of alleles work normally. In model II, gametic selection is caused by the interaction between gamete and parent genotypes; gametes carrying X_2 are aborted when produced on a plant of genotype X_1X_2, and gametes carrying Y_2 on a plant of genotype Y_1Y_2 are aborted. In both models, characteristics distinguishing indicas and japonicas were assumed to be controlled by loci A and B that are linked with loci X and Y, respectively. Genotypes A_1—B_2B_2 and $A_2A_2B_1$— are assumed to show characteristic combinations typical of indicas and japonicas, respectively.

In the initial simulation, populations of two genotypic compositions were considered. In the first, a mixture (population) of four genotypes—$A_1A_1X_1X_1B_1B_1Y_1Y_1$, $A_1A_1X_1X_1B_2B_2Y_2Y_2$, $A_2A_2X_2X_2B_1B_1Y_1Y_1$, and $A_2A_2X_2X_2B_2B_2Y_2Y_2$—with an equal frequency of 0.25 each was considered to represent the state where all possible combinations of the genes responsible for indica-japonica differentiation grow in adjacent areas. In the second, a mixture (population) of indica and japonica types—$A_1A_1X_1X_1B_2B_2Y_2Y_2$ and $A_2A_2X_2X_2B_1B_1Y_1Y_1$—with an equal frequency of 0.5 was simulated to represent a state where indica and japonica types grow side by side. Genotypic compositions in the advanced generations of these populations were calculated with some possible combinations of linkage intensity and outcrossing rate. The results of the simulations were summarized by the average genotypic frequencies over 50 replicate runs.

Table 1 shows the results under a condition involving 0.1 of the recombination frequencies and 0.05 of the outcrossing rate. In both models of gametic selection, genotypes of indica and japonica types (A_1—B_2B_2 and $A_2A_2B_1$—) decreased as the generation advanced, while genotypes A_1—B_1—showed a remarkable increase. Similar results were obtained in other combinations of the variables. Thus hybrid sterility as modeled here is not likely to be a mechanism causing differentiation and isolation of indica and japonica.

Table 1. Change of genotypic frequencies in advance of generations under different types of gametic selection and initial states.[a]

Model of gametic selection	Generation	Genotypic frequency			
		$A_1—B_1—$	$A_1—B_2B_2$	$A_2A_2B_1—$	$A_2A_2B_2B_2$
Model I	0	0.25	0.25	0.25	0.25
	100	0.541	0.230	0.209	0.020
	200	0.566	0.220	0.200	0.014
	0	0	0.5	0.5	0
	100	0.314	0.397	0.243	0.046
	200	0.339	0.370	0.252	0.039
Model II	0	0.25	0.25	0.25	0.25
	100	0.392	0.186	0.185	0.237
	200	0.427	0.193	0.178	0.203
	0	0	0.5	0.5	0
	100	0.108	0.387	0.505	0
	200	0.107	0.410	0.483	0

[a] Recombination frequencies between loci A and X, and between loci B and Y are 0.1. Outcrossing rate is 0.05.

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Effects of genotype x environment interaction on late-heading Japanese rice

T. Sato

Japanese rice *(Oryza sativa* L.) genotypes EG1, EG2, EG3, EG4, EG5, EG6, and EG7 have the genetic constitution $E_1E_1e_2e_2e_3e_3$, $e_1e_1E_2E_2e_3e_3$, $e_1e_1e_2e_2E_3E_3$, $E_1E_1E_2E_2e_3e_3$, $e_1e_1E_2E_2E_3E_3$, $E_1E_1e_2e_2E_3E_3$, and $E_1E_1E_2E_2E_3E_3$, respectively. The flowering responses of the seven genotypes were analyzed under four conditions:

- short daylength (10-h photoperiod) (SD) and high temperature (28 °C) (HT)
- long daylength (14 h) (LD) and HT
- SD and low temperature (18 °C) (LT)
- LD and LT

The time to panicle initiation (PI) of these genotypes varied from 42 to 116 d under LD, HT. Under SD, HT, however, PI occurred in 35-38 d. Furthermore, the total number of leaves on the main culm under LD, HT appreciably differed among EG lines. Under SD, HT, the differences were fewer (Table 1). Therefore, there were significant differences among the seven genotypes in the photoperiod-sensitive phase, and not in the basic vegetative phase. Low temperature increased the difference in leaf number in EGI and EG4, which have gene E_1, by 2.5 and 1.5, respectively, while the difference in EG3 and EG5, which have gene E_3, was 0.5 and 0.6, respectively. These results indicate that genotypes having E_1 are more photoperiod sensitive under LT than ones having E_3.

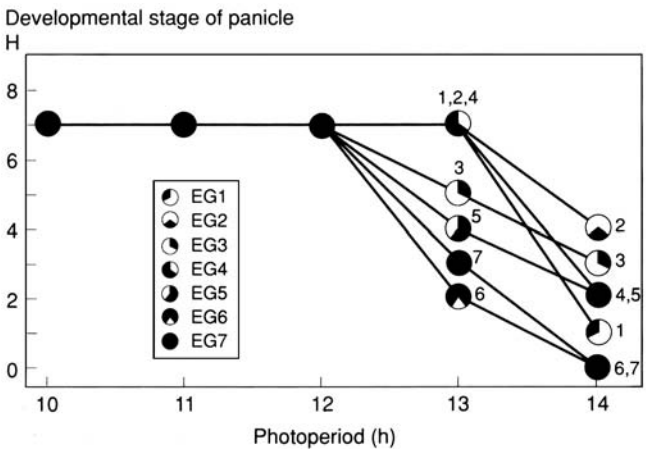
Genotypes EG1, EG2, and EG4 (without the E_3 gene) had a critical photoperiod between 13 and 14 h under 23 °C; the other genotypes, between 12-13 h (Fig. 1). However, under excessive photoperiod (14 h), the panicles of EGI and EG4 developed more than those of EG3 and EG5.

Thus, late-heading genes E_1, E_2, and E_3,control, not the basic vegetative phase but the photoperiod-sensitive phase. Furthermore, the difference in flowering behavior of E_1, E_2, and E_3 genes under optimal, critical, and excessive photoperiod may partly explain the complex pattern of heading behavior when the seven genotypes are grown at different latitudes in Japan.

Table 1. Effect of photoperiod and temperature on number of leaves on main culm in 7 genotypes.[a]

Genotype	SD, HT (1)	LD, HT (2)	Difference (2-1)	SD, LT (3)	LD, LT (4)	Difference (4-3)
EG1	10.7 ± 0.8	12.8 ± 0.4	2.1	9.6 ± 0.5	14.2 ± 0.4	4.6
EG2	10.2 ± 0.4	12.3 ± 0.5	2.1	9.6 ± 0.7	12.0 ± 0.0	3.4
EG3	10.3 ± 0.5	13.8 ± 0.4	3.5	10.0 ± 0.0	14.0 ± 0.0	4.0
EG4	10.2 ± 0.8	12.2 ± 1.0	2.0	9.5 ± 0.5	12.0 ± 0.0	3.5
EG5	10.3 ± 0.5	13.3 ± 0.5	3.0	9.4 ± 0.5	13.0 ± 0.0	3.6
EG6	11.0 ± 0.0	18.8 ± 0.4	7.8	10.6 ± 0.5	16.2 ± 0.4	5.6
EG7	10.7 ± 0.5	20.7 ± 0.6	10.0	9.9 ± 0.3	16.3 ± 0.5	6.4

[a]SD = 10 h light, LD = 14 h light, HT = 28/28 °C, LT = 18/18 °C.



1. Effect of day length on panicle initiation and development of 7 genotypes at 23 °C. H = heading. Stages after Suge and Osada 1967. Numbers by symbols refer to genotype: 1= EG1, etc.

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Variation in chlorophyll proteins between wet and dry season rice varieties

N. Watanabe, R.B. Austin, and C.L. Morgan

Rice leaves respond at several levels of organization to the light regime in which they grow. Some varieties are well adapted to low-light, monsoon environments and others to high-light, "summer" environments. Tu et al (1988) found that Gui-Chao 2, a modern "monsoon" variety from China, was less able to adjust to high irradiance than were two varieties from Texas, Bellemont and Lemont. In high light, the Texas varieties developed thicker leaves with a greater light-saturated rate of photosynthesis than those of Gui-Chao 2.

We investigated whether these varieties also differ in their responsiveness to light at the level of the organization of the thylakoid membrane. Plants were grown in compost in a controlled environment with a constant temperature of 28 °C and relative humidity of 85%. Half of the plants were grown at an irradiance (400-700 nm) of 370 µmol quanta/m^2 per s (high light), and the other half at 75 µmol quanta/m^2 per s (low light).

When the plants had 3-4 fully expanded leaves, thylakoids were extracted from the leaves for separation by nondenaturing gel electrophoresis of the chlorophyll proteins and subsequent quantification of the distribution of total chlorophyll. Measurement of the steady state, light-saturated rates of photosynthesis of fully expanded 3d or 4th leaves were made using an infrared gas analysis system.

Samples of the leaves were also analyzed for chlorophyll content and chlorophyll a:b ratio.

The system for separating chlorophyll proteins resulted in negligible destruction of protein complexes; only 1.2% of the total chlorophyll was found to be released from the complexes. Seven chlorophyll proteins were evident and were allocated on the basis of molecular weight and chlorophyll a:b ratio into three classes; photosystem I (PSI), the core system of photosystem II (CC II), and the light-harvesting protein associated with photosystem II (LHC II). The proportion of total chlorophyll in PSI was 32% and varied little with variety or light treatment. As expected from work with other species, the chlorophyll a:b ratio was highest in light-grown plants, and this was associated with, and probably caused mainly by, a higher ratio of CC II/LHC II. Under high light, this ratio averaged 41.5% for the Texas varieties but only 34% for Gui-Chao 2. Under low light, the difference was reversed: 28 and 34%, respectively (Table 1). Both Texas varieties had a higher rate of light-saturated net photosynthesis than Gui-Chao 2, although this

Table 1. Maximum light-saturated rate of photosynthesis (Pmax), chlorophyll a:b ratio, ratio of the chlorophyll in the core complex of photosystem II to that in the light-harvesting chlorophyll protein of photosystem II, and relative density of PS II reaction centers for 3 rice varieties:

Genotype	Pmax (µmol CO_2/m^2 per s)	Chlorophyll a:b ratio	CC II/LHC II	CC II/m^2
Plants grown at 370 µmol quanta/m^2 per s				
Gui-Chao 2	30.1	3.681	0.34	88.9
Lemont	31.0	3.756	0.44	98.0
Bellemont	33.7	3.739	0.39	107.3
Standard error	0.7	0.034	0.02	5.2
Plants grown at 75 µmol quanta/m^2 per s				
Gui-Chao 2	–	3.465	0.34	–
Lemont	–	3.397	0.27	–
Bellemont	–	3.609	0.29	–
Standard error	–	0.034	0.02	–

[a] Relative density of PS II reaction centers was calculated assuming that number of chlorophyll molecules associated with reaction centers was constant between genotypes. Calculation was total leaf chlorophyll (mol/m^2) × chlorophyll in CC II, expressed as fraction of total leaf chlorophyll.

difference was significant only for the comparison between Bellemont and Gui-Chao 2 (Table 1).

Thus the two variety groups represented by a “monsoon” variety from China and two “summer” varieties from Texas differ in their capacity to adjust the relative amounts of light-harvesting and core complex chlorophyll in photosystem II. This difference is reflected in their chlorophyll a:b ratios, as has been found in other species.

These results are consistent with the hypothesis that the “monsoon” varieties under monsoon conditions (i.e., low light) have a greater electron transport capacity through photosystem II than do the “summer” varieties, while the opposite is true when the plants are grown under high light.

This may represent a genetic difference between the variety groups. Selection for the character could, perhaps, most easily be done by measuring the chlorophyll a:b ratios of the leaves.

Facilities available for this work did not allow the varieties to be grown at the irradiances encountered in the field during the summer season, i.e., 1000-2000 µmol quanta/m^2 per s. Under those conditions the differences in photosynthesis, chlorophyll a:b ratio, and CC II/LHC II ratio would probably have been greater than we observed.

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Genotypic difference in response to light interruption in Japanese rice varieties

Y. Okumoto, T. Tanisaka, and H. Yamagata

In rice, a short-day plant, the formation of floral primordia is suppressed by interruption of the dark period (light interruption, LI). Yamagata et al (1986) showed that the degree of suppression is significantly affected by the genotype for heading time, being much larger in plants with the E_1 gene than in those without E_1. In this study, the effect of the genotype for heading time on the response to LI was further analyzed.

Seedlings of 7 tester lines (EG lines), which differ in genotype for 3 late heading-time genes (E_1, E_2, and E_3), and 17 Japanese varieties that possess E_1 (Table 1) were grown under 24-h daylength for 50 d, then subjected to 10-h daylength treatments with or without LI for 30 d. LI was a 1-h illumination in the middle of the dark period; the LI treatment was 6.5 h dark + 1 h light + 6.5 h dark. Immediately after treatment, five plants were examined for the developmental stage of the young panicle on the main culm following the criteria in Table 2.

The results are shown in Table 1 along with the estimated genotypes of the materials. Twelve varieties (1-1 2) responded to LI strongly as expected, but 4 varieties (13, 14, 16, and 17) did as weakly as ones without E_1. The former have gene Se^n($=Lm^n$, Yokoo and Kikuchi 1977), while the latter are supposed to have the early-heading gene Se^e ($=Lm^e$, Yokoo and Fujimaki 1971). These results indicate that E_1 and Se^n control the response to LI through complementary action. Further analysis is needed to explain the strong response to LI of variety 15, which is supposed to have both E_1 and LM^e.

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Table 1. Response of young panicle development to light interruption (LI) in 7 EG lines and 17 varieties.

Line or variety		Estimated genotype[a] (in haploid state)		Developmental stage of young panicle[b]	
				With LI	Without LI
EG 1		$E_1e_2e_3$	Sen	1.6	H
EG 2		$e_1E_2e_3$	Sen	7.8	H
EG 3		$e_1e_2E_3$	Sen	7.7	H
EG 4		$E_1E_2E_3$	Sen	0.5	8
EG 5		$e_1E_2E_3$	Sen	7.4	H
EG 6		$E_1e_2E_3$	Sen	0.7	8
EG 7		$E_1E_2E_3$	Sen	0.1	8
1	Manryou	$E_1e_2e_3$	Sen	3.0	H
2	Nipponbare	$E_1e_2e_3$	Sen	2.0	8
3	Norin 6	$E_1E_2e_3$	Sen	1.2	8
4	Norin 8	$E_1E_2e_3$	Sen	1.2	8
5	Norin 22	$E_1E_2e_3$	Sen	3.0	H
6	Futaba	$E_1e_2E_3$	Sen	2.0	8
7	Kinmaze	$E_1e_2E_3$	Sen	3.0	H
8	Chuuseishinsenbon	$E_1e_2E_3$	Sen	3.0	H
9	Zuihou	$E_1e_2E_3$	Sen	1.3	8
10	Akebono	$E_1e_2E_3$	Sen	1.1	7.4
11	Shiranui	$E_1e_2E_3$	Sen	1.3	8
12	Houyoku	$E_1e_2E_3$	Sen	0.5	8
13	Norin 1	$E_1e_2e_3$	Sen	8.0	H
14	Kiyonisiki	$E_1e_2e_3$	(Sen)	6.9	H
15	Koshihikari	$E_1E_2e_3$	Sen	1.2	8
16	Fujisaka 5	$E_1e_2E_3$	Sen	8.0	H
17	Fujiminori	$E_1e_2E_3$	(Sen)	8.0	H

[a]Genotypes in parentheses have not yet been proven by crossing with tester lines. [b]At the end of 10-h daylength treatment. See Table 2.

Table 2. Developmental stage of young panicle (partly after Suge and Osada 1967).

Developmental stage	Situation of panicle	Panicle length (mm)
0	Vegetative stem primordia, before neck node differentiation	–
1	Differentiating stage of primary rachis branch primordia	–
2	Differentiating stage of secondary rachis branch primordia	0.5-0.9
3	Early differentiating stage of flower primordia	1-3
4	Late differentiating stage of flower primordia	3-15
5	Differentiating stage of pollen mother cell (PMC)	15-50
6	Early stage of reduction division of PMC	50-100
7	Late stage of reduction division of PMC	100
8	Completing stage of pollen	ca. 200
H	Heading just finished	ca. 200

Notes

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Differentiation between populations of wild rice (*Oryza rufipogon*) coexisting with *Leersia hexandra* and *Ipomoea aquatica*

Y. Shimamoto and H. Morishima

Wild rice *Oryza rufipogon* Griff., a relative of cultivated rice *O. sativa* L., is widely distributed throughout tropical and subtropical Asia. The great interest in surveying and collecting wild relatives of rice for use as genetic resources prompted us to investigate some of their quantitative characteristics in their natural habitat in relation to ecological, especially biotic, conditions. In Thailand, the major companion species of wild rice, except the weedy type found in ricefields, are *Leersia hexandra* Sw. and *Ipomoea aquatica* Forsk, both perennials (Morishima et al 1984). Most wild rices coexisting with these species can be identified by perennial growth habit, including intermediates.

Various characters of the perennial wild rices collected in Thailand were recorded in their natural habitats and under controlled conditions. Forty-eight wild rice populations, consisting of 20 accompanying *Leersia,* 13 with *Ipomoea,* and 15 with other species, were surveyed with respect to their features and the ecological conditions in their natural habitat. The wild rices accompanying *Ipomoea* grew in shallower water, were bigger, and had larger panicles. Those with *Leersia* were shorter and coexisted with a lesser diversity and frequency of companion plants.

In 20 of the 48 wild rice populations, some plants reached heading under short-day conditions. But only three from populations accompanying *Ipomoea* did so, although more than half from populations with *Leersia* and other species did so. This suggests that flower bud initiation in wild rice may differentiate populations coexisting with major companion species.

On the other hand, under controlled conditions the wild rices accompanying *Leersia* were taller and had larger panicles than those with other species. This positive response of plant height in populations with *Leersia* was similar to that of annual populations of wild rice observed by Shimamoto and Sano (1986).

The awn and anther, which are key characters to discriminate annual wild rices from other species (Morishima et al 1984), were smaller in populations with *Leersia* than in populations with other species under controlled conditions. In wild rices with *Leersia,* panicle and awn length varied greatly from population to population under both natural habitat and controlled conditions. Furthermore, in these two characters the regression coefficients of natural habitat on controlled conditions were positive and significant. Therefore, in wild rices with *Leersia,* variations in panicle and awn size may be genetic.

In contrast, although variations in some characters occurred among wild rice populations accompanying other species, no relationship was found between growth in natural habitat and that under controlled conditions.

Wild rice populations may thus be differentiated by their response to biotic conditions, specifically to major companions. This information may be very important when surveying wild plants in their natural habitats.

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Rice aroma: methods of evaluation and genetics

Shih-Cheng Lin

Aroma is an important economic character in breeding rice for high eating quality. Because of unreliable means of evaluation, some earlier results derived from genetic studies are questionable (Reddy and Sathyanarayanaiah 1980, Tripathi and Rao 1979). Moreover, specious conclusions (e.g., scented rice is dominant over nonscented) have been extensively reported in some authoritative publications. This paper introduces effective, reliable, and rapid ways to ascertain and evaluate genetic information on rice aroma.

Available rapid methods of evaluation include 1) soaking the green leaf in 1.7% KOH solution in a test tube without cutting it to pieces (Sood and Siddiq 1978), and 2) smearing the partially scraped surface of a single brown rice kernel with about 1.0% I-KI solution; if the tester fails to evaluate by smelling, he can repeat scraping and smearing the same sample within 30 s. Since evaluation of seed is one generation before evaluation of the leaf, the results of seed evaluation are complementary and more reliable. In linkage studies one can evaluate the aroma character segregation with F_2 seeds produced from F_1 plants. The scented and nonscented populations are then raised separately for recording associated marker characteristics with higher efficiency. For this purpose the evaluation for aroma of F_2 seeds is done by cutting a small portion (less than 1 mm) of the vegetative side (opposite to the embryo) and grinding in a small mortar with a drop of I-KI to liberate the aroma (if scented) instead of scraping and smearing for evaluation. Seed or brown rice with slight wounding is capable of germinating and growing up to a normal but a bit smaller plant without changing its genetic behavior.

In 23 crosses of 10 scented varieties with nonscented ones, all gave nonscented F_1 hybrids, meaning scented is recessive; the F_2 population segregated to 3 nonscented: 1 scented offspring—a monogenic Mendelian ratio with c^2_c, values around 0-1.589 and P values around 1-0.25, fitting at a very high level. In 5 F_3 generations, the segregation of 17 families at a ratio of 1 purely scented: 2 segregating: 1 purely nonscented gave c^2_c values of 0.350-1.408 and corresponding P values of 0.75-0.25, still fitting at a very high level. In recent years, studies on the inheritance of rice aroma have tended to produce similar conclusions (Huang and Zou 1989, Song et al 1989, Sood and Siddiq 1978).

Table 1. Segregation for aroma in F_2 of some crosses.

Cross	Nonscented (no.)	Scented (no.)	c^2_c
			(9:7)
Ai-Xiang-Nuo/Xi-Xiang	25	23	0.181
Xi-Xiang/Jing-Xiang 1	127	95	0.048
Xi-Xiang/Xin-Yi-Xiang-Nuo	310	229	0.300
Xi-Xiang//Lian-Xiang 1/ Xin-Yi-Xiang-Nuo	160	114	0.428
			(13:3)
Xi-Xiang/Basmati 370	187	49	0.502
Zao-Xiang 17/Xi-Xiang	176	50	1.474

In 16 scented/scented crosses, most F_1 hybrids were scented, and there was no segregation for scent in the F_2 populations, showing scent genes from both parents to be allelic. In other crosses (Table 1), the F_1 hybrids were all nonscented, and their F_2 populations showed digenic segregation for nonscented: scented in ratios of 9:7 or 13:3, meaning that scent genes from both parents are non-allelic. Therefore, Jing-Xiang 1, Lian-Xiang 1, Xin-Yi-Xiang-Nuo, Ai-Xiang-Nuo, Zao-Xiang 17, Xiang-Dao 80-66, IET4699, Basmati 370, Dian-Rui 507, etc. possess the same gene, named sk_1, but Xi-Xiang has another gene, tentatively named sk_2.

The linkage study revealed that one scent gene (sk,) is independent of waxy, liguleless, long empty glume, purple glume, purple stigma, and purple pericarp genes. Further studies on linkage relations are in progress.

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Application of computer image analysis to characterization of plant type in rice—response to spacing

K. Hinata and M. Oka

Computer image analysis of a side-view photograph of a plant (PPP method of Oka and Hinata 1988,1989) was applied to the characterization of plant type of rice grown under different plant spacing.

Three cultivars—Koshihikari, Sasanishiki, and Milyang 23—were grown in a single planting at intervals of 18 x 18 cm (close spacing), 24 x 24 cm (standard), and 36 x 36 cm (wide spacing). The methods of photographing and data processing have been detailed in prior papers (Oka and Hinata 1988,1989). Distributions of shoot density and leaf angle are illustrated for two cultivars at heading and harvest (3 wk after heading) in Figure 1.

Although plant shape varied according to spacing, traits of the shape of each cultivar were expressed in every spacing plot. The ratio between the width of upper leaf distribution and that of the lower was also distinctive for each cultivar. Another characteristic investigated was the percentage of dense area to total profile area in side view. Milyang 23 showed higher values for this characteristic than the other cultivars in every plot and at every stage.

Mean leaf angle at the middle part was highly variable according to spacing, stage, and cultivar. Narrow spacing made leaves more erect. Leaves at harvest were more inclined than those at heading. Milyang 23 tended to have more erect leaves than did the others.

This preliminary study suggests a close curvilinear regression between real leaf area and projected shoot area. The results indicate that plant type is a highly heritable character, and that traits of cultivars are expressed under different spacing conditions. Plant type analysis can be utilized in selecting for high efficiency of sunlight utilization by rice plants.

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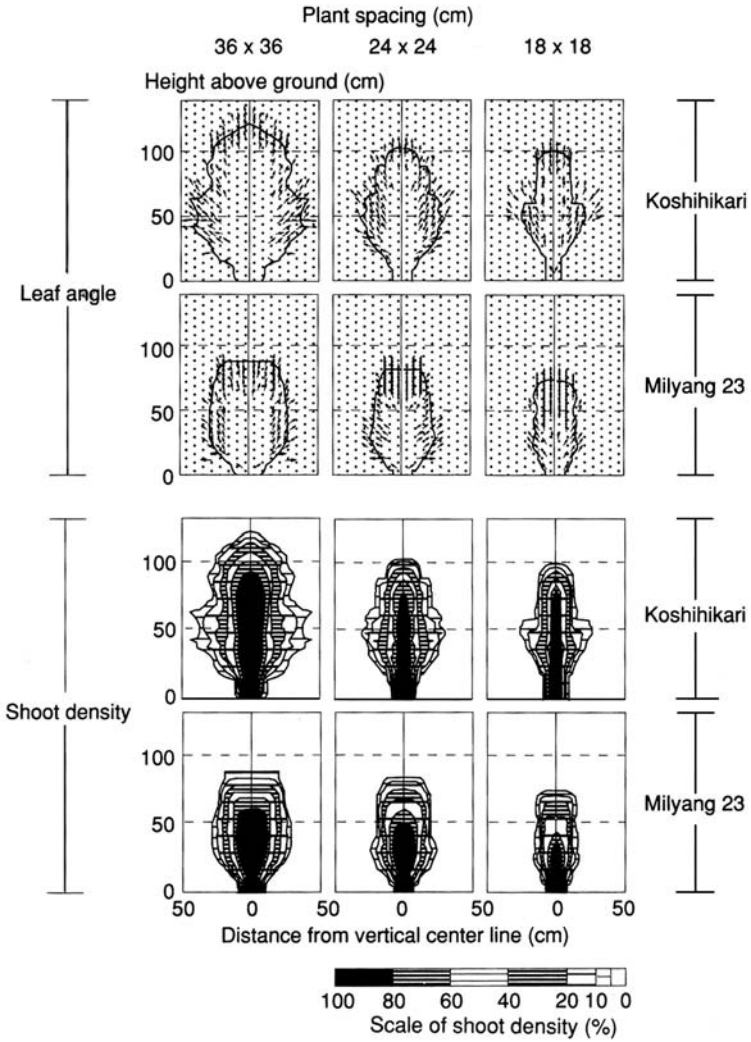

1. Two-dimensional distribution of shoot density and leaf angle at heading of Milyang 23 and Koshihikari cultivated under 3 plant-spacing regimes. Shoot density is shown by contour lines and leaf angle by arrows.

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Genetic analysis of embryo length in rice *Oryza sativa* L.

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Genetic analysis of embryo length was done in two 9 x 9 and 8 x 8 diallel sets of F_1 and F_2 crosses of indica rice cultivars, 5 of which (Pokkali, Nep Non Tre, Sugdasi, Thatno sabanet, and Etale) had long embryos and 4 (BRIO-I-87, Palman 46, Intan Gawri, and UPR231-28-1-2) had short embryos. Estimates of genetic parameters following Hayman's (1954) method showed significant additive and nonadditive gene action, and the latter appeared to be due solely to dominance. Short embryo was dominant over long embryo, and average dominance was within the range of incomplete dominance. Positive and negative alleles were unequally distributed at the loci exhibiting dominance. Combining ability analysis by Griffing's (1956) method also indicated the significance of both additive and nonadditive effects, and the former appeared to be more important than the latter. Narrow sense heritability (0.78 in the F_1 and 0.94 in the F_2 diallel) indicated that additive gene action was more important in the inheritance of the trait. On the basis of the general combining ability effects of the parents and the mean performance of the crosses, hybrids Pokkali/Intan Gawri, Nep Non Tre/Sugdasi, Nep Non Tre/Intan Gawri, and Sugdasi/Palman 46 showed greater selection potential for longer embryo.

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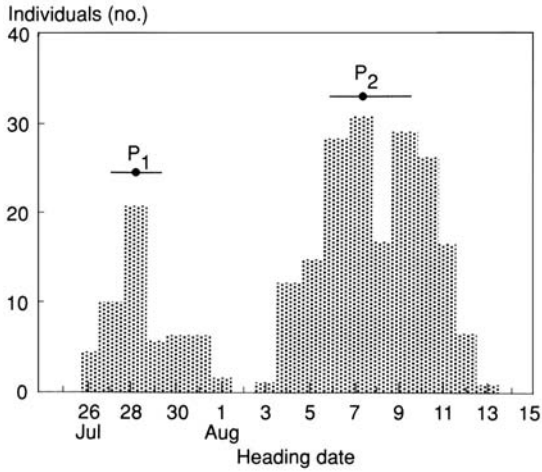
Induced mutations for identifying and characterizing genes in rice: I. Three useful mutants and their characteristics

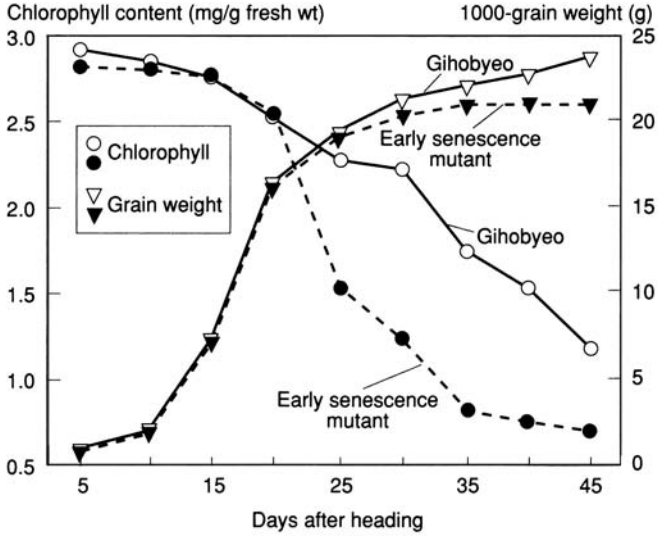
Moo Young Eun, Yong Gu Cho, Yong Kwon Kim, and Tae Young Chung

Induced mutation techniques were applied to develop mutants as suitable materials for identifying and characterizing rice genes. Rice seeds of eight cultivars were treated with ^{60}Co g-ray (25 krad) in 1986. During the M_2-M_5 generations, many fixed mutants were successively selected as research materials for gene analyses and isogenic line breeding. Among them were three useful mutants, as follows:

- Mutant 89032, originating from Gihobyeo, is early flowering(10d), with Gihobyeo's good plant type and high yield potential. Segregation for heading date showed a good fit to a 1:3 ratio, indicating that early flowering is controlled by a single recessive gene (Fig. 1). Single-gene control of early flowering, and high yields in preliminary yield trials in four regions suggested that the mutant would be highly useful as a new gene source for molecular genetic studies and for breeding of early-flowering varieties.
- Early senescence mutant 89043, selected from Gihobyeo, has morphological characteristics so similar to that of the original plant that it cannot be distinguished until about 20 d after heading. After that, senescence comes suddenly (Fig. 2), indicating a great decrease (40%) in leaf chlorophyll content within 5 d, and earlier maturation. The mutant could be useful for studying the biochemistry, physiology, and genetics of plant senescence.
- Large-grain mutant 89012, whose 1,000-grain weight is about 150% that of the original variety, was selected from Sangpungbyeo (Table 1). The mutated glabrous character of the mutant indicates the same gene as for the existing glabrous character in FI 168. Major isozyme bands for malic enzyme in the mutant differed from those in the original variety, whereas those for esterase, phosphoglucose isomerase, hexokinase, and b-amylase were the same. The mutant's low panicle number, high fertility ratio, and uniform and high germination ability at 20 °C would be highly desirable for direct seeding.



1. Frequency distribution of heading date in F_2 population of early-flowering mutant/Gihobyeo cross. Segregation showed a good fit to 1:3 ratio, indicating that early flowering is controlled by a single recessive gene.



2. Changes in total chlorophyll content and 1,000-grain weight during ripening.

Table 1. Agronomic characteristics of large-grain mutant and Sangpungbyeo.[a]

Character	Cultivar or line		LSD
	Sangpungbyeo	Large-grain mutant	
Heading date	12 Aug	12 Aug	ns
Culm length (cm)	63.0	104.5	**
Panicles (no./hill)	14.3	8.0	**
Spikelets (no./panicle)	80	100	**
100-grain weight (g)	23.7	34.5	**
Fertility (%)	89.4	91.0	**
Yield (g/hill)	29.5	28.7	ns
Amylose content (%)	18.7	18.4	ns
Alkali digestibility			
10%	3.0	4.0	**
1.6%	6.0	7.0	**
Brown rice protein (%)	7.6	7.7	ns
Phenol staining (2%)			
Hull	–	–	ns
Brown rice	–	–	ns

[a] ns = not significant, ** = significant at the 1% level by *t*-test.

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Reliable, simple method for producing rice plants through anther culture

Gun-Sik Chung, Sae-Jun Yang, and Byeong-Geun Oh

To save time and labor and to standardize anther culture techniques in vitro, a one-step culture method without transferring the callus to plant regeneration medium and other techniques were applied for high-volume rice breeding (Chung and Sohn 1986, Heu and Koh 1986, Pulverand Jennings 1986). Cold pretreatment (12°C, 15 d) was applied before anther plating at the appropriate pollen stage throughout this experiment. Callus formation and green plant percentage were calculated on the basis of anthers plated.

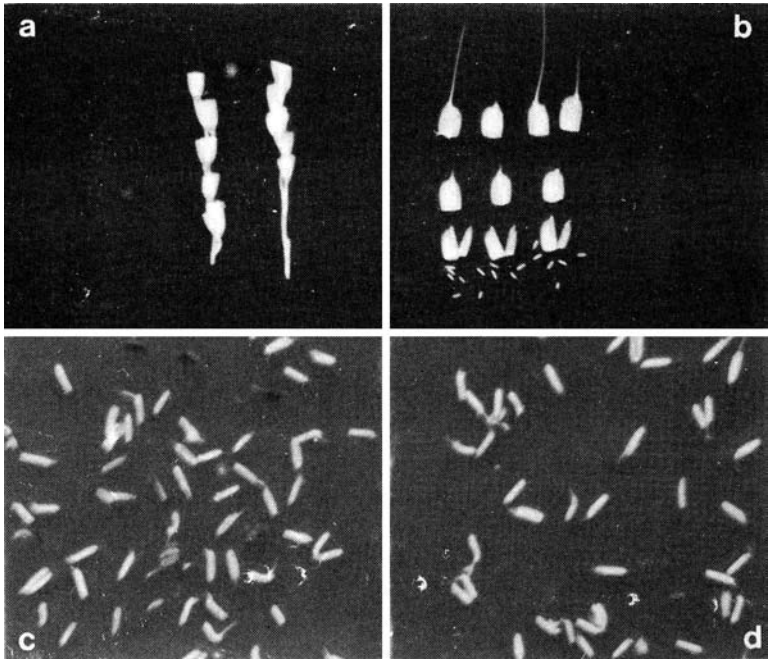
Clipping the basal part of the spikelet to extract anthers was more effective, not only for callus formation but also for anther plating efficiency, than clipping the upper part of the spikelet (Table 1). With clipping the basal part and dripping on the surface of the medium, less anther browning occurred (Fig. 1).

N6-Y1 medium (Chung and Sohn 1986) supplemented with 1 mg naphthaleneacetic acid (NAA) and 4 mg kinetin/liter gave a higher green plant percentage than other combinations of NAA, kinetin, and abscisic acid (Ling et al 1988, Moon et al 1988a). A 1.2% agar concentration gave the highest green plant percentage (Moon et al 1988b).

Variation in green plant percentage occurred according to the type of culture vessel in one-step culture. The highest green plant percentage was obtained with a culture bottle with a wide mouth (95 x 160 mm). Although the green plant percentage in the one-step culture was still low compared with that of the two-step culture, this technique will permit reconsideration of high-volume haploid rice breeding before pollen culture has become practical (Table 2, Fig. 2) (Jia et al 1987).

Table 1. Callus formation with 2 cutting methods for extracting anthers from Nagdongbyeo.

Part of spikelet clipped	Anthers (no.)	Callus (%)	Anthers plated (no./h)
Upper	1,000	25.1	400
Basal	1,000	29.0	800

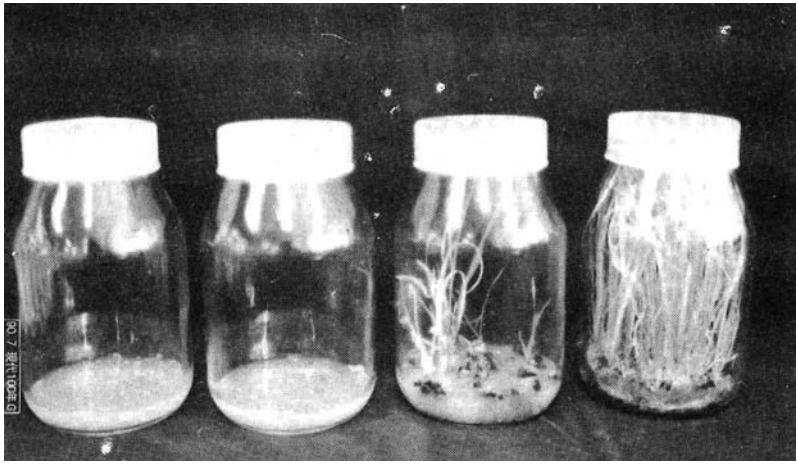


1. (a) Clipping upper part of spikelets. (b) Clipping basal part of spikelets. (c) Browning anthers after (a). (d) Fresh anthers after (b).

Table 2. Efficiency of 1-step and 2-step rice anther culture.

Variety	1-step culture [a]		2-step culture [b]		A/B
	Anthers (no.)	Green plants (A) (%)	Anthers (no.)	Green plants (B) (%)	
Nagdongbyeo [c]	500	7.5	500	9.0	0.83
Samgangbyeo [d]	900	1.2	700	2.0	0.60

[a]N6-Y1 + 1 mg NAA + 4 mg kinetin + 12 g agar per liter. [b]Callus: N6-Y1 + 2 mg NAA + 1 mg kinetin + 5 mg ABA + 8 g agar per liter. Plant regeneration: N6-Y1 + 0.2 mg indoleacetic acid + 1 mg kinetin + 8 g agar per liter. Japonica type. [d]Tongil type.



2. Rice pollen plants by 1-step culture method.

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Internode elongation system of Nepalese rice

G.L. Shrestha

The internode elongation system of Nepalese rice germplasm including four wild species from different parts of the country was investigated in Parwanipur, Nepal (27° 04'E, 84° 58'N, 115 m altitude)(Shrestha and Vaughan 1989).

Among the wild species, *Oryza officinalis* Wall et Watt showed the longest culm (206 cm), followed by *O. rufipogon* Griff (167 cm), *O. nivara* Sharma et Shastry (57.5 cm), and *O. granulata* Nees et Arn. ex Watt (38 cm) (Fig. 1). The first three wild species have a normal internode elongation system, with the first internode from the top being the longest one, followed by decreasing lengths. In *O. granulata,* the second and third internodes from the top have the same length (11 cm).

Among local cultivars, the strongly photoperiod-sensltlve late group (A) has the longest culm (125.9 cm), followed by the tall upland early group (B) (102.9 cm), the tall lowland early group (C) (79.5 cm), and the dwarf early Gamadi group (D) (34.8 cm) (Fig. 2)(Shrestha 1986). The first three groups demonstrate the normal internode elongation system of Group I of Murai et al (1982), whereas the panicle-enclosing Gamadi group shows the complete reduction of the top internode. The Gamadi type of internode elongation system has been proposed as a new group—Group V—of internode elongation system in rice. This character seems to be important in the evolutionary processes of the rice plant.

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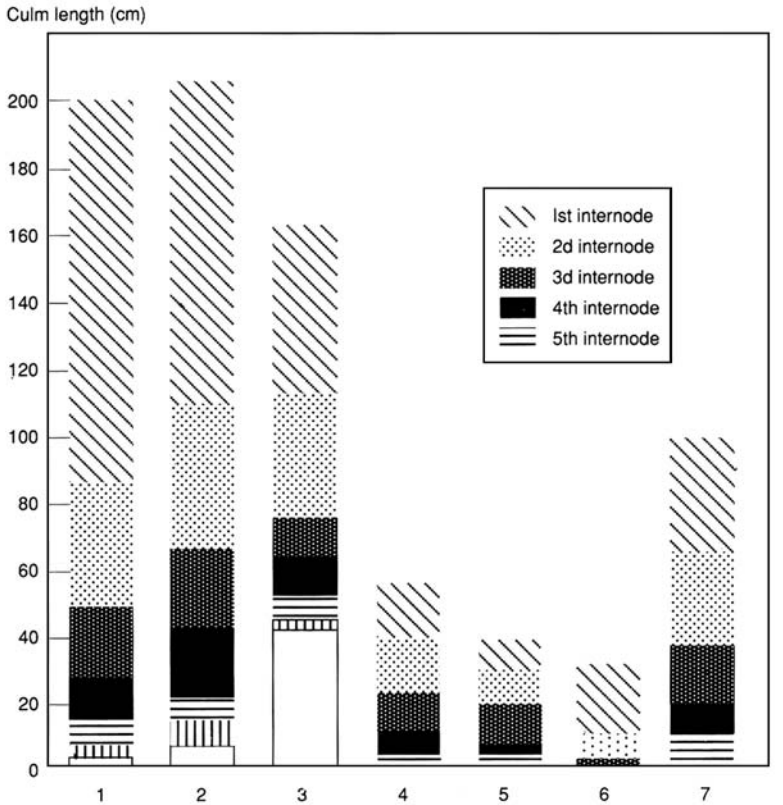
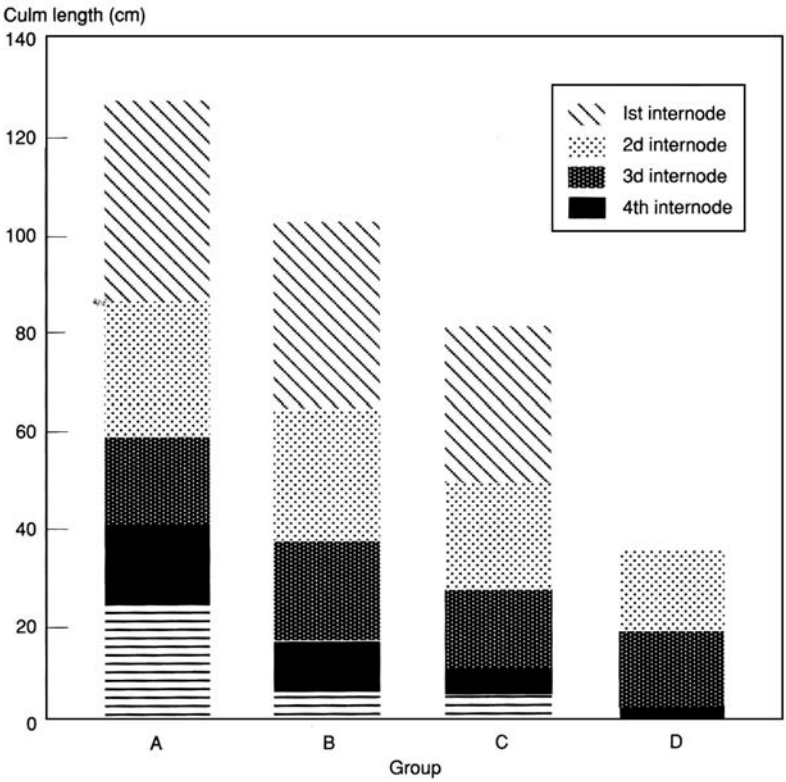


1. Internode elongation system in wild rices of Nepal. 1. *O. alta,* 2. *O. officinalis,* 3. *O. rufipogon,* 4. *O. nivara,* 5. *O. granulata,* 6. Gamadi, and 7. Masuli.



2. Comparative elongation of groups of local rice varieties.

Notes

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Inheritance of high-density rice grain and its relation to other panicle characters

S. Mallik, A.M. Aguilar, and B.S. Vergara

Demographic forecasts emphasize the worldwide need for a rice production increase of 3% per annum in the year 2000. Increasing the number of high-density (HD) grains in rice panicles, which have higher test weight and head rice recovery, may be an approach for attaining higher yields. To facilitate incorporation of this character into new rice varieties, the nature of gene action, heritability, and the relationship between HD grains and other panicle characters were studied.

In set I, 6 HD grain parents having at least 30% HD grains, 2 low-density grain parents, F_1s, and F_2s of IR32307-75-1-3-l/IR30, IR34615-75-1-1/IR29725-135-2-2-3, IR29692-117-1-2-2-2/IR30, IR30/IR35337-61-2-2-2, IR34615-75-1-1/IR32419-102-3-2-3, and IR30/IR32385-37-3-3-3 were used. In set II, parents, F_1, F_2, and backcrosses of Rewa 353-4/IR28211-43-1-1-2 were used.

The HD grams having specific gravity >1.20 in primary branches (Pb) and secondary branches (Sb) of panicles were determined by floating the grams in salt solution at maturity. The HD grain index (HDI) was calculated by dividing the number of HD grains by the total number of spikelets.

Variable gene action from no dominance to overdominance through partial and complete dominance was observed. Multiple genes with dominance and additive effects controlled HDI on Pb and Sb, though dominance effects were greater than additive effects.

Among the six crosses in set I, IR30/IR32385-37-3-3-3 was most promising, having the highest HDI value in the F_1 as well as the F_2.

The frequency distribution of F_2 plants for HDI relating to Pb and Sb was a continuous unimodal curve (Fig. 1), slightly negatively skewed. The F_2 plants transgressed both the parental limits, typical of the involvement of multiple genes with opposing effects.

Heterosis and heterobeltiosis for HDI were significantly positive for Pb and Sb.

Heritability estimates were high to moderate, and the number of effective factors did not exceed two for any cross. Of the parents, IR30 was promising for increasing HD grains.

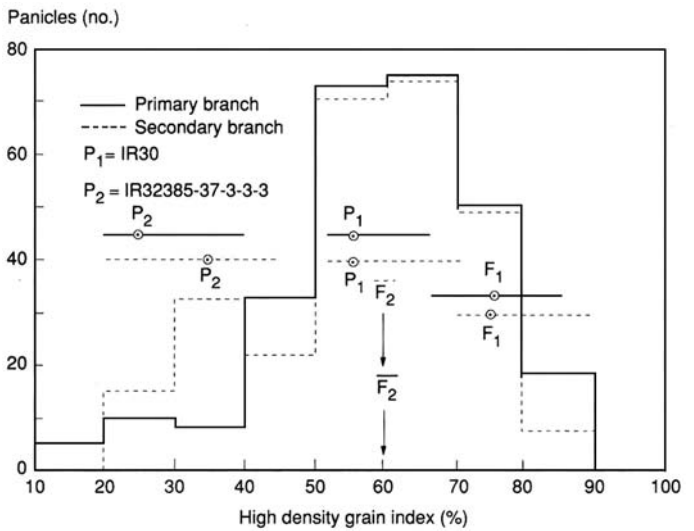
Rewa 353-4/IR28211-43-1-1-2 manifested 50% heterobeltiosis for number of HD grains, but HDI was very close to that of the higher parent. The backcross with the

better parent produced more HD grains than the backcross with the lower parent (Table 1).

Only the additive effects were highly significant (Table 2). Of the epistatic effects, only additive/dominance was significant.

The similar sign of h (dominance) and l (dominance/dominance) indicated the absence of duplicate epistasis. Heritability estimates were high for both Pb and Sb. The number of effective factors was 13.6 for Pb and 6.1 for Sb.

Significant additive effects, high heritability, low number of effective factor pairs, and significant correlation show the possibility of constructing rice varieties with higher number of HD grains following recurrent selection, which effectively utilizes the dominance and additive effects. IR30 may be a good parent for this approach.



1. Distribution and means of parents, F_1, and F_2 plants by high-density grain index in IR30/IR32385-37-3-3-3, 1987 wet season.

Table 1. Mean values of HD grains for 6 generations in Rewa 353-4/IR28211-43-1-1-2. IRRI, 1989.

Generation	HD grains (no. per plant)		Total HD grains (no.)	Mean HDI
	Pb	Sb		
P_1	244 ± 4.7	399 ± 9.4	643	33.8
P^1	530 ± 5.3	398 ± 6.1	928	53.2
F^2_1	731 ± 5.0	719 ± 3.8	1450	54.7
BC_1	411 ± 7.3	440 ± 7.5	851	38.9
BC_2	173 ± 4.6	215 ± 7.2	388	18.8
	695 ± 9.2	561 ± 8.9	1256	62.7

Table 2. Estimates of gene effects for number of HD grains, IRRI, 1989.[a]

Parameter	Pb	Sb
m	411** ± 73.2	440** ± 75.1
d	-522** ± 103.2	-346** ± 114.6
h	436 ± 363.9	-113 ± 383.4
i	92 ± 358.3	-208 ± 377.7
j	-379** ± 110.6	-346** ± 126.6
l	408 ± 521.9	891 ± 563.6
Heritability	85.2	83.5
Effective factors	13.6	6.1

[a] ** = significant at the 1% level.

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Restriction fragment length polymorphism analysis of wide-cross progenies having brown planthopper resistance gene(s) introgressed from *Oryza officinalis* into *O. sativa*

K.K. Jena, G.S. Khush, and G. Kochert

Eleven breeding lines (Jena and Khush 1989) derived from a cross between *Oryza sativa* (IR31917-45-3-2) and *O. officinalis* (Acc. no. 100896) were analyzed for restriction fragment length polymorphism (RFLP) linked to the region for brown planthopper (BPH) resistance introgressed from *O. officinalis* into *O. sativa.* Genomic DNA of the 11 breeding lines as well as of the parents was digested with 5 restriction enzymes *(Eco*RI, *Eco*RV, *Hin*dlll, *Bam*Hl, and *Pst*I) and Southern blotted onto Gene Screen Plus membranes. A total of 127 mapped RFLP clones (McCouch et al 1988) occurring at approximately 20-cM intervals throughout the genome showed polymorphism between the parents for most of the enzymes. The introgressed lines showed monomorphic banding patterns similar to those of the *O. sativa* parent for 98% of the probes surveyed, indicating that most of the genome of the breeding lines was derived from that parent. Of the 13 RFLP clones mapped on chromosome 4, clone KG214 revealed *Eco*RI fragments characteristic of the *O. sativa* parent for 5 lines that are susceptible to BPH, and 3 lines had a novel restriction fragment (5.8 kb) that was not present in either parent. However, three lines showed fragments characteristic of the *O. officinalis* parent (Fig. 1). lntrogressed lines having the *O. officinalis* fragment or the novel type fragment correlated with BPH resistance. This result indicates that BPH resistance gene(s) introgressed from *O. officinalis* might be present in the region around RFLP marker RG214 on chromosome 4. Further experiments are under way to determine the linkage between BPH resistance genes and RFLP marker RG214. This molecular marker may be useful in transferring BPH resistance gene(s) of *O. officinalis* from introgressed lines by conventional plant breeding and to monitor for BPH resistance in an early generation.

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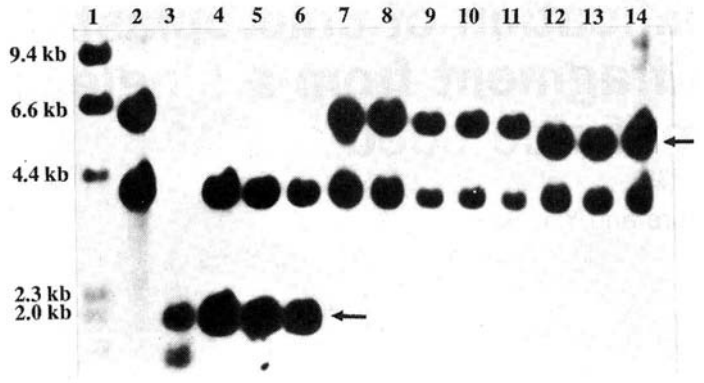

1. Banding patterns of RFLP marker RG214 in 11 introgressed lines having different reactions to BPH. Southern blots contained genomic DNA from *O. sativa* (lane 2), *O. officinalis* (lane 3), and 11 introgressed lines (lanes 4-14) that was digested with *Eco*RI and hybridized to radiolabeled RFLP marker RG214. Lanes 4-6 are resistant lines showing presence of *O. officinalis* band (arrow), lanes 7-11 are susceptible lines with *O. sativa* bands, and lanes 12-14 are resistant lines with novel band (arrow).

Notes

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Amplification of chloroplast DNA fragment from a single ancient rice seed

I. Nakamura and Y-I. Sato

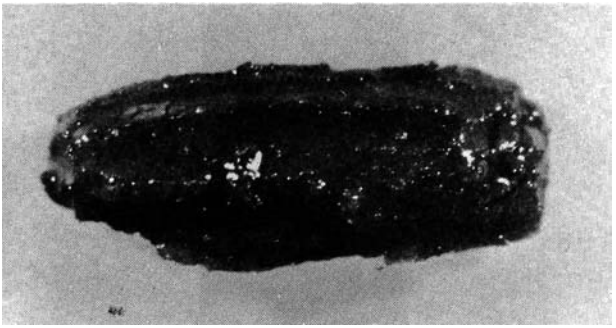
Ancient rice seeds have been found in many archaeological excavations in East Asia. If DNA fragments can be recovered from these seeds, the phylogenic view of rice can be broadened, because DNA analysis of an ancient seed gives us more information than morphological analysis does. Polymerase chain reaction (PCR) technology enables us to amplify DNA isolated from museum specimens and archaeological finds (Higuchi et al 1984, Pääbo 1985). Golenberg et al(1990) successfully extracted DNA and amplified the chloroplast gene (*rbc*L) from the fossilized leaves of 17- to 20-million-yr-old *Magnolia* species. Our work investigated whether a chloroplast DNA (ctDNA) fragment could be amplified from a single ancient rice seed.

Ancient rice seeds (1,200yr old) were found in the Ishikawa-Joriteki-lseki excavation of Nagano Prefecture (central Honshu) in Japan (Fig. 1).

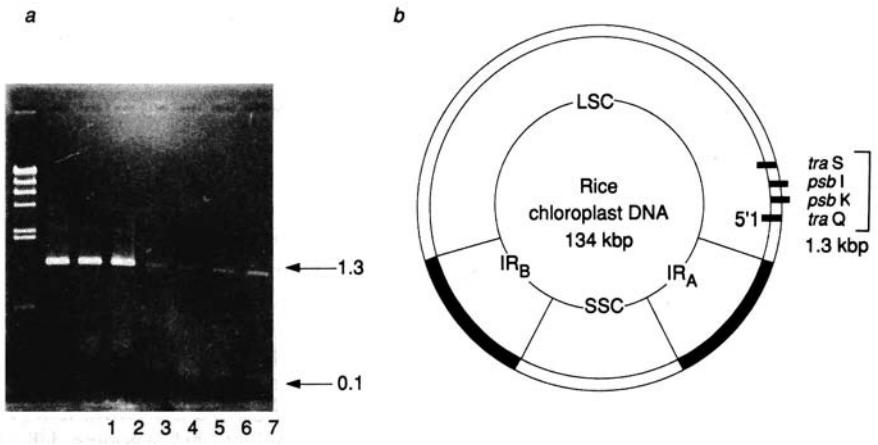
DNA extraction from a single seed is a modification of the method for plant DNA minipreparation of Dellaporta et al (1983). DNA was also extracted from living seeds and leaves of two varieties: T65 (japonica) and Ac. 144 (indica).

5' and 3' primers for the amplification were 20-mers based on the sequence of rice chloroplast *trnQ* and *trnS* genes, respectively, as reported by Hiratsuka et al (1989) (Fig. 2). Thermostable DNA polymerase-mediated amplification of ctDNA fragments was carried out as follows. A total volume of 25 µl reaction mixture was constituted from 2.5 µl of 10 x PCR buffer, 4 µl of four 2'-deoxynucleoside-5'-triphosphates, 2 µl of 5' primer, 2 µl of 3' primer, 5 ng of template or ancient DNA, and 1 unit of *Thermus thermophilus* DNA polymerase. The reaction mixture was incubated in a thermal programer (B 631, Kyo-ritsu Bio, Japan) for 45 cycles consisting of 1 min at 94 °C, 1 min at 37 ºC, and 2 min at 72 °C. Four microliters of reaction mixture was analyzed by 0.8% agarose gel electrophoresis. Amplified fragments were checked by restriction and Southern blot analysis.

DNA (50-100 ng) could be extracted from a single ancient rice seed. This was half of the amount extractable from a single living seed by the same procedure (Fig. 3). The DNA fragments from the ancient seed were recovered as 15- to 20-kbp fragments and were a little smaller than intact DNA. Since these ancient seeds have sunk in water-saturated soil, water must have protected the DNA from strand cut and modification by oxidation, preserving it extremely well.

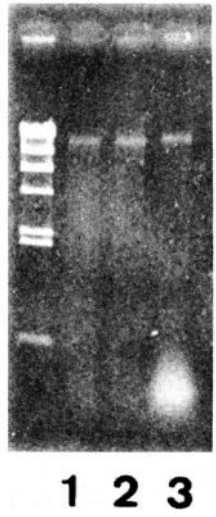


1. Ancient rice seed (AD 800) from central Honshu, Japan.



2. a) Amplication of ctDNA fragments from a single ancient rice seed. Lane 1 = T65 leaf, lane 2 = Ac. 144 leaf, lane 3 = T65 seed, lanes 4 and 5 = 90-yr-old seed, lanes 6 and 7 = 1,200-yr-old seed. b) Design of primers (5' and 3') for amplification of rice ctDNA used in PCR reactions. 1.3-kbp fragment was expected to be amplified with this primer pair.

Amplified products by PCR reaction were fractionated through agarose gel (Fig. 2). A unique product (1.3 kbp) expected from the sequence was amplified in lanes 1 (T65 leaf), 2 (Ac. 144 leaf), and 3 (T65 seed). A fragment of the same size was also seen in lanes 4-7 (2 different aged ancient seeds) by ethidium bromide staining. A labeled 1.3-kbp chloroplast fragment of T65 leaves was hybridized with 1.3-kbp fragments amplified from ancient seeds as well as from living seeds and leaves. Restriction



3. Total DNA extracted from single seed. Lanes 1 and 2 = ancient seed, lane 3 = T65 seed (2x dilution).

analysis showed that these 1.3-kbp fragments were correctly amplified from ctDNA and were not differentiated among T65 (japonica), Ac. 144 (indica), and ancient rice even by 4 base cutters.

Ishii et al (1 988) reported differences between indica and japonica ecospecies in rice using restriction fragment length polymorphism (RFLP) of ctDNA. These RFLP markers can be applied to distinguish the ecospecies of an ancient rice by DNA analysis of its excavated seed. Although we suspect that part of a DNA fragment (1.8 kbp) of rice phytochrome gene could be amplified from ancient DNA, it seems difficult to amplify and recover a fragment of the single-copy gene from this DNA. In practice, the DNA analysis of ancient seeds should be focused on multicopy genes such as chloroplast genes. Now, we can amplify and analyze the specific-sequence, 1.3-kbp fragment covering the *trn*Q, *psb*K, *psb*I, and *trn*S genes of an ancient ctDNA segment corresponding to 1 % of the total rice chloroplast genome (134 kbp). The DNA analysis of the ancient rice seed described here can greatly contribute to the investigation of the origin and varietal diversification of rice cultivars.

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Citation information: International Rice Research Institute (1991) Rice genetics II. P.O. Box 933, Manila, Philippines.

Closing Reports, Participants, and Index of Varieties and Lines

Overview of the Second International Rice Genetics Symposium

K.J.Frey

Two decades ago, I described the research program of the International Rice Research Institute, and the similar program of its sister institute, the Centro Internacional de Mejoramiento de Maiz y Trigo, as grand, worldwide, social and humanistic experiments on harnessing the world's genetic resources of maize, rice, and wheat to feed an already hungry but expanding human population. Later, research on soils, climate, economics, and sociology was added to the repertoire of disciplines used in these grand experiments. At the Second International Rice Genetics Symposium, we have had an assessment, not of how well that IRRI experiment is succeeding, because we know it has been eminently successful; rather we have learned how researchers at IRRI and their colleagues worldwide are changing the face of research at IRRI to permit this institution to continue its grand social and humanistic mission.

As at the First International Rice Genetics Symposium, we heard a number of researchers describe the Mendelian inheritance of morphological and pest resistance traits of the rice plant. Such studies provide the grist for making varietal improvements in the near future. The addition of new alleles to the rice breeding gene pool from several related species was described. Research results were given on the genetic control of tolerance for drought, iron chlorosis, and salinity, characters that promise to make future rice varieties better adapted and more tolerant of production stresses. Male sterility that is photoperiod- and thermosensitive promises to make hybrid rice seed more readily available in food-deficit countries.

What set the Second International Rice Genetics Symposium apart from all other rice conferences, however, was the compilation of research results given on tissue and cell culture, molecular genetics of the cytoplasmic and nuclear genomes of rice, development of a rice genomic map via restriction fragment length polymorphism, and transformation. Fully half of the program was devoted to the exciting new areas of molecular biology and biotechnology of rice. With this new knowledge and accompanying techniques, plant breeders will be able to create a new rice plant with characteristics unimagined when IRRI began. And their work will be accomplished with precision at an order of magnitude greater than was possible a decade or two ago.

IRRI has built an immense worldwide network of rice researchers. At this symposium, oral reports were presented by scientists from 14 countries, and poster papers from 15 countries were presented. Truly, the world plant science community is committed to research on rice—research that will enhance IRRI's mandate to strengthen rice-based agriculture to better feed the human population of the tropics.

The Second International Rice Genetics Symposium was educational; it provided opportunity for exchanging information; it created new personal linkages; it was well conducted; and yes, it was a grand affair and a great success. For all of this, the rice research community and others (like me) who attended this conference owe Dr. Lampe, Dr. Khush, and the entire IRRI staff a debt of gratitude. We give you our resounding thanks.

Closing remarks

F.A. Bernardo

I am happy to be in the company of the world's most distinguished scientists and scholars who have been devoting their lives to the advancement of knowledge in rice genetics. Any scientist working on rice is serving the interests of about 3 billion rice-eating people at present, and anyone pursuing research on rice genetics is helping pave the way for the development of improved rice varieties to feed 4.5 billion people in the year 2020.

Five years between two world symposia on rice genetics is short; yet, we have witnessed tremendous developments as revealed by the quantity and variety of papers and posters presented during this symposium:

- Work done in China, India, Japan, the US, and other countries—not to mention at IRRI—has shed more light on the inheritance of important characters such as grain weight and embryo length; resistance to rice blast, tungro, gall midge, and brown planthopper; and tolerance for drought and salinity.
- At IRRI, research results have shown the successful introgression of genes for resistance to bacterial blight and blast from *Oryza minuta* to *O. sativa.*
- In China, further work on wide compatibility genes has confirmed their ability to eliminate sterility in subspecific F_1 hybrids or crosses between indicas and japonicas.
- The development in Japan of H89-1, a thermosensitive genetic male sterile line, offers exciting opportunities to revolutionize hybrid rice seed production without using cytoplasmic male sterile lines and restorers.
- Improved or new techniques in tissue and cell culture as a result of studies done in China, India, the USSR, Belgium, Japan, and the United Kingdom and at IRRI provide greater opportunities for using those techniques as tools in rice improvement work.
- The papers from advanced countries on the molecular genetics of cytoplasmic genomes and nuclear genomes have given us a better understanding of the complexity of the genetic structure and inheritance in rice. Further progress in genetic mapping, aided by modem tools in biotechnology such as restriction fragment length polymorphism, has been encouraging and must accelerate.

- The search for more efficient or effective transformation of genetic materials through improved techniques and new technologies such as "biolistics" goes on. This is an exciting area where rapid progress can be expected within the next few years. Transformation is both a science and an art in laboratory work. It offers tremendous possibilities to accelerate crop improvement. Plant breeders await successful results in rice. Successful transformation materials could make plant breeding work easier. They provide possible dramatic shortcuts to commercial exploitation of improved varieties.

Contributions to this symposium are so numerous that I cannot cite all the significant research results. The symposium brought eminent rice scientists together for 4 days of intensive discussions. There were 64 papers and 70 posters. It has been a rare opportunity for about 300 scientists coming from 25 countries and working on conventional genetics and molecular genetics of rice to interact with each other.

I am happy to know that a system of numbering rice chromosomes and linkage maps has been agreed upon. This should help expand the frontiers of knowledge in rice genetics. It is gratifying that useful discussions about the nomenclature of rice genes took place. 1 would urge the rice workers to follow the rules of gene nomenclature and the recognized system of chromosome and linkage group numbering.

The Rice Genetics Cooperative (RGC) established during the First International Rice Genetics Symposium in 1985 has provided an excellent forum for bringing rice geneticists together and for providing leadership in preparing rules for gene nomenclature and linkage groups and chromosomes. The *Rice genetics newsletter* (RGN), published by the RGC, is an excellent medium for exchanging information. The RGC also plays an excellent role in monitoring rice gene symbols. I hope you will continue to give your full cooperation to the RGC to help further the cause of rice genetics.

I wish to make a special acknowledgment of the distinguished scientists and leaders who contributed much to the success of this symposium. Special thanks go to

- *Dr. K.J. Frey* for providing an overview of the symposium. Dr. Frey has organized many international symposia, and his insights are very valuable.
- *Drs. H.I. Oka, Y. Futsuhara,* and *T. Kinoshita* from Japan, who served as members of the organizing committee for the symposium and played a leading role in organizing the RGC; Dr. Oka, particularly, has devoted much time to the editorial affairs of the RGN.
- *Dr. E.C. Cocking* for his keen interest in advancing the frontiers of rice science and for bringing several of his colleagues to the symposium.
- *Dr. Gary Toenniessen* of the Rockefeller Foundation (RF) for encouraging many participants in the RF network on rice biotechnology to attend this symposium and for providing them financial support.
- *Dr. Ray Wu* of Cornell University for his active participation in RGC activities and for his wise counsel.
- *Dr. Gurdev S. Khush* for serving as the workhorse at IRRI in making all the preparations and attending to all the details required for the success of this symposium.

- *The members of the organizing committee* of the symposium for their hard work.

Finally, I wish to thank all the participants for their contributions and for their keen interest. If you suffered any inconveniences, we ask for your forgiveness. We hope you will have a nice trip back home and carry fond memories of companionship with your colleagues.

Best wishes to all of you, and may you have more power and more success in your scientific endeavors until we meet again at the Third International Rice Genetics Symposium in 1995.

Committee reports

Report of the meeting to discuss coordination of gene symbols for rice isozyme loci, 16 May 1990

PRESENT: H. Morishima, J.C. Glaszmann, A. Ghesquiere, G. Second, J.-L. Pham, D.S. Brar, B. de los Reyes, R. Ishikawa, and T. Nagamine

The discussions during the meeting led to the following agreements:

- Forty-nine isozyme loci (including six tentative ones) are now known in rice. Of these, 30 have been associated with specific chromosomes.
- The rules for allele designation suggested by Glaszmann et al (1988) were accepted. The major alleles frequently found in *O. sativa* are given small numbers (slow band, allele 1; fast band, allele 2; etc.). Rare alleles are numbered according to the order of discovery.
- Locus/allele nomenclature will be compiled in tabular form and published in the *Rice genetics newsletter* (RGN), volume 7. The table will include information on marker line, plant organ used, buffer system, isozyme diagram, and chromosome location if known.
- Isozyme alleles found only in wild species will not be included in the compilation. Such alleles should be designated by letters of the alphabet.
- Researchers should exchange marker lines for allelism tests. This will promote the use of common allele designations.

Report of the meeting on gene symbolization, nomenclature, and linkage groups in rice, 16 May 1990

PRESENT: T. Kinoshita, H.I. Oka, Y. Futsuhara, G.S. Khush, H.K. Wu, M.H. Heu, G. Toenniessen, N. Iwata, R. Wu, H. Morishima, M.E. Takahashi, S.K. Min, H.K. Tsai, S.S. Virmani, D. Senadhira, and others

The list of gene symbols compiled by T. Kinoshita and published in RGN3 was reviewed, and problems of gene symbolization were discussed. The following agreements were reached:

- Guidelines for naming biochemical loci will be prepared by a small group headed by R. Wu.
- Efforts will be made to coordinate the restriction fragment length polymorphism (RFLP) mapping of rice chromosomes so that a unified RFLP map becomes available.
- The gene symbols for heading date proposed by T. Tanisaka will be further examined by concerned scientists.
- H.I. Oka's suggestions regarding the nomenclature of japonica and indica rices will be further examined, along with the nomenclature of species and subspecies of rice and related genera.
- A small meeting of scientists interested in the problems of rice gene symbolization will be organized in 1991 or 1992.

Report of the meeting to discuss coordination of rice RFLP mapping, 16 May 1990

PRESENT: M. Kawase, T. Kinoshita, G.S. Khush, S. McCouch, S. Tanksley, G. Toenniessen, M. Yano, and A. Yoshimura

It was the consensus of the group that the maps developed in S. Tanksley's laboratory and by the Japanese group should be coordinated. M. Yano suggested that the groups exchange and map clones from the ends of each chromosome. Dr. Tanksley thought that this might be extended to 5-10 markers for each chromosome to provide better correlation of the two maps.

The issue was raised of working from a common mapping population in the future. This would ensure that all mapped clones could be ordered on a single cross. Currently, both groups are working from vegetatively propagated populations. Dr. Yano indicated that they are considering exchanging mapping populations. Dr. Tanksley said that the Chinese scientists are developing doubled haploid populations that could be shared by scientists for RFLP mapping in the form of seed.

The question of probe availability arose. Clones developed in Dr. Tanksley's laboratory were supported by Rockefeller Foundation funding and are generally available. Japanese clones are not currently available, but once the paper describing the map is published, the concerned researchers expect to be able to distribute a subset of 70 clones (at 10- to 20-cM intervals). A. Saito is the group leader of the Japanese RFLP mapping project. It was suggested that Dr. Tanksley write to the RFLP mapping group, proposing specific methods for coordinating maps so that formal discussion of ideas can be discussed in Japan and to allow time for a thoughtful response.

Report of the meeting to discuss the chromosome numbering system in rice, 15 May 1990

PRESENT: T. Kinoshita, Y. Futsuhara, G.S. Khush, N. Iwata, H.I. Oka, K. Takeda, H.K. Wu, M. Yano, and A. Yoshimura

Shastry et al (1960) numbered rice chromosomes according to their length at the pachytene stage of meiosis, the longest chromosome being 1 and the shortest being 12. Nishimura (1961) assigned arbitrary numbers (I-XII, later 1-12) to rice chromosomes involved in translocations in the order in which the translocations were discovered. Kurata and Omura (1978) numbered the chromosomes based on their length at somatic prophase, from K1 (longest) to K12 (shortest).

Two primary trisomic series were used to associate linkage groups of rice with respective chromosomes. Khush et al (1984) used primary trisomics of indica variety IR36 and associated the linkage groups with 12 chromosomes. The extra chromosomes of the IR36 trisomics were identified at the pachytene stage of meiosis. Iwata et al (1984) associated linkage groups of rice with respective chromosomes using the primary trisomics of japonica variety Nipponbare. The extra chromosomes of the Nipponbare trisomics were identified at mitotic prophase. When the results of the two studies were compared, linkage group-chromosome associations did not always agree.

Meanwhile, on the basis of discussions among geneticists in Japan, at IRRI, and elsewhere, it was decided to hold an International Rice Genetics Symposium at IRRI in May 1985. Problems related to gene symbolization and chromosome numbering were discussed during the symposium. An interim committee chaired by C.M. Rick and with R. Riley, N. Iwata, G.S. Khush, T. Kinoshita, N. Kurata, H.I. Oka, R. Seetharaman, and H.K. Wu as members was established to examine the chromosome numbering system in rice. The committee recommended that the chromosome numbering system based on length at the pachytene stage of meiosis proposed by Shastry et al (1960) be followed. This recommendation was accepted by the general body.

Discussions among participants of the symposium also led to the birth of the Rice Genetics Cooperative for promoting international collaboration in rice genetics. The Cooperative gave top priority to resolving discrepancies in the chromosome-linkage group associations determined by Khush et al (1984) and Iwata et al (1984). It was decided that the IR36 trisomics should be reexamined to see if any error was made in

the identification of extra chromosomes of some trisomics. Consequently, R.J. Singh, representing the group of Dr. Khush, and N. Kurata, representing the group of Dr. Iwata, spent 3 wk at IRRI in June 1986 to examine the IR36 trisomics together. They confirmed the earlier identification that trisornics 1, 2, 5, 7, 9, and 10 have chromosomes 1, 2, 5, 7, 9, and 10, respectively. However, no agreement was reached regarding the identification of extra chromosomes of the remaining six trisomics because of lack of time. However, Dr. Kurata continued the examination of the six trisomics in Japan; Dr. Wu of Taiwan was also asked to examine these trisomics.

During the meeting of the Coordinating Committee of the Rice Genetics Cooperative held at IRRI on 22-24 October 1987 and attended by Y. Futsuhara, G.S. Khush, T. Kinoshita, T. Matsuo, H.I. Oka, D. Senadhira, H.K. Wu, and T. Tsuchiya, new results were reviewed, and it was agreed that the extra chromosome of trisomic 4 is number 3 and the extra chromosome of trisomic 8 is number 8. It was agreed to continue the examination of the remaining four trisomics (3, 6, 11, and 12). Results of their investigations were reported by Kurata (1988) and Wu and Chung (1988). Dr. Khush and his colleagues continued the reexamination of IR36 trisomics. All the available data were reviewed by Drs. Iwata, Kinoshita, and Khush during the latter's visit to Kurashiki City, Japan, in January 1990. A tentative agreement was reached regarding the identification of extra chromosomes of the remaining four trisomics; the extra chromosomes of trisomics 3, 6, 11, and 12 are 6, 12, 11, and 4, respectively. This agreement was accepted during the meeting of the ad hoc committee on 15 May 1990 and approved by the general body on 18 May 1990. This agreed upon system of numbering chromosomes and linkage groups and corresponding trisomics is shown in Table 1. From now on, the linkage group corresponding to chromosome 1 will be designated as linkage group 1; that corresponding to chromosome 2, as linkage group 2; and so on.

Table 1. The agreed upon system of numbering rice chromosomes and their relationships with linkage groups.

Chromosome number	Trisomics		Karyotype		Translocations (Nishimura [1961])	Linkage group
	Khush et al (1984)	Iwata et al (1984)	Kurata and Omura (1978)	Wu and Chung (1988)		
1	1	0	K1	1	3	III
2	2	N	K2	3	8	X
3	4	M	K3	2	5	XI + XII
4	12	E	K4	4	11	II
5	5	L	K9	5	2	VI + IX
6	3	B	K6	6	6	I
7	7	F	K11	7 (11)	10	IV
8	8	D	K7	9	12	sug
9	9	H	K10	8	1	v + VII
10	10	C	K12	10	7	fgl
11	11	G	K8	11 (7)	9	CIII
12	6	A	K5	12	4	d-33

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Election of officers of Rice Genetics Cooperative

New officers of the Rice Genetics Cooperative were identified for the 1990-95 term on the basis of discussions with many participants. The following officers were proposed and approved by the general body on 18 May 1990:

Coordinating committee

M.E. Takahashi (Japan), Chairman
K.J. Lampe (IRRI), Co-chairman
G.S. Khush (IRRI), Secretary and Editor
H.I. Oka (Japan), Editor
Y. Futsuhara (Japan), Secretary
D. Senadhira (IRRI), Treasurer
T. Kinoshita (Japan)
Shao Kai-min (China)
G. Toenniessen (USA)
M. Jacquot (France)
R.S. Paroda (India)
M.H. Heu (Korea)

Standing committees

Gene Symbolization, Nomenclature, and Linkage Groups
T. Kinoshita (Japan), Convenor
G. Takeda (Japan)
H.K. Wu (Taiwan, China)
R. Seetharaman (India)
J.N. Rutger (USA)

Genetic Stocks
N. Iwata (Japan), Convenor
D.A. Vaughan (IRRI)
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Index of varieties and lines[1]

[1] Names in parentheses are full forms of the main entries. Names in brackets are those recorded in the International Rice Germplasm Center.

A

B

G

H

I

J

K

L

M

N

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V

W

X

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Z