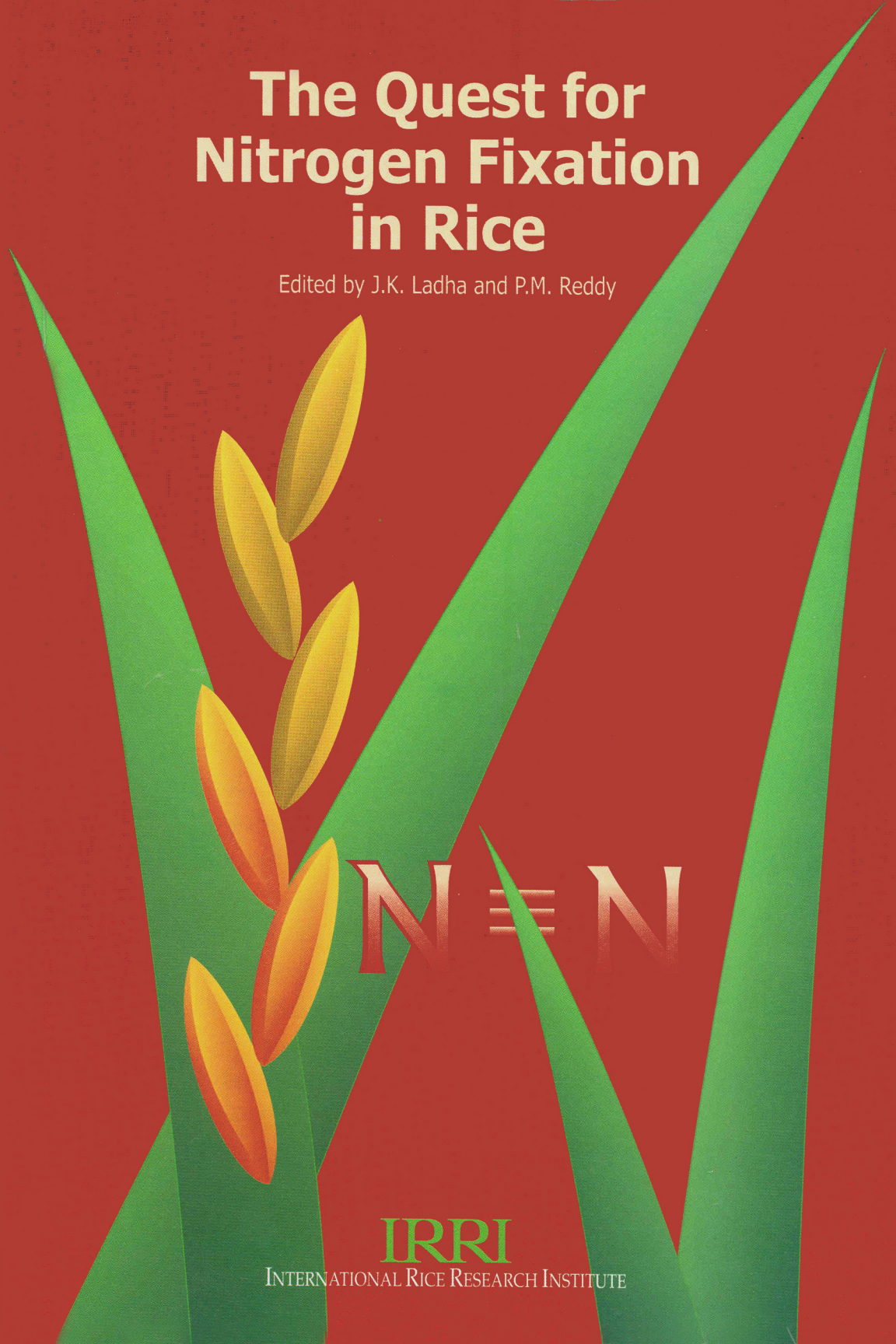
The Quest for Nitrogen Fixation in Rice

Edited by J.K. Ladha and P.M. Reddy



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International Rice Research Institute

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Foreword

The world's population has surpassed 6 billion. And the world will need about 60 percent more rice than today's global production to feed the extra billions who will rely on it within the next three decades. Because nitrogen is the critical nutrient for agricultural production, global agriculture now relies heavily on N fertilizers derived from nonrenewable energy resources such as petroleum and natural gas to meet the demand for food grain. It is estimated that twice as much fixed nitrogen will be required to increase rice production to supplement the food requirements of people in 2020. Awareness is growing, however, that such an increase in agricultural production needs to be achieved without endangering the environment. Industrially produced N fertilizer depletes nonrenewable resources and, if not managed properly, can pose a hazard to humans and the environment. Environmentally adverse inputs must be reduced to meet the challenge of feeding the world's growing population of rice consumers on a sustainable and equitable basis. Nowhere is this challenge more serious than in supplying environmentally friendly N resources for crop production. For this, biological nitrogen fixation (BNF) has the advantages of lower cost and reduced environmental hazards and is more consistent with the development of sustainable agriculture.

To achieve food security through sustainable agriculture, the requirement for fixed nitrogen must be increasingly satisfied by BNF rather than by industrial nitrogen fixation. In view of the importance of BNF to sustainable agriculture. it is imperative to improve existing BNF systems and develop nitrogen-fixing nonlegume crops, particularly important cereal crops such as rice. To meet this challenge, IRRI organized a think-tank workshop in 1992 to assess the feasibility of nitrogen fixation in rice. Based on the research strategies recommended, IRRI launched a global collaborative initiative in 1993, the Frontier Project on Nitrogen Fixation in Rice. The long-term goal of this project is to enable rice plants to fix their own nitrogen. Rice, as a model cereal plant, is particularly well suited for such investigations. In addition, emerging knowledge of functional genomics and new biotechnology tools will enormously benefit such research endeavors. This Frontier Project involves a committed group of scientists from several research institutes around the world. The project also has a working group, through which IRRI facilitates communication among scientists worldwide with active research interests in nitrogen fixation in rice and other cereals. Since the project began, remarkable scientific progress has been made in providing new knowledge. The working group periodically reviews the progress to evaluate how such new knowledge has furthered the probability of success in meeting this long-term goal. Two BNF working group meetings were held previously, at

IRRI (Philippines) in 1995 and at the National Institute for Biotechnology and Genetic Engineering (Pakistan) in 1996, and the reports of the meetings were widely disseminated. In the recently held third BNF working group meeting at IRRI, 9-12 August 1999, several significant results emanating from the Frontier Project were presented. This book features the proceedings of the meeting, encompassing the latest research advances made in developing nitrogen-fixing rice. We hope that this book will encourage more research in this area.

Ronald P. Cantrell
DIRECTOR GENERAL

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Many researchers contributed to the success of the Third Working Group Meeting of the Frontier Project on Assessing Opportunities for Nitrogen Fixation in Rice, upon which this book is based. In conjunction with the Working Group meeting, a project review was also held. We thank Frans J. de Bruijn, chairperson of the Working Group, and John Bennett, chairperson of the Project Review Task Force, for their help in developing the program of the meeting. We also thank other distinguished scientists, who chaired the sessions at the meeting, and rapporteurs for their contributions. We are grateful to the Review Panel members, Alfred Puhler (chairperson), John Beringer, Robert H. Burris, David Layzell, and Naoto Shibuya, and to the Project Review Task Force members, Darshan S. Brar, Osamu Ito, and Guy J.D. Kirk, for their contributions to the discussions. Most important. we wish to thank all the participants for making this meeting successful. Thanks are also due to Ella Figueroa, Flor Junsay, and Minnie Bandian for their excellent secretarial support during the meeting.

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The potential role of biological nitrogen fixation in meeting future demand for rice and fertilizer

David Dawe

Rice is the most important crop in Asia, accounting for nearly half of caloric intake in many countries. Future demand for rice will increase at approximately the rate of population growth in the next 20 years, which is projected to be slightly more than 1% per year in Asia. Nitrogen is a key input in rice farming, but the cost of mineral nitrogen fertilizer relative to the value of rice production is typically low in both irrigated and rainfed systems (just 4-7%). This low share is not due to a preponderance of subsidies, which are much less common today than in the past. More important factors are the sharp decline in inflation-adjusted world prices of urea over the past 40 years and the fact that labor is by far the most important cost in rice farming. The low share of N fertilizer in gross returns implies that farmers will not adopt rice varieties with enhanced nitrogen fixation capacity if there is even a small yield penalty associated with such capacity. It also implies that the environmental benefits from reducing nitrogen runoff are likely to be more important than the financial benefits for farmers due to reduced mineral N consump tion. Because a large share of rice is grown under irrigated conditions, or in rainfed environments with continuous flooding, it will be important to develop biological nitrogen fixation that can operate under flooded conditions.

The importance of rice to Asian consumers is well known, but the magnitude of this importance is staggering. Rice accounts for more than 70% of total caloric intake for the average citizen in Myanmar and Bangladesh, and it accounts for nearly two-thirds in Vietnam. Even in relatively wealthier countries such as Thailand and Indonesia, it still accounts for nearly 50% of caloric intake (FAO 1998). Furthermore, these figures are for the average citizen, which means that for many citizens (primarily the poor) the share is even higher. Rice is also important to millions of small Asian farmers who grow the crop on millions of hectares throughout the region, and to the many landless workers who derive income from working on these farms. These considerations argue for the strong need to increase rice productivity through continued research.

Future demand for rice

Although the importance of rice declines with economic growth, demand for rice will continue to grow for many years, and this growth could occur at a pace that exceeds growth in supply. If this scenario were to materialize, prices would increase, and this could endanger the food security of millions of poor Asians. The role of rice research is to increase supply at a rate that exceeds growth in demand through increased farm-level productivity.

Many factors affect growth in rice demand, but the main ones are (1) population growth, (2) income growth, and (3) urbanization. Income growth affects demand for rice, but the effect depends on the level of income (Timmer et al 1983). When people are poor, increased income leads to increased consumption of rice, but the magnitude of this effect declines as income rises. Eventually, people reach a threshold level of income after which rice consumption actually begins to decline in absolute terms because people desire a more balanced diet when income permits. As they grow richer, they tend to consume more meats, poultry, fish, fruits, and vegetables. Total caloric demands do not increase substantially, however, so the increased calories from these other foods crowd out calories from rice. Several countries in Asia have already reached the point at which per capita consumption of rice is declining. The average Japanese citizen, for example, consumed approximately 110 kg of milled rice per year in the early 1960s. By the mid-1990s, however, consumption had declined to about 60 kg per person. Similar trends have already taken hold in the relatively high-income countries of South Korea, Malaysia, and Thailand, where per capita consumption of rice peaked in the 1970s. In middle-income countries such as China, Indonesia, and the Philippines, per capita consumption has not started to decline significantly, but it appears to have reached a peak and is unlikely to increase further. In poorer countries such as India and Bangladesh, however, per capita consumption will likely increase in the future. The net effect of all these conflicting trends is likely to be slightly positive growth in aggregate Asian rice demand due to rising incomes, but the effect will probably not be large.

Urbanization affects growth in rice demand in a negative fashion. Compared with the rural population, urban dwellers at the same level of income consume less rice for at least two separate reasons. One is the greater variety of foods available in urban areas, which tends to lead to diet diversification. The second factor is that urban residents typically have employment that is less physically demanding, leading to a lower demand for starchy staple foods such as rice. Asia is becoming steadily more urbanized, and this will have a negative effect on rice demand. For example, the proportion of the population living in urban areas in Indonesia was 31% in 1990, but is projected to rise to 57% by 2020 (Hossain 1999). The net effect of urbanization is also likely to be small. Since it is opposite in sign to the effects of income growth, the two factors will more or less negate one another for Asia as a whole. This was not always true in the past, but is likely to be true in the next 20 years.

This leaves population growth as the major driving force behind growth in rice demand. Population growth has been steadily declining in Asia for the past few de-

cades. In the 1960s, population grew at an average annual rate of 2.3%, but this has slowed substantially to just 1.4% from 1990 to 1997 (FAO 1998). Population growth projections from the United Nations estimate that this trend will continue, with average annual population growth in Asia slowing to a rate of 1.08% per year from 1995 to 2020 (UN 1997). This implies that growth in rice demand in the next 20 years will be much slower than it was in past decades. Thus, estimates made by Rosegrant et al (1995) in the IMPACT model of the International Food Policy Research Institute (IFPRI) are that demand for rice will grow by approximately 1.0% per year until 2020. Of course, this slower growth in demand does not guarantee food security for Asia because rice production gains will also be more difficult to come by in the 21st century.

Prices will also affect the quantity of rice demanded by consumers, but this effect tends to be small because rice is a staple food and is so important to consumers that its demand is relatively insensitive to changes in prices. Furthermore, long-term forecasts of prices are notoriously difficult to make, so these effects are not discussed further.

The role of nitrogen fertilizer in rice production

In terms of cost, nitrogen fertilizer is the single most important purchased material input in rice production. This importance stems from the fact that nitrogen is the major factor limiting growth under most conditions. The development of fertilizer-responsive varieties in the Green Revolution, coupled with the realization by farmers of the importance of nitrogen, has led to high levels of N fertilizer use on rice. The use of nitrogen per hectare on rice is especially high in countries where most rice is grown under irrigated conditions because fertilizer use is much higher in the irrigated ecosystem than in rainfed, upland, or flood-prone ecosystems. For example, a recent study by Harris (1998) estimates that rice farmers in South Korea apply about 170 kg N ha^{-1} . In China, nitrogen use amounts to about 140 kg ha^{-1} . In contrast, in countries where the proportion of irrigated area is not as large, nitrogen fertilizer use is lower. For example, in Thailand, average N use on rice amounts to less than 40 kg ha^{-1} .

World prices for mineral fertilizers, including urea, have fallen rapidly in real terms during the past 40 years (a decline in real prices means that prices have increased less rapidly than the prices of other goods and services). In the early 1960s, world market prices for urea were approximately US$500 t^{-1} (prices are in 1998 US$), but by 1998 they were only US$121 t^{-1} , a decline of about 75%. Coupled with the development of modern varieties, these declining prices have encouraged farmers to use more nitrogen, and this is reflected in statistics for aggregate N consumption. In developing Asia, total use of N fertilizer was less than 1.5 million tons of fertilizer in 1961 (FAO 1998). By 1996, use had increased to nearly 47 million tons, an increase of more than thirtyfold! (These figures are in tons of fertilizer, not tons of N. They also include use on all crops, not just rice.)

Despite the tremendous use of nitrogen fertilizer on rice, the share of nitrogen costs in the total value of production is perhaps not as large as is commonly believed.

This fact has been established in many surveys of farm enterprises. For example, recent data collected from representative farm enterprises in several key intensive irrigated rice-growing regions showed mean shares of just 3.5% in West Java, Indonesia, 6.7% in the Central Plains of Thailand, and 6.8% in the Mekong Delta of Vietnam (these data are averages of six seasons during the period 1994-98).

The share is comparable in typical rainfed systems. Using data from Table 6 in Pandey (1998) and average rainfed yields cited in IRRI (1997), the average share of N fertilizer in production value ranges from 2.7% in Bangladesh to 7.2% in Cambodia. Even in a very intensive rainfed system in Ilocos Norte. Philippines, Lucas et al (1999) find that total fertilizer costs are only 12% of the production value of rice. Taking rough account of P and K use on these farms would lower the share of N fertilizer to roughly 7.7%.

The share of N fertilizer in total costs must be larger because total costs are less than gross returns. At the intensive irrigated sites mentioned in the previous paragraph, N costs account for about 17% of total costs in the Central Plains of Thailand, 15% in the Mekong Delta, and 8% in West Java, Indonesia. The share is about 17% for the intensive rainfed site in Ilocos Norte described in Lucas et al (1999).

The figures for some of these countries might be larger were it not for fertilizer subsidies, which artificially lower the cost share of fertilizer. For example, the figures cited above for West Java, Indonesia, were distorted by the subsidies in place during the recent economic crisis. The government-mandated retail price for urea in 1998 was perhaps only half the free-market price at that time. Thus, one might argue that removal of the subsidy could have doubled the cost share of N fertilizer, but even this would only make the cost shares cited above for Indonesia comparable with cost shares at the other sites. This counterfactual is an exaggeration, however, as farmers would have decreased their use of fertilizer had the government removed the subsidy.

Furthermore, Indonesia during the recent crisis is an unusual example. In other countries, N fertilizer subsidies have been progressively reduced or eliminated altogether, as in the Philippines, Bangladesh, Thailand, and Vietnam (Hossain and Singh 1998). This has occurred because of the general movement toward globalization, as well as because of more tightly constrained government budgets. Even in the case of Indonesia, the above-cited subsidy did not reach many farmers, and black market prices were considerably higher than the government-mandated price. Acknowledging this reality, the Indonesian government doubled the official retail price for fertilizer in 1999, thus effectively ending any attempts at subsidy. In conclusion, because N fertilizer subsidies have been largely phased out, and because farmers tend to increase fertilizer use in response to the subsidies when they do exist, the "true" cost share of N fertilizer in production value is typically less than 7%.

Future demand for nitrogen fertilizer on rice

Estimating future demand for nitrogen use on rice is more complex than estimating future demand for rice. Several factors are worth mentioning. First, increased future demand for rice will, assuming constant prices, lead to increased future rice produc-

tion. Holding other factors constant (to be discussed below), an increased supply of rice will lead to increased N fertilizer requirements.

Trying to quantify this effect is difficult, however, because it depends on how future yield increases will be realized (aggregate rice area harvested is not likely to increase substantially, so production increases will need to be met by higher yields). The higher yields that will be necessary in the future can come in one of two fundamentally different ways. First, if farmers apply more fertilizer, but use the same seeds and the same technology, then increased yields will result from movements along the nitrogen response function. These increases in yield tend to be fairly small because most farmers operate in the region where the response function is fairly flat. Because the yield increases that are feasible from simply applying more N fertilizer with current technologies are so small, this scenario is not likely to drive future N fertilizer demand.

On the other hand, if new seeds and new technologies are adopted, then yield increases can arise even if no additional fertilizer is applied. For example, both the new plant type (NPT) and hybrid rice promise higher yields without necessarily requiring additional quantities of mineral N fertilizer. But it is difficult to predict how farmers will react to these technologies. For example, farmers may decide to increase fertilizer rates with the adoption of these seeds in an attempt to push yields still higher. If this happens, it is not easy to speculate by how much fertilizer use will increase. Alternatively, it is possible that farmers will decide to cut back on applications of N fertilizer while still taking advantage of the new seeds to increase yields above current levels. Ultimately, the behavior of farmers will depend on future prices of N fertilizer and rice and the shape of the response function of the new seeds. None of these are known with any certainty. As a result, any future forecast of N use on rice will have a very large degree of uncertainty associated with it.

Another complicating factor is that the price of fertilizer will also affect the quantity of fertilizer demanded. The price effect for fertilizer is potentially more important than for rice because Asian farmers respond more to fertilizer prices than Asian consumers do to rice prices. (In economics jargon, the price elasticity of demand for fertilizer is greater than for rice.) As noted earlier, world market prices for urea have declined substantially during the past 40 years. If this trend continues, this will encourage farmers to use even more fertilizer. On the other hand, if world petroleum prices increase substantially in the next few decades because of a shortage of fossil fuels, urea prices will likely also rise because the price of natural gas is a key component of urea fertilizer production costs. Debate is intense regarding future world petroleum prices, but no attempt to enter that debate will be made here (Science, 2 I August 1998). Suffice it to say that forecasting future world commodity prices has historically been very difficult.

Finally, one might also suppose that the relative amounts of irrigated and rainfed rice area will affect the demand for N fertilizer. Holding constant the level of production, however, the relative amount of irrigated area will only affect aggregate N use on rice to the extent that rice production per unit N (the partial factor productivity, or PFP of N) is different for irrigated and rainfed systems. Comprehensive statistics on

the PFP-N in different ecosystems are lacking in most countries, but there are such data for the Philippines. For irrigated rice, the PFP-N is roughly 53 kg paddy kg^{-1} N, while in the rainfed system it is only 43 kg paddy kg^{-1} N. Thus, the larger the share of irrigated area in total area, the lower will be the total demand for N fertilizer on rice, holding the level of production constant. This may be somewhat counterintuitive because irrigated farms use more N per land area. But irrigated farms also produce considerably higher yields, so the total N requirement to produce a given quantity of rice is less on irrigated land than on rainfed land.

A quick calculation shows that this effect is probably exceedingly small, however. During the period from the late 1970s to the early to mid- l990s, the share of irrigated rice area in total rice area in Asia increased from 51% to 55% (Huke and Huke l997), despite the slowdown in growth of irrigated area. If the share of irrigated area were to increase to 60% by 2020 (and this is by no means certain due to increasing competition for water), then the figures for PFP cited above would imply a reduction in N use at constant levels of production by just about 1% relative to a situation where the share of irrigated area remained at 55%. Such a small change over such a long period of time is truly inconsequential.

Potential benefits of biological nitrogen fixation

Biological nitrogen fixation (BNF) may generate benefits because of higher yields, lower use of fertilizer N, or both. Whether or not BNF can generate higher yields is highly speculative at this point. Such yield increases could potentially be due to improved congruence between plant N demand and supply, resulting in an enhanced C sink and photosynthesis. If BNF does give higher yields, the benefits to farmers and society are unambiguous and potentially very high. On the other hand, BNF may also give rise to a yield penalty because the energy requirement of nitrogen fixation may necessitate translocation of some carbon away from the grain. This concern may be mitigated to some extent because rice is low in protein. This implies that much lower rates of nitrogen fixation will be required (relative to legumes), with correspondingly less drain on the plant's photosynthates (Reddy and Ladha 1995).

BNF, if realized in rice without a yield penalty, will certainly reduce the amount of mineral N fertilizer required to achieve the high yields necessary to feed tomorrow's growing population. This will bring at least two classes of benefits: financial benefits to farmers and environmental benefits to society at large.

The potential financial benefits to farmers may not be particularly important, however, especially if there is a yield penalty as discussed above. For example, if BNF were to reduce use of mineral N fertilizer by half, then based on the figures cited earlier for N fertilizer costs as a share of production value, this would lead to increased profits of about 2.0–3.5% of the value of production. If the yield penalty exceeds this figure, then BNF would not be profitable for farmers. Thus, in the irrigated ecosystem, where yields average about 5 t ha^{-1}, farmers would be unwilling to tolerate a yield loss of even 200 kg ha^{-1}, even if they were able to save 50% on their fertilizer costs! In the rainfed ecosystem, the absolute ceiling on the acceptable yield

penalty would be even lower because fertilizer costs as a share of production value are roughly comparable with those on irrigated farms while yields are considerably lower. For example, a farmer achieving a yield of 2.3 t ha^{-1} (a typical yield for rainfed farmers) would be able to tolerate a yield penalty of at most 80 kg ha^{-1}! Some farmers may benefit by not having to take out expensive loans to pay for fertilizer, but again this benefit will be of little value if there are even small yield penalties due to BNF.

The environmental benefits of BNF in rice are likely to be more substantial than the financial benefits to farmers. In many areas, the share of water pollution caused by nitrogen runoff from fertilizers is very high. Thus, a 50% reduction in the use of mineral N fertilizer would have significant health, environmental, and economic benefits such as an improved quality of drinking water and restoration of habitats for aquatic life. Furthermore, there would be a key advantage in that these environmental benefits could be realized without the need for enactment of legislation, which is often politically complex, or without the need for enforcement of laws, which is especially difficult in poor countries with millions of small farmers. Instead, as farmers plant the new seeds that allow the rice plant to fix atmospheric nitrogen, they will have incentives to reduce fertilizer use on their own without any need for government action. This scenario assumes that there is no yield penalty because of BNF.

If BNF does suffer from a yield penalty, this will seriously hinder adoption in the near future. Furthermore, the environmental benefits may also be reduced if there is a yield penalty, as farmers may compensate for the lower yields by increasing the total N used by the plant (i.e., the reduction in mineral N application will be less than the quantity of N supplied by BNF). Or, lower yields because of BNF may encourage larger areas to be planted to rice so as to keep production levels constant (if this does not happen, then rice prices will rise, with adverse effects on consumption by the poor, who cannot afford the higher prices). In the longer term, however, if the poor countries of Asia prosper and poverty rates continue to decline, politicians and society may be willing to accept a yield penalty in exchange for significantly reduced nitrogen loads into the environment. When that occurs, BNF in rice could play an extremely important role in improving the environment because rice occupies so much land area and rice farmers use so much nitrogen fertilizer.

The type of BNF that is achieved will also have an important effect on benefits. After several years of key research, it appears that nonnodular BNF is likely to have very low potential in terms of the amount of N_2 fixed relative to the potential of nodular association or *nif* gene transfer to the plant. Some debate remains whether or not it will be possible to achieve nodular BNF in a rice plant that can prosper under flooded conditions, though some legumes in nature are known to have functional root nodules under flooded conditions (Ladha et al 1990). Stem nodules may also be able to support BNF in rice grown under flooded conditions. Either of these two developments would allow BNF to be used over a wider area because most rice is either irrigated or grown in rainfed environments where flooded conditions prevail for a substantial part of the growth period. Upland rice accounted for just 13% of world rice area in 1991 (IRRI 1997) and the area planted to upland rice in Asia decreased substantially from the late 1970s to the early 1990s (Huke and Huke 1997). If upland

rice area continues to decrease and BNF cannot be adapted to irrigated and rainfed rice, the application domain will be severely restricted in the future. These considerations suggest that future research should focus on BNF that can be achieved under flooded conditions.

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Retrospective on biological nitrogen fixation

Robert H. Burris

In 1838, Boussingault reported that leguminous plants fixed atmospheric nitrogen, but it was not until the convincing 1888 report of Hellriegel and Wilfarth that the validity of this concept was generally accepted. The biochemistry of the process was not investigated seriously until the 1930s when the influence of nitrogen and oxygen pressures and the inhibitory action of hydrogen were established. The application of ^{15}N as a tracer in the 1940s indicated that ammonia is the key intermediate in fixation. Achievement of consistent cell-free nitrogen fixation in the 1960s substantiated the role of ammonia and showed that both an Fe protein and a MoFe protein are involved in the fixation process with energy derived from ATP. In 1992, the tertiary structure of the MoFe protein was established. Recent investigations have emphasized genetic aspects of the nitrogenase system.

Biological nitrogen fixation has been with us for a long time, and was exploited for centuries without any recognition of how it was functioning. The Romans practiced mixed cropping with legumes and nonlegumes following their observations that such a practice enhanced the productivity of the nonlegumes. Probably the first person to really demonstrate clearly what was going on was Boussingault (1838). He reported that in a mixture of legumes and nonlegumes the total nitrogen of the plants increased, and he suggested that the legumes were fixing nitrogen from the air for use by the plants. This explanation of observations by a Frenchman did not conform to a German interpretation by Liebig, and Liebig pronounced that the nitrogen increased in the legumes merely because they had larger leaves to absorb ammonia effectively from the air. Of course Liebig did not believe it necessary to do any experiments to validate his suggestions because, after all, he was the leading organic chemist of his day, and he had spoken. Not surprisingly, the French did not share his unsupported allegations, so the French Academy set up a commission to test whether Boussingault or Liebig was right.

Experiments by committee do not necessarily go smoothly, and the committee had its problems in this case. The committee members decided they had to do things

properly, so they painted a big metal sheet to provide a clean base for their experimental pots. The young plants in their pots were placed on the sheet, and fumes from the paint killed them promptly. So the committee reconvened, evaluated the situation, and started a new experiment distanced from fresh paint fumes. The plants prospered and, not surprisingly, the French commission concluded that their colleague Boussingault was correct in his conclusions that the legumes increased in nitrogen content, whereas the nonlegumes did not, and that the German position of Liebig was incorrect. Skeptics remained, however, and questions about the validity of nitrogen fixation by legumes were raised periodically.

A group at the Rothamsted Experiment Station in England decided that they should do the crucial experiment to put the argument to rest. So Lawes, Gilbert, and Pugh (1861) cleaned their pots meticulously, heated their soil to a high temperature, and planted their legumes and nonlegumes. At the end of the growth period, they analyzed not only their plants and soil, but even broke up the pots and analyzed them for nitrogen. Their precautions were apparently so effective that they destroyed all the rhizobia. To most people, their carefully executed experiments furnished a definitive answer, but as always a few skeptics raised questions and performed their own experiments. In the United States, Atwater (1885) ran experiments at the Connecticut Experiment Station that indicated positive fixation by legumes.

It is interesting, however, that the definitive experiments supporting fixation came not from the United States or France but from Germany, the home of Liebig. In 1886, a meeting took place in Germany that interestingly enough was chaired by Gilbert of the Lawes, Gilbert, and Pugh experiment at the Rothamsted station. Here Hellriegel and Wilfarth (1888) reported that they had observed nitrogen fixation by peas, but that fixation was inconsistent in their early experiments. When the peas fixed nitrogen, however, they were always nodulated. The root nodules were loaded with bacteria and, when these bacteria were placed on pea seeds at the time of planting, all the pea plants were nodulated and grew vigorously without added nitrogen. The experiments were very convincing.

It was several years before the Rothamsted group repeated the Hellriegel and Wilfarth experiment, but, when they did, they verified the findings and established the validity of nitrogen fixation in leguminous plants (Lawes and Gilbert 1892). After the Lawes, Gilbert, and Pugh experiments in 1861, Pugh had gone back to Penn State University. We don't hear much about his experiments there. This is not surprising, however, because he got mixed up in administration and became the president of Penn State University. If you visit Penn State today, you will find a Pugh Street named in his honor, so he is not forgotten. This reminds me of a more recent and perhaps even more impressive honor. After Stanley B. Prusiner received the Nobel Prize for his discovery of prions, he returned to the University of California, where he was signally honored by being given a permanent parking space right on the campus.

Thus it was established that fixation occurred in a symbiotic association between leguminous plants and the root nodule bacteria. Winogradsky (1893) demonstrated that the anaerobic microorganism *Clostridium pasteurianum* was capable of fixing N_2 by itself, and Beijerinck (1901) reported that the aerobic *Azotobacter*

chroococcum likewise could use N_2 as its sole source of nitrogen. Although these free-living organisms had less impact than the rhizobia on practical agriculture, they certainly were much easier to use than the symbiotic organisms in many types of laboratory experiments.

Many investigators tried to get the rhizobia to fix nitrogen as free-living organisms, and many reported positive results. An extensive literature built up on reported fixation by free-living rhizobia. The organisms were grown under a wide variety of conditions on many different media. When someone reported nitrogen fixation. others would repeat the experiments with negative results. At the University of Wisconsin in the Bacteriology Department, E.B. Fred had reported fixation, but his student Lois Almon was embarrassed by her inability to repeat the results of the boss. It was not until 1975 that three groups of investigators (Kurz and LaRue 1975, Pagan et aL 1975, McComb et al 1975) reported reproducible fixation by free-living rhizobia in a single issue of *Nature.* The organisms fixed N_2 if supplied succinate as a substrate under a low concentration of oxygen.

Although early investigators could not get the rhizobia to fix N_2 consistently by themselves, many studies were completed on the culture and physiology of the rhizobia. It turned out that there was a specificity of the rhizobia for host plants, and the organisms were classified on the basis of the plants they were capable of infecting. The rhizobia were placed in cross-inoculation groups, e.g., *Rhizobium trifolii* included organisms that could nodulate the true clovers, and *R. japonicum* could nodulate soybeans. Not all rhizobia that infected a given plant were equally effective in nitrogen fixation, and this was referred to as strain variation. Likewise, there was a difference of effectiveness of a single bacterial strain; this was called host-plant specificity.

When it was recognized that the rhizobia could fix nitrogen from the air to nourish the legumes, it became apparent to farmers that one should be certain that the rhizobia were present to infect their legumes. As one could not be certain that the soil would supply a reliable inoculum, production of inoculants for leguminous crops became a commercial operation. This is not to say that commercial inoculants were always effective. In fact, many of them were produced by unreliable operators and were of no value. This led to testing of inoculants by state agricultural agencies in the United States. Any company whose inoculants proved ineffective in tests one year could expect poor acceptance of their inoculant the following year. Liquid and agar cultures were sold, but these were largely replaced by inoculants grown in liquid culture and then transferred to a peat-based powder. These stayed moist and retained good viability.

So the early years of the 20th century were spent in isolating rhizobia and studying their cultural characteristics and applicability as inoculants. There was little interest in the biochemistry of biological nitrogen fixation. Dean Burk studied in Germany with Meyerhof and published some of the first biochemical studies on fixation (1928). His measurements were not very accurate, but he got the studies going with free-living N_2 fixers.

Perry W. Wilson came to the University of Wisconsin as a graduate student in bacteriology and studied with E.B. Fred and W.H. Peterson. After joining the staff of

the Department of Bacteriology, he began studies on the biochemistry of nitrogen fixation. Rather than using the free-living N_2 fixers, he investigated symbiotic N_2 fixation, particularly in red clover. The symbiotic system posed many problems not encountered with the free-living fixers. The clover plants had to be grown under bacteriologically controlled conditions and, for studies of the influence of nitrogen, oxygen, and carbon dioxide, they had to be grown in closed chambers. Clover seeds were surface-sterilized, germinated aseptically in petri dishes. and transferred to sterile 9-L serum bottles that carried a sand substrate with a nitrogen-free nutrient solution. These stoppered bottles could be evacuated and then filled with a gas mixture that would allow testing of the effect of the pN_2, the PO_2, or the pH_2. The photosynthesizing red clover plants signaled that they had to be supplied CO_2 when a cresol red indicator in 0.001 M sodium bicarbonate in a suspended vial in the bottle turned color. With this rather demanding experimental setup, Wilson was able to establish that the pN_2 supporting half-maximum fixation (Michaelis constant for fixation) was about 0.05 atm N_2. Helium was used as the diluent gas to bring the pressure in the bottles to one atmosphere.

On an occasion when the supply of helium was depleted, hydrogen was used as the diluent gas on the logical assumption that it would be inert in the nitrogen-fixing reaction. When the inoculated red clover plants were harvested, those under an atmosphere of hydrogen were all stunted relative to comparable plants without a hydrogen atmosphere. The first guess was that this probably could be attributed to an impurity in the tank H_2. But other tanks of hydrogen gave the same response. So H_2 was generated with zinc and HCI, with aluminum and NaOH, and electrolytically on the assumption that these certainly would not carry the same toxic impurity. All were inhibitory, so the conclusion had to be accepted that the H_2 per se was an inhibitor of biological nitrogen fixation (Wilson 1940). Study of its inhibitory properties established that it was a specific competitive inhibitor. Many years later, Guth and Burris (1983) showed that H_2 inhibits N_2 fixation in an ordered sequential reaction in which H_2 must bind to nitrogenase before N_2 binds.

Although evidence in support of specific intermediates in biological nitrogen fixation was not pushed vigorously in the 1930s, Winogradsky (1930) had suggested that fixation first formed ammonia. His conclusions were based on studies with free-living N_2 fixers, and he reported that azotobacter released ammonia into the culture medium. As this could also arise from decomposition of organisms in the medium after an extended period, it was not taken as definitive evidence in favor of ammonia as the first product of nitrogen fixation. A.I. Virtanen in Helsinki, Finland, had developed a method for ensiling legumes, such as clover and alfalfa, that were rather low in carbohydrate. This was a great boon to the dairy industry in Finland, and Virtanen was furnished with a well-supported laboratory. In his research on legumes, he observed that some legumes excrete nitrogen into the soil. His isolation of these products led him to conclude that aspartic acid was a major excretion product, and from this he argued that the first product of N_2 fixation was hydroxylamine. He postulated that hydroxylamine combined with oxalacetic acid to form the corresponding oxime,

and that the oxime in turn was reduced to aspartic acid. To support this hypothesis, he reported the presence of oxalacetic acid in his plant material.

Other investigators had difficulty embracing the hydroxylamine hypothesis because they could not get their plants to excrete aspartic acid. P.W. Wilson was among those interested in the first product of nitrogen fixation, but he was unable to get his legumes to excrete aspartic acid. He went to Helsinki and set up experiments in Virtanen's greenhouses. Virtanen attributed the lack of demonstrable excretion in their joint experiments to the unfavorable fall and winter weather. So, when Wilson returned to his Wisconsin lab, he brought back samples of Virtanen's seed, his inoculant strain of the rhizobia, and even a large container of virtanen's soil. The reconstructed system did not excrete in Wisconsin. As Virtanen insisted on the importance of seasonal variation, Wilson established his Marathon experiments. These required that his graduate students set up a new excretion experiment at the beginning of each month to observe whether seasonal variation really was definitive for excretion. Peas at Wisconsin refused to excrete nitrogen; they held on to it tenaciously to support their growth. The evidence in support of the hydroxylamine hypothesis was rather indirect, and several labs other than Wilson's lab were unable to repeat the Virtanen experiments. Virtanen summarized his work in this field and on silage production in his book *Cattle Fodder and Human Nutrition* (1938).

I enjoyed seven weeks in Virtanen's lab in the summer of 1954. Virtanen was a very gracious host, and he had a very helpful and energetic lab group. We were no longer arguing about nitrogen excretion, but some time earlier he had published a short paper in *Suomen Kemistilehti* questioning our report that nitrous oxide was a specific and competitive inhibitor of nitrogen fixation (he claimed it inhibited nitrate use). His report with Sinikka Lundbom was so brief that we were unable to repeat his experiments without more details about how they were run. So, after arriving in Virtanen's lab. I asked Sinikka how the experiments were done, and she gave me the details. They had set up the experiment with *Azotobacter vinelandii* as their nitrogen fixer, had put it under various atmospheres with and without nitrous oxide, and had measured the N content and nitrate of the cultures after six days. I said, "Your oxygen would be depleted by this rapidly respiring organism after about a day, so what you were measuring after six days was an oxygen effect in your various atmospheres and this had nothing to do with an effect of nitrous oxide." I asked who had designed the experiment, and she said, "The professor." When I said, "Let's go talk with him about it," she said, "Oh, no. My voice even changes when I talk with the professor." That apparently was a side effect of Virtanen being the only Nobel laureate in Finland. In any case, we repeated the experiment with proper control of the pO_2 and verified with three independent methods of analysis (respirometry, total N analysis, and mass spectrometry with ^{15}N as a tracer) that nitrous oxide was a specific competitive inhibitor. Virtanen published the results (Mozen et al 1955). Later he repeated the experiment with his original faulty methodology and got his original faulty results. These results he published as a retraction of his retraction. I often cited this to my students as a method to enlarge your publication record not by one but by three papers. But Virtanen

was a very pleasant fellow whose AIV silage[1] did great things for Finnish agricultural production. As a person who never enjoyed foreign languages, I was greatly impressed with his ability to deal with seven languages.

As indicated, the hydroxylamine hypothesis of nitrogen fixation never had strong backers outside Virtanen's lab, but evidence supporting other suggestions was also weak. Wayne Umbreit in Wilson's lab and I often talked about the potential application of ^{15}N as a tracer in biological nitrogen fixation. The only report of isotope work in the literature was a short paper by Ruben, Hassid, and Kamen (1940) in which they used radioactive ^{13}N as a tracer and reported that the nonlegume barley fixed nitrogen. ^{13}N has a half-life of about 10.5 minutes, and the experimental exposure of the barley was 20 minutes. There were reports in the literature that nonlegumes such as barley fixed nitrogen, but, as these results were highly questionable, the tests with ^{13}N as a tracer were also inadequate to convince everyone that barley fixed N_2. Schoenheimer, Rittenberg, and colleagues had used ^{15}N as a tracer to establish clearly the dynamic state of proteins in the body, but investigators had not applied ^{15}N in studies of biological nitrogen fixation. As this seemed a logical application of this stable isotopic tracer, I wrote up a proposal to the National Research Council and was awarded a postdoctoral fellowship to test ^{15}N as a tracer in fixation studies.

Professor Harold Urey had concentrated ^{15}N by an exchange reaction between ammonium ion and ammonia gas, and he had supplied the ^{15}N-enriched material that other investigators had used in their studies. I showed up in Urey's lab at Columbia University in the fall of 1940 and explained the proposed application of his tracer material. He grasped the possibilities immediately and suggested that my first experiment should be to test whether an exchange reaction would complicate or invalidate the use of ^{15}N as a tracer in fixation reactions. He indicated that the way to test for exchange would be to run the fixation test under a nonequilibrium mixture of the molecular species of N_2, and he pointed out that one could produce a nonequilibrium mixture by mixing N_2 gases with different concentrations of ^{15}N. If you take normal N_2 and mix it with isotopically enriched N_2, you will produce a nonequilibrium mixture of $^{14}N^{14}N$, $^{14}N^{15}N$, and $^{15}N^{15}N$. If an exchange reaction occurs that breaks these NN bonds, they will reassemble into a mixture that more closely approaches equilibrium, as can be established readily by mass spectrometric measurement.

So, Miller, a student in the lab, and I generated N_2 from ^{15}N-enriched material, mixed it with normal N_2 to give a nonequilibrium mixture, and exposed N_2-fixing *Azotobacter vinelandii* under the mixture. The organisms fixed well, but there was no equilibration of the N_2 toward an equilibrium distribution of the molecular species

[1] Arturri Ilmari Virtanen (AIV) found that, by adding inorganic acids such as sulfuric or phosphoric acids to ground alfalfa at the time of ensiling, he could preserve it as forage, and it was acceptable to cattle. This allowed year-round production of milk, and hence year-round production of cheese (primarily "Swiss" cheese), so this AIV silage was of great importance to Finnish farmers.

(Burris and Miller 1941). I reported to Professor Urey that there seemed to be no exchange reaction. He was delighted with this result not only because I could use ^{15}N as a valid tracer in the projected experiments but also because he could use the results to kid Hugh Taylor at Princeton University. Hugh Taylor was a distinguished physical chemist who had asked Urey for some ^{15}N so that a postdoctoral student (incidentally, a student in physical chemistry from Wisconsin) could run some experiments on the exchange reaction in N_2-fixing organisms. It tickled Urey that he could tell Taylor to start thinking up a new postdoctoral project because the reaction he was proposing to study didn't exist.

Because we also wanted to check the report by Rubin and others that barley fixed $^{13}N_2$, we rigged up an apparatus so that barley and red clover could be exposed simultaneously to $^{15}N_2$ in an interconnected vessel with the plants separated only by a cotton plug barrier. Instead of exposing them for 20 min as in the ^{13}N experiments. we exposed them for 42 and 56 d. When the experiment ended, the clover had a generous enrichment in ^{15}N, whereas the barley had no detectable enrichment above normal abundance (Burris 1941).

We thought that the distribution of ^{15}N in various products from a N_2-fixing organism might give some hint of the first product of fixation. Schoenheimer (1942) and colleagues had followed details of N metabolism in animals with ^{15}N as a tracer and had developed methods for recovering a variety of amino acids. I exposed a rather large culture of *A. vinelandii* to ^{15}N-enriched N_2 for 4.5 min, recovered the cells, and hydrolyzed them with acid to release the constituent amino acids. The people in Schoenheimer's lab helped me to recover solid derivatives of the amino acids from the hydrolysate so that their ^{15}N concentration could be determined mass spectrometrically (Burris 1942). The highest ^{15}N concentration among the amino acids was in glutamic acid, not in aspartic acid as would be required by Virtanen's hydroxylamine hypothesis. The high level in glutamic acid was compatible with the participation of ammonia as the first product of fixation, as ammonia could be combined with a-ketoglutaric acid and then reduced to glutamic acid by glutamic dehydrogenase. Actually, the assimilation of the ammonia is via the glutamate-oxoglutarate aminotransferase (GOGAT) system, a system unknown at the time of the experiment. In subsequent comparable experiments in the following years, it was demonstrated that essentially the same ^{15}N distribution occurred in *Clostridium pasteurianum, Rhodospirillum rubrum,* soybean nodules, *Bacillus polymyxa,* and *Mastigocladus* laminosus that had been exposed to ^{15}N, for a short period. All the data were therefore compatible with the idea that the initial product of N_2 fixation was ammonia.

Because the early report by Rubin, Hassid, and Kamen that barley fixed ^{13}N-enriched N_2 proved incorrect, when Kamen and Gest observed that *R. rubrum* fixed N_2, they thought it would be advisable to check their results with $^{15}N_2$. So they put a bottle of the growing culture of the organism into an illuminated box, boarded a train, and plugged the light-box into the only electrical outlet in the men's room of the train. They arrived in Madison (Wisconsin) about noon and by about 1:30 p.m. we had their *R. rubrum* culture under ^{15}N-enriched N_2. After a few hours, we harvested the culture, recovered the cells, digested them, and distilled off the ammonia. This was converted

to N_2 and analyzed mass spectrometrically the next day. The N2 recovered was clearly enriched in ^{15}N, so Kamen and Gest (1949) had added the photosynthetic bacteria to the list of free-living N_2-fixing organisms.

A variety of experiments had been supportive of ammonia as the "key intermediate" in N_2 fixation, that is, the compound that marked the end of the fixation process and the beginning of assimilation of the fixed nitrogen. But many investigators wanted more direct evidence of the role of ammonia, and a logical approach to this seemed to be to develop cell-free preparations from bacteria that would effect fixation without the complications of the metabolism of the intact cell. There had been attempts to achieve cell-free fixation in the past, and Bach, Jermolieva, and Stepanian (1934) in Russia had reported success in getting cell-free preparations of the azotobacter to fix N_2. This observation was of such importance that it induced Dean Burk of the Fixed Nitrogen Laboratory in Washington to travel to Russia to find out how to run the experiments. Unfortunately, while Burk was in Bach's lab, neither Burk nor any of Bach's group could repeat the demonstration of cell-free fixation. ^{15}N as a tracer offered a specific and sensitive means to repeat the earlier attempts to demonstrate cell-free fixation. Sometimes, we could show cell-free fixation with ^{15}N, but fixation was not consistent. As we could measure an increase in 0.003 atom % ^{15}N excess with the mass spectrometer, we accepted five times this value, or 0.015 atom % ^{15}N excess, as a positive indication of fixation. Our fixation was often much better than this, and Hoch and Westlake (1958) reported values of 0.823, 0.929, and 1.251 atom % ^{15}N excess in extracts from *C. pasteurianum* exposed for 1 h to 95 atom % ^{15}N, at a pN_2 of 0.05 atmosphere.

Carnahan et a1 (1960), from the DuPont labs, reported consistent fixation with cell-free preparations from *C. pasteurianum* that had been dried at about 40 "C in a rotary evaporator. They supplied generous levels of pyruvate to the extracts and operated anaerobically with this organism. The investigators reported that added adenosine triphosphate (ATP) was inhibitory to fixation. The important point was that the results were consistent, and we reproduced them in our first trial with their method (Schneider et a1 1960).

When we obtained consistent fixation with cell-free preparations from *C. pasteurianum,* it was then possible to look for the early products of fixation without the confusion of the many reactions in the intact cell (Schneider et al 1960). We exposed the cell-free preps to ^{15}N-enriched N_2 and after 3 h we found 52.06 atom % ^{15}N excess in the ammonia recovered. As this was a far higher concentration than in any other product recovered, it gave substantial support to our contention that ammonia was the first demonstrable product of biological nitrogen fixation.

Despite the report that ATP was inhibitory to biological nitrogen fixation in cell-free preparations, Jack McNary (McNary and Burris 1962) thought that he should check this out himself, and he concluded that, far from being inhibitory, it was an absolute requirement for fixation. His report met with a surprising amount of skepticism and was challenged frequently until the challengers actually repeated McNary's work carefully and then embraced the concept.

The possession of a consistent cell-free preparation opened the field to purification of the nitrogenase enzyme system. Mortenson (1965) reported that the enzyme consisted of more than one component, and it soon became apparent that there were in fact two components. Because several labs engaged in purification studies, it is hard to say who should get credit, as improvements in the preparations were often in parallel among labs. Names for the two components of the nitrogenase proliferated, but fortunately most of these have now faded from use. We prefer the term "nitrogenase" for the complete complex, "dinitrogenase" for the MoFe unit that binds and reduces the N_2, and "dinitrogenase reductase" for the Fe unit that transfers electrons to the MoFe unit. Component 1 or protein 1 is often used to designate the MoFe protein, and component 2 or protein 2 for the Fe protein. The abbreviation Cp2 can be used conveniently to designate the Fe protein of *C. pasteurianum,* etc.

Nitrogenase is not limited to N_2 as a substrate; in fact, it is a remarkably versatile enzyme (Burris 1991). It was recognized that nitrogenase could produce H_2 from H^+. but the first substrate other than H^+ and N_2 to be reported was N_2O. This was followed by the indication that cyanide and methyl isocyanide could also serve. Schollhorn observed that azide and acetylene were reduced by nitrogenase (Schollhorn and Burris 1966), and quite independently Dilworth also observed acetylene reduction (Dilworth 1966). Cyclopropene, cyanamide, and diazarine can also serve as substrates. Most of these substrates have been only laboratory curiosities, but the reduction of acetylene to ethylene has developed into a useful tool in the study of nitrogenase. Ethylene is easily detected at low concentration by gas chromatography, so its formation can be followed in the laboratory or field with relatively simple equipment. The acetylene reduction assay for nitrogenase has become a standard method for studying many aspects of nitrogen fixation.

It had been accepted since the 1930s that molybdenum was needed for biological nitrogen fixation, but it was unclear how it functioned. When Brill's group started investigating the genetics of the system, Shah and Brill (1977) found that they could extract an iron-molybdenum component from the system, and they named this unit FeMoco, or iron-molybdenum cofactor. They succeeded in extracting FeMoco and reinserting it to reactivate the nitrogenase system. Subsequently, Shah et al (1994) achieved the in vitro synthesis of FeMoco and purified and characterized the NifB-cofactor.

There had been hints that other metals might function in nitrogenase. and Bishop and coworkers (1980) found that vanadium-iron and iron-iron centers might also serve in place of FeMoco. This was another report that was denied by other research groups until they conducted careful tests on their own and found that Bishop was right.

Few question the predominant importance of symbiotic nitrogen fixation in leguminous plants for practical agriculture. But for the lab investigator, free-living organisms such as the azotobacter and clostridial groups are most attractive for research because they grow rapidly and are relatively easy to culture and control. The symbiotic system of nonleguminous plants plus actinorhizal organisms occupies several important niches in ecosystems. In our base in Wisconsin, the alder and its actinorhizal associate are dominant along our trout streams. *Comptonia peregrina* is

found in our sandy Lake County. In many arid countries, trees with the actinorhizal system are used for plantings along roadways and as windbreaks because they do well with a limited supply of water. Several of the dominant shrubs of the western U.S. desert bear actinorhizal nodules. This group of plants was given little attention because of the difficulty of culturing the actinomycetes from the root nodules. After Baker, Torrey, and Kidd (1979) reported the culture of these actomycetes, interest in the group developed rapidly.

Another group of fixers that have sparked interest relatively recently are the so-called associative nitrogen fixers. These do not form nodules on roots, but they grow on or in the roots, fix N_2, and furnish some fixed nitrogen to the plant. Beijerinck (1925) described *Spirillum lipoferum* and reported that it could fix nitrogen. Interest in this organism died when Schroder (1932) reported that the cultures were contaminated. Becking (1963) once more isolated *S. lipoferum* (now known as *Azospirillum lipoferum*) and reported that pure cultures were able to fix N_2. Reports by Okon and colleagues (1988) gave evidence that *Azospirillum* in association with roots of some nonlegumes enhanced their growth. After a burst of activity on associative fixation, Okon and others reported that the enhanced growth with *Azospirillum* perhaps was more because the organism excreted plant growth factors than because it excreted nitrogen.

One of the most interesting cases of associative nitrogen fixation is the mutual action between *Acetobacter diazotrophicus* and sugarcane. *A. diazotrophicus* can grow within the sugarcane and fix enough N, so that the cane needs no external addition of nitrogen fertilizer. Boddey and associates (1991) have calculated that this association can fix up to 150 pounds of nitrogen per acre in a growing season. Cane is now grown commonly in Brazil without the addition of nitrogen fertilizer. *A. diazotrophicus* is a remarkable organism. It can grow in the very high sugar concentration in sugarcane, and it is commonly cultured in the laboratory on 10% sucrose. Even more remarkable is its ability to fix nitrogen at low pH. Most bacteria fix nitrogen best around neutrality and cease fixation if the pH drops to 5. But *A. diazotrophicus* fixes down to pH 3, which has 100 times the H ion concentration than at pH 5.

As indicated, ATP is required for biological nitrogen fixation. Under ideal conditions, 16 ATP are required for each N_2 fixed (Winter and Burris 1968), but under real conditions the requirement is probably between 20 and 30 ATP per N_2 fixed. This is a very high energy demand, and many nitrogen-fixing organisms have devised schemes to save energy by turning off nitrogen fixation when it is not needed. The best-defined system is one discovered by Ludden when he was studying fixation in the photosynthetic bacterium *R. rubrum* (Ludden and Roberts 1989). Cell-free nitrogen-fixing preps had been made from many organisms, but preps from *R. rubrum* were inconsistent. Ludden found that the trouble was that the organism turned fixation off when darkness, during the preparation of extracts, robbed it of its abundant energy source from light. In other organisms, the addition of ammonium ion or another readily available nitrogen source turned fixation off, and fixation resumed when the fixed nitrogen was exhausted. Ludden and colleagues established that there was an inactivating enzyme in *R. rubrum* and several other nitrogen-fixing organisms.

They termed the switch-off enzyme DRAT (dinitrogenase reductase ADP-ribosyl transferase) and termed a reactivation enzyme DRAG (dinitrogenase reductase-activating glycohydrolase). Inactivation was effected by adding an ADP-ribosyl group to arginine 100 of dinitrogenase reductase, and reactivation was effected by removing this group with the glycohydrolase.

In the nitrogenase reaction, electrons can be passed to N_2 to form NH_3, or they can be passed to hydrogen ions to form H_2. An increase in the pN_2 will favor production of NH_3 at the expense of production of H_2. The question was often raised whether at an extremely high pressure of N_2 the production of H_2 would be completely suppressed. Nobody seemed to want to rig up an experiment to test this possibility. Finally, Frank Simpson built a "bomb" with 24-karat gold seals that he could pressurize to more than 50 atmospheres (Simpson and Burris 1984). When he placed a cell-free nitrogenase prep in the chamber together with ATP and other needed reactants and ran fixation at a pN_2 of 50 atmospheres, he found that 27% of the electrons were still used for H_2 production despite the very high pN_2. Extrapolation of the data indicated that, at infinite pN_2, 25% of the electrons would still be used for H_2 production rather than for N_2 fixation.

It was not until 1992 that the tertiary structure of nitrogenase was established, when Kim and Rees (1992a,b) published the structure in *Nuture* and *Science.* The structure indicated was of the dinitrogenase, that is, the subunit that includes the FeMoco center. The structure of dinitrogenase reductase must fit to dinitrogenase to form the nitrogenase complex that allows electron transfer between the Fe and MoFe units.

Recent years have seen research emphasize the genetics of the nitrogenase system. As both the Fe protein (dinitrogenase reductase) and the MoFe protein (dinitrogenase) are involved in fixation as well as in auxiliary systems, it is apparent that the genetics can become complicated. More than 20 genes are involved with the nitrogenase system. The structural genes are *H, D,* and *K,* with *H* defining dinitrogenase reductase and *D* and *K* coding for dinitrogenase. The roles of many of the genes for nitrogen fixation have now been defined, and many genes have been modified in studies of their specific roles in the formation and function of the nitrogenase system.

Now back to the theme of the week! How do we make rice fix nitrogen? Rice has had a few million years to devise a system without accomplishing the feat, whereas legumes plus rhizobia have worked it out, as have the actinorhizal systems. The associative nitrogen fixers and the free-living nitrogen fixers also offer us models. They have much in common as far as the enzymology goes—participation of Fe, Mo, and V and using ATP for their energy source. But the systems are all labile to oxygen, so how do we get the high levels of ATP needed without oxygen to interfere? Anaerobic operation requires quantities of substrate that are prohibitive in a productive system. The symbiotic systems have developed the clever trick of using hemoglobin with an affinity for oxygen high enough to keep it from inactivating the O_2-sensitive nitrogenase but still providing adequate O_2 to support the ATP-generating respiratory system. Blue-green algae have developed heterocysts that shield the nitrogenase from the adjacent photosynthesizing cells that are generating O_2 while they capture energy

from light. The azotobacter apparently respire at such a rapid rate that they keep the pO_2 under control, but this is very wasteful of energy. The roots of rice growing in water may be at a pO_2 compatible with N_2 fixation, but, if the N_2 fixers are outside the roots, the products of N_2 they fix may be washed away, so we must devise a way to get the fixing organisms to thrive inside the roots while the plant provides them with adequate substrates to support fixation and provides them with a pO_2 adequate to support fixation but not high enough to inactivate their nitrogenase system. The fact that sugarcane plus *A. diazotrophicus* can fix adequate N for cane growth is encouraging, so maybe we should generate a high-sugar rice. Of course, we will do this at the expense of the abundant starch that we want the rice to deposit in its seed.

It doesn't appear that the solution to the problem of rice fixing its own nitrogen will come easily. But I have now been retired for 15 years, so I'll let you solve it.

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Frontier Project on nitrogen fixation in rice: looking ahead

Kenneth S. Fischer

Rice is the world's most important food, particularly for the poor. The future need for rice (more than a 30% increase by 2020) to be grown with less land in the intensive rice areas, and with new technologies in the rainfed systems, demands that science explore new frontiers.

IRRI has begun a set of Frontier Projects that explore the feasibility of applying new knowledge and methods to transfer novel traits from other crops to rice. One of these projects is "Nitrogen Fixation in Rice." In 1992, a think-tank workshop concluded that IRRI, working closely with advanced research laboratories, should explore the feasibility of N_2 fixation in rice in a few well-defined approaches: nonnodular symbiosis, nodular symbiosis, transferring *nif* genes, and CO_2 fixation and N-use efficiency.

A BNF (biological nitrogen fixation) working group for rice was formed to monitor research progress and the free exchange of information and materials. Significant scientific progress has been made in providing new knowledge. Yet this alone is not enough to maintain the project. The Frontier Projects must be periodically assessed on how such new knowledge has furthered our probability of success in meeting the long-term goal. This chapter describes the rationale for IRRI's support of these projects with a long-term, ambitious, and problematic outcome.

Rice is the staple food for nearly half the world's people, most of them living in Asia, many of them among the poorest in the world. The rice research community, including the International Rice Research Institute (IRRI), has been successful, so far, in helping provide this staple food to expanding populations. But alarming indications of poverty and malnutrition, unabated environmental degradation, and high population growth put pressure on the thin margin between the crop's supply and demand. Rice scientists continue to believe that they can find ways to grow enough rice for the expanding population for the coming decades, sustain higher rice production, and maintain the natural resource base and protect the environment.

Present trends suggest that tomorrow's rice lands will be under even more pressure than today. In Asia, total demand for cereals will increase 30% by 2020. Diversi-

fication of current rice fields to other crops will also increase. Tomorrow's technologies and management practices must produce more rice on less land in the intensive lowlands, increase rice productivity of the rainfed lowlands where most of the rural poor live, and develop productive and sustainable systems for the uplands to avert overexploitation of the soil and water resource base in the lowlands.

New Frontier Projects

In ecosystems of Asia—intensive irrigated lowlands, extensive rainfed lowlands, and the vulnerable uplands—rice is the dominant crop. New technologies based on sound economic and ecological principles need to be developed for rice, for the near and distant future. Because of our new knowledge on DNA sequences, gene function, and new biotechnology tools, we can dramatically change breeding in the 21 st century. It may be possible to use many of the discoveries in the basic sciences to solve problems that have proved intractable using traditional breeding methods. Breeders can also contemplate introducing traits that were previously not considered for rice, although they were well known in other crops. Realizing this need, IRRI identified four areas of research of a long-term nature (see IRRI Medium Term Plan 1994- 1998). They are designated as New Frontier Projects, and there are four:

- N_2 fixation in rice, to decrease our dependency on nonrenewable resources and to increase N-use efficiency.
- Apomixis, to make the benefits of heterosis available to poor rice growers.
- Perenniality in rice, to protect the uplands where rice is grown by the poor.
- Biological weed management, to make available an integrated approach to weed management.

IRRI uses the term New Frontier Project to signify a certain type of upstream research. This research is scientifically risky because it enters uncharted territory, but is likely to have enormous impact if successful. IRRI is learning how to recognize such a project, when to start it, and how to build a team of researchers from partner institutes to provide the necessary critical mass of expertise. This research may take 15 years or more to enter the pipeline that carries IRRI's more downstream products and recommendations from the laboratory to national agricultural research systems (NARS) and eventually into farmers' fields.

Initial studies in the Frontier Project aim at exploring the feasibility of achieving the set goal. The success of this initial phase must be judged in terms of whether it clarifies the way we define the problem, whether it clarifies alternative ways forward and discards earlier hypotheses, and whether it creates a sense of scientific excitement to attract the best collaborators to the effort.

A key element of the Frontier Project is that IRRI plays a small but pivotal role in focusing basic research in many laboratories on the needs of rice. IRRI has only a small investment (less than 5% of its research effort) in the Frontier Projects, relying on the collaborative efforts of basic laboratories to undertake most of the research. IRRI has also sought, on behalf of the working groups of these projects, additional funds to facilitate the activities of the groups.

Our stakeholders, including donors, advanced institute collaborators, and, most importantly, the NARS of Asia, have endorsed this new emphasis by IRRI on more basic and upstream research. This is in recognition of four important trends:

1. The growing strength of NARS in applied research.
2. The increasing relevance of basic research to international agriculture.
3. The development of new tools that facilitate the application of basic discoveries to agricultural problems.
4. The emergence of a global network for rice research.

IRRI recognizes that the Frontier Projects are scientifically risky and often explore new areas of basic science. For these projects to be effective, it is important that scientists have an environment where they can make mistakes and are not constrained in their creativity by fear of failure. But equally important to the projects is the capacity to recognize quickly nonproductive areas of research. This requires scientific management that ensures critical and nontrivial experiments to test well-designed hypotheses and experiments that give a clean result. Because the outcome and the time frame for the Frontier Projects are uncertain, it is all the more reason to follow methods that can monitor and reexamine the feasibility of success at defined periods.

IRRI has begun each Frontier Project by convening a "think-tank'' workshop to bring the world's best together to explore the possibility, assess the feasibility, and define the nontrivial experiments required to increase our knowledge base. Each project is being reviewed to reexamine the probability for success. One such project is the assessment of "N_2 Fixation in Rice."

The New Frontier Project "Nitrogen Fixation in Rice"

Why nitrogen fixation in rice?

Nitrogen is the nutrient that most frequently limits agricultural production. Global agriculture now relies heavily on N fertilizers, which are developed using fossil fuel as the energy supply. In the tropics, lowland rice yields 2–3.5 t ha^{-1} using naturally available N derived from biological nitrogen fixation (BNF) by free-living and plant-associated diazotrophs (Watanabe and Roger 1984, Ladha et a1 1993) and from mineralization of soil N (Bouldin 1986, Kundu and Ladha 1994). This system was sustainable for thousands of years while it supported approximately 10 persons ha^{-1}.

For higher yields to support a rapidly increasing population, however, additional N must be applied. In Western agriculture, the rapid use of N fertilizer began in the 1940s (Frink et a1 1999; Table 1). In Asia, it did not take place until the mid-1960s, when the modern rice varieties that could respond to N fertilizer were developed. Today, world use of N is around 80 million metric tons. Although there are opportunities to increase the efficiency of N use in most crops, including rice (Dobermann and White 1999), the question remains: What will be the N requirements to feed the 10 billion people projected by 2070?

Frink et a1 (1999) projected worldwide N use by 2070 to be nearly 2.5 times today's use. Their projections are based on a model with the components of population, gross domestic product (GDP), the proportion of GDP to agriculture, and N use

Table 1. N resources and their use in world agriculture.

Date	Use
2000 BC onward	BNF of rice paddies (supports 10 persons ha–l)
19th century	Nitrate deposits (guano)
20th century	N from air (Haber-Bosch process)
1930	N use: 1.3 Tg N
1940	N use: 3.0 Tg N
1988	N use: 80.0 Tg N
1990	N use: 77.0 Tg N
1995	N use: 80.0 Tg N
2070 (projected)	N use: 180.0 Tg N

Source: Frink et al (1999).

Table 2. Change in components that determine N use by 2070.

Component	Estimated change
Population growth (no.)	0.8%
GDP[a] per person ($ no.$^{-1}$)	1.8%
Crop produced per GDP (t $\$^{-1}$)	–1.0%
N used per crop (N t^{-1})	–0.5%
No. x $ no.$^{-1}$ x t $\$^{-1}$ x N t^{-1}	1.1%
1990	77 Tg N
2070	180 Tg N

[a]GDP = gross domestic product.
Source: Frink et al (1999).

per unit of agricultural output (Table 2). Of the four components of the Frink et a1 (1999) estimate, the one most amenable to change is N efficiency. Under the current methods of growing rice, more than half, and often as much as 70%, of the N applied is lost, thus causing economic (private) and environmental (public) costs. Under today's methods of growing rice, at least a doubling of the 10 million tons of N used today to produce the rice required by 2020 will be needed (IFA-IFDC-FAO 1992, IRRI 1993). Excess N in the global system in its various forms augments greenhouse effects, diminishes ozone levels, promotes smog, contaminates drinking water, acidifies rain, eutrophies rivers, and stresses ecosystems (Socolow 1999). Because of the adverse impact of excess N on the global system, Socolow (1999) argues that management of the food-nitrogen connection should be given as much importance as management of the carbon-energy connection during the Green Revolution, a task that has already been undertaken at the global level.

In addition to these global concerns about the increase in N use, there is also a need to reduce the level of poverty. Fifty percent of the rice lands in Asia are rainfed. They provide the source of food (up to 60% of daily calories) for most of the poor who live in these areas. And Asia has the largest share of the world's poor! Current

rice yields in these rainfed areas are around 2 t ha^{-1}, and these have not benefited from Green Revolution technology. Under these rainfed conditions, many farmers use little or no N fertilizer because of the high risk associated with N-fertilizer use under variable water conditions and high costs to poor farmers, among other factors.

Three basic approaches can be used to increase the efficiency of N use (and reduce the riskiness to poor farmers). One is to increase the yield of rice per unit of N uptake by the plant. Improvements from plant breeding have raised the efficiency of N use from 40 kg grain kg^{-1} N in IR8 to 53 kg grain kg^{-1} N in IR72 (S. Peng, personal communication; Ladha et a1 1998). The second approach is to regulate the timing of N application based on the plant's need (precision farming), thus increasing the efficiency of the plant's use of applied N (Cassman et a1 1998). IRRI is working with many NARS to develop a site-specific management system that can increase N efficiency at the farm level (Dobermann and White 1999). The third approach is to increase the ability of the rice system to fix its own N (Bennett and Ladha 1992, Ladha and Reddy 1995, de Bruijn et al 1995). The third approach is a long-term strategy, but it has large environmental benefits while helping poor farmers. Furthermore, farmers more easily adopt a seed-based technology than they do crop and soil management practices that are associated with additional costs.

Feasibility of a Frontier Project on nitrogen fixation in rice

In 1992, IRRI organized a think-tank workshop to assess the feasibility of (symbiotic) N_2 fixation in rice (Khush and Bennett 1992). The experts attending the meeting concluded that exploratory research would clearly be needed to assess the feasibility of novel approaches and that rice was an excellent plant model system, and they identified four major short- and long-term approaches:

1. Nonnodular associations: improve the associations between rice and N_2-fixing soil bacteria. This includes achieving colonization and invasion of rice roots by suitable diazotrophs.
2. Nodular associations (legume-like symbiosis): lay the foundation for the engineering of rice plants capable of "nodulation." This approach includes identifying compatible rhizobia and varieties of rice and examining the defense response of rice to find ways to avoid responses that would inhibit symbiosis or the N_2-fixation process.
3. Transferring N_2-fixation *(nif)* genes: transform rice with *nif* genes to ensure the expression of nitrogenase, protection of nitrogenase from inactivation by oxygen, and an energy supply for N_2 fixation without compromising yield.
4. CO_2 fixation and N-use efficiency: increase the understanding of nitrogen metabolism in rice and the impact of N_2 fixation on carbon and energy budgets.

Considerable interest and support for this project were generated after the think-tank workshop. Following the recommendations of the think-tank experts, a New Frontier Project, "Nitrogen Fixation in Rice," was developed in 1994 (Ladha et al 1997) and included in IRRI's 1994-1998 and 1998-2001 medium-term plans. The long-term objective of this project is to enable rice plants to fix their own N. This

research project involves a committed group of scientists from several research disciplines and institutes around the world. The project has a working group, through which IRRI facilitates communication among scientists worldwide with active research interests in nitrogen fixation in rice and other cereals. The first and second BNF working group meetings were organized at IRRI (Philippines) and in Faisalabad (Pakistan) in 1994 and 1996, respectively. The exciting papers presented at the first two working group meetings as well as those presented at the current meeting represent a comprehensive picture of achievements made in assessing opportunities for N_2 fixation in rice and other nonlegumes. This work is summarized in the strategy paper "Steps toward nitrogen fixation in rice" prepared for the BNF Project Review Team and Third Working Group Meeting (Ladha and Reddy, this volume).

Notwithstanding these scientific achievements, an important concept of the Frontier Project is that the work be reviewed critically at regular intervals to reassess the feasibility of helping refine the studies to meet the long-term goals. A project of this magnitude carries the risk of failure. But biotechnological innovations may eventually lead to the accomplishment of N_2 fixation in rice. A project of the nature of N_2 fixation in rice therefore elicits diverse views of its probability for success. The following highlights just some of that diversity:

- The Kendall report to the World Bank (Kendall et al 1997)—"At some point in the future, N_2 fixation may be transferred to crops such as corn and rice, but such an achievement must be seen as a far-off goal."
- Ausubel(l986)—"Although there are many potential obstacles in the transfer of functional N_2-fixation genes to cereals, my personal opinion is that cereals will eventually be engineered to fix N_2. I do not see any theoretical reasons why it cannot be accomplished. It is simply an extremely complex engineering job, which, if left to nature alone, might take a few million more years. Hopefully, man will be able to speed up that process."

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Steps toward nitrogen fixation in rice

Jagdish K. Ladha and Pallavolu M. Reddy

Nitrogen is the most important nutrient input required for rice production. A major goal of biological nitrogen fixation (BNF) research has been to extend the nitrogen-fixing capacity to cereal plants such as rice. If a BNF system could be assembled in the rice plant, it could increase the potential for nitrogen supply because fixed nitrogen would be available to the plant directly, with little or no loss. Such a system could also enhance resource conservation and environmental security, besides freeing farmers from the economic burden of purchasing fertilizer nitrogen for crop production.

To achieve nitrogen fixation in rice, the International Rice Research Institute (IRRI) launched a global collaborative initiative, the Frontier Project on Nitrogen Fixation in Rice, about six years ago. An international working group, consisting of research scientists with diverse backgrounds and approaches and committed to reducing the dependency of rice on mineral nitrogen resources, was established to review, share research results and materials, and catalyze research in the frontier project.

The strategies enabling rice to fix its own nitrogen are complex and long-term in nature. Nonetheless, in the past six years, worldwide collaborative efforts have led to remarkable progress in the areas of rice-endophytic diazotroph associations and determination of the genetic predisposition of rice for symbiosis with rhizobia. In addition, concerted efforts are also under way to elucidate the genetic, biochemical, and physiological requirements for the assembly and function of the nitrogenase enzyme complex in plant cells. In the next five to ten years, we envisage even greater progress in achieving nitrogen fixation in rice.

Nitrogen is the nutrient that most frequently limits agricultural production. Global agriculture now relies heavily on petroleum-based nitrogen fertilizers, which are expensive inputs, costing agriculture more than US$45 billion per year, and fertilizer is vulnerable to political uncertainties and economic fluctuations in oil markets. Manufacturing fertilizer for today's global needs requires fossil fuel energy equal to about 100 million t of oil per year. Oil is a nonrenewable resource and its oxidized products

pose hazards to human health and to the environment (Bockman 1997). Moreover, fossil fuel reserves are finite and therefore unsustainable in the long term.

Rice needs 1 kg of nitrogen to produce 15–20kg of grain. Lowland rice in the tropics can use enough naturally available N to produce 2–3t ha^{-1} . For higher yield, additional N must be applied. Before the advent of chemical fertilizers, rice productivity depended almost entirely on biological nitrogen fixation (BNF; Fig. 1). During the Green Revolution, and since the 1960s, the application of chemical nitrogen fertilizers has boosted rice yields by 100–200%to match the demands of the increasing human population. In the next 25 years, we must produce nearly 70% more rice than the 460 million t of today (IRRI 1993). To enhance rice production from the present 8 t ha^{-1} to 12 t ha^{-1} in 2020 ("green revolution 2") would require an increase in fertilizer application from 220 to 400 kg ha^{-1} (Fig. 2). At current levels of N-use efficiency, we will require approximately double the 10 million t of N fertilizer currently used each year for rice production.

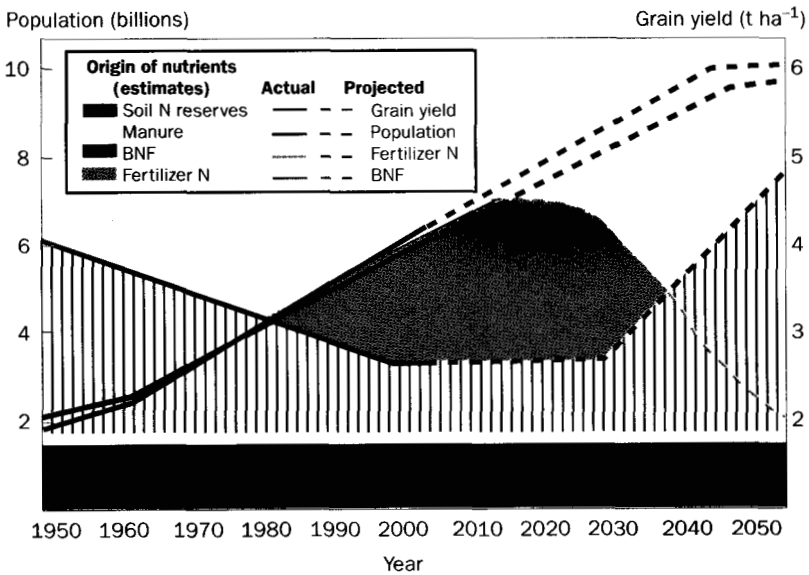

Fig. 1. Global trends in population growth and cereal production, and relative contributions of nitrogen from various sources. (Modified from Bockman et al 1990.)

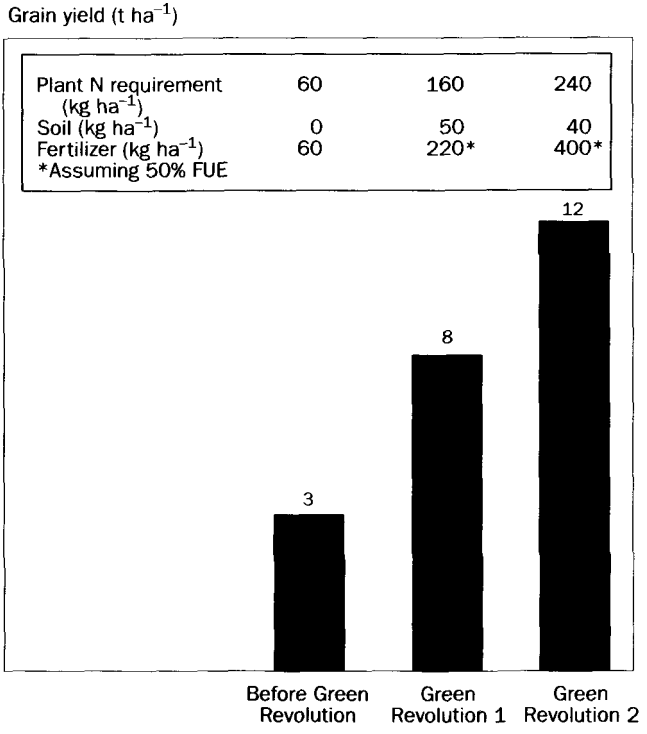

Fig. 2. Rice yields and fertilizer requirements. FUE = fertilizer-use efficiency.

Conventional biological nitrogen fixation: biofertilizer technology and limitations to use in rice-based cropping systems

Conscious of the need and the predicted demand for nitrogen fertilizers to enhance rice production, the International Rice Research Institute (IRRI) began research on conventional BNF systems in the mid-1970s and expanded it further in the '80s and '90s to find solutions to provide rice with additional inexpensive and environmentally safe fixed nitrogen (Fig. 3). This research succeeded in developing more accurate methods to measure nitrogen fixation and develop biofertilizer technologies, particularly green-manure technology, for application in rice-farming systems (Ladha et a1 1988, Roger 1996, Watanabe 1987). Simultaneously, an active program was begun to train scientists in national agricultural research systems (NARS) to strengthen their research on biofertilizer technology. IRRI also established germplasm for the dissemination of biofertilizer materials such as diazotrophic bacteria/cyanobacteria, *Azolla,* and aquatic green-manure legumes to NARS and researchers from developed countries worldwide (Watanabe et a1 1992).

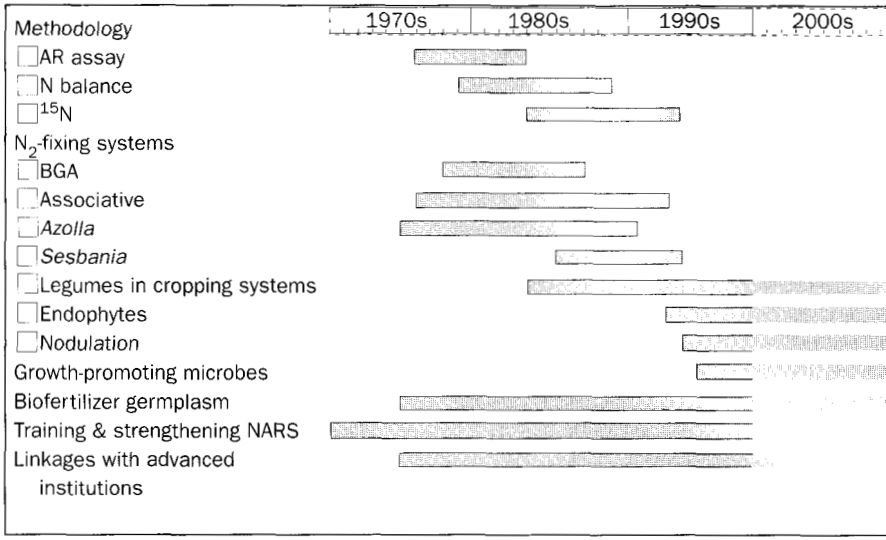

Fig. 3. The biological nitrogen fixation program at IRRI. AR = acetylene reduction, BGA = blue-green algae, NARS = national agricultural research systems.

Diverse free-living/associative nitrogen-fixing microorganisms (aerobes, facultative anaerobes, heterotrophs, phototrophs) grow in wetland rice fields and contribute to soil N. These BNF systems include heterotrophic bacteria in the root zone and in the soil, and cyanobacteria and photosynthetic bacteria that inhabit floodwater and the soil surface. Heterotrophic bacterial BNF estimated from N-balance studies averaged 7 kg N ha^{-1} (App et al 1986). Using available N of a stabilized ^{15}N-labeled soil as a control, Zhu et al (1984) estimated that, with no fertilizer and the photodependent BNF controlled, heterotrophic BNF contributed 16–21% of rice N, or 11-16 kg N ha^{-1} $crop^{-1}$. Incorporation of straw in soil increased BNF by 2–4kg N t^{-1} straw applied (Santiago-Ventura et al 1986).

In favorable conditions, blue-green algae can fix 2040 kg N ha^{-1} $crop^{-1}$ (Roger and Watanabe 1986). Since the discovery of the importance of cyanobacteria in nitrogen gains under flooded conditions, many inoculation experiments have been conducted using indigenous or cultured cyanobacteria to improve soil fertility and grain yields of rice. Cyanobacterial inoculation is a low-cost technology, but many constraints restrict its success. Factors such as phosphate deficiency, the presence of high concentrations of nitrogen in floodwater, low pH, and arthropod grazer populations can limit the growth and BNF activities of cyanobacteria in rice fields (Grant et al 1986, Roger and Watanabe 1986). Alleviation of these constraints by the application of phosphate, biocides (such as neem), and lime. and deep placement of fertilizer nitrogen stimulates BNF, but is only sufficient to marginally enhance rice production.

Aquatic plants such as the water fern *Azolla* and semiaquatic legumes *Sesbania, Aeschynomene,* or Astragalus are recommended green-manure plants for rice, and they fix nitrogen symbiotically. The potential of *Azolla* and *Sesbania* to produce yields

of 6–8 t ha^{-1} is roughly equivalent to an application of 100–200 kg N ha^{-1} as urea (Ladha et al 1992, Watanabe and Liu 1992). Farmers, however. usually have no economic advantage in choosing *Azolla* or *Sesbania* over fertilizer nitrogen because additional costs of labor. land opportunity, irrigation, seed/inoculum, phosphate, and pesticides make the use of *Azolla* and *Sesbania* uneconomical. Excellent opportunities exist, however, for integrating dual- or multipurpose legumes (grain, forage, and green-manure) in the postmonsoonal period following rice cultivation.

Need for *in planta* biological nitrogen fixation: looking ahead

Rice suffers from a mismatch of its nitrogen demand and the nitrogen supplied as fertilizer, resulting in a 50–70% loss of applied N fertilizer, which eventually contributes to the degradation of the environment by groundwater contamination. nitrous oxide emission, etc. A regulated supply of N is likely to maximize the yield potential of rice. and this could be accomplished through two basic approaches. One is to coordinate the timing of N application based on the plant's need. This can partially increase the use efficiency of applied nitrogen. The other is to increase the ability of the rice plant to fix its own nitrogen (Ladha and Reddy 1995, Ladha et al 1998). If a BNF system could be assembled in the rice plant, it could amplify the potential for nitrogen supply because fixed nitrogen would be available to the plant directly, with little or no loss. Such a system would give farmers no additional economic burden. This latter approach is a long-term strategy with large public and environmental benefits that would simultaneously help resource-poor farmers. If half of the N fertilizer applied to the 120 million ha of lowland rice could be obtained from biologically fixed nitrogen, the equivalent of about 7.6 million t of oil would be conserved annually.

A global collaborative initiative

In 1992, the International Rice Research Institute organized a workshop to assess the feasibility of developing nitrogen-fixing capability in rice (Khush and Bennett 1992). The experts attending the meeting identified four major short- and long-term approaches: (1) improving the endophytic associations between rice and nitrogen-fixing bacteria, (2) laying the foundation for the engineering of rice plants capable of forming legume-like symbiosis and nodules with rhizobia, (3) transforming rice with genetic mechanisms to ensure the expression of nitrogenase and protect nitrogenase from inactivation by oxygen, and (4) refining the processes to enhance N-use efficiency in rice. The workshop participants acknowledged the challenge of achieving (endo)symbiosis and nodulation and nitrogen fixation in rice through genetic engineering and agreed to work toward that goal (Table 1). Based on the workshop recommendations, IRRI developed a New Frontier Project to coordinate worldwide collaborative efforts among research centers committed to reducing the dependency of rice on mineral nitrogen resources (Ladha et al 1997a). An international BNF working group was also established to review and share research results and materials and to catalyze research. Two BNF working group meetings were held, at IRRI (Philip-

Table 1. Universities and research institutions engaged in collaborative and independent research on nitrogen fixation in rice and other cereals.

Country	Institution[a]	Research theme with rice[1]/wheat[2]
Australia	Australian National University	Endophyte/rhizobial interaction[1,2]
	University of Sydney	Associative diazotroph interaction[1,2]
Belgium	Katholieke Universtiet Leuven	Associative diazotroph interaction[1]
Brazil	EMBRAPA	Endophyte interaction[1]
China	Chinese Academy of Agricultural Sciences	Endophyte/rhizobial interaction[1,2]
	University of Shandong	Endophyte/rhizobial interaction [1,2]
Egypt	Sakha Agricultural Research Station	Rhizobial interaction[1]
France	INRA-CNRS,Toulouse	Rhizobial interaction[2]
Germany	Max-Planck Institute, Marburg	Endophyte interaction[1]
India	Banaras Hindu University	Endophyte interaction[1]
Japan	National Institute of Agrobiological Resources	Rice *ENOD* homologues, chitin recognition and defense response[1]
Pakistan	NIBGE	Endophyte interaction[1]
Philippines	International Rice Research Institute	Endophyte/rhizobial interaction, legume *ENOD* gene expression in rice, rice *ENOD* homologues[1]
	University of the Philippines at Diliman	Endophyte interaction[1]
Switzerland	ETH	*ENOD* gene expression in ricel
UK	John Innes Centre	Expression of nif genes in plant cells[1]
	University of Dundee	Endophyte/rhizobial interaction[1]
	University of Nottingham	Rhizobial interaction[1,2]
USA	Michigan State University, East Lansing	Endophyte/rhizobial interaction[1]
	University of Arizona, Tucson	Endophyte interaction[1]
	University of California, Davis	Endophyte/rhizobial interaction[1]
	University of Tennessee, Knoxville	Legume *ENOD* gene expression in rice[1]

[a] EMBRAPA = Empresa Brasileira de Pesquisa Agropecuaria; ETH = Eidgenossische Technische Hochschule Zurich; INRA-CNRS = Institut National de la Recherche Agronomique-Centre National de la Recherche Scientifique; NIBGE = National Institute for Biotechnology and Genetic Engineering.

pines) in 1995 and at the National Institute for Biotechnology and Genetic Engineering (Pakistan) in 1996. The reports of the meetings were widely distributed. In addition, a proceedings comprising the deliberations of the second working group meeting was published (Ladha et a1 1997b).

Frontier project on "Assessing Opportunities for Nitrogen Fixation in Rice": research advances (1994-99) and future directions

Research in the frontier project "Assessing Opportunities for Nitrogen Fixation in Rice" began in 1994 on two major fronts—identifying and improving endophytic diazotroph associations in rice, and determining the genetic predisposition of rice for forming an endosymbiotic association with rhizobia. In addition, R. Dixon, Norwich (U.K.), is investigating the prospects for and problems associated with the expression of *nif* genes in plant cells (Dixon et a1 1997 and this volume).

Endophytic diazotroph associations

Diazotrophs such as *Acetobacter diazotrophicus* and *Herbaspirillum* spp. grow endophytically in the stems and leaves of sugarcane. Evidence shows that A. diazotrophicus is the main contributor of endophytic BNF, which, according to nitrogen-balance studies, was as high as 150 kg N ha^{-1} yr^{-1} in sugarcane (Dobereiner et al 1993). Another nitrogen-fixing endophyte of considerable interest is *Azoarcus.* This diazotroph inhabits the roots of Kallar grass *(Leptochloa fusca),* which yields 20-40 t of hay ha^{-1} yr^{-1} without the addition of any N fertilizer in saline-sodic, alkaline soils having low fertility (Sandhu and Malik 1975, Sandhu et al 1981). Inoculation experiments with *Herbaspirillum* spp. in nonsterilized soil under greenhouse conditions have shown that these endophytic diazotrophs can be readily introduced into the rice plant by applying bacterial cultures on seeds before germination (Olivares et al 1993). Infection occurs through the roots as well as stomata and the diazotrophs are translocated through the xylem to all parts of the plant. *Herbaspirillum* spp. inoculated into rice seedlings growing in nitrogen-free Hoagland solution, containing ^{15}N-labeled nitrogen, showed ^{15}N dilution amounting to a 40% increase in total plant nitrogen. These investigations suggest that endophytic diazotrophs have a considerable potential to contribute to the productivity of nonlegumes, including rice. With a methodical approach, scientists at IRRI and collaborating institutions are pursuing the following research initiatives to explore and evolve strategies to improve rice-endophytic diazotroph associations:

- Identify specific and predominant endophytic diazotrophs from rice.
- Determine the mode of invasion and extent of colonization by endophytic diazotrophs.
- Assess the contribution of endophytic diazotrophs to rice growth and yield through nitrogen fixation and growth promotion.

First, the method for surface sterilization of rice tissues was standardized to enable the proper enumeration and isolation of putative endophytes (Barraquio et al 1997, Stoltzfus et al 1997). Subsequently, several diazotrophic endophytes were isolated from different cultivated and wild rice species, and their genetic diversity was studied using polymerase chain reaction-based techniques (Barraquio et al 1997, Stoltzfus and de Bruijn, this volume, Stoltzfus et al 1997). The bacteria isolated from rice also included certain novel diazotrophic endophytes belonging to *Serratia* and *Alcaligenes* species (Gyaneshwar et al, unpublished, Hurek et al, this volume) as well as, on some occasions, the rhizobia1 strains (Yanni et al 1997). Interestingly, *Alcaligenes* species inhabiting rice were always seed-borne endophytes, whereas *Serratia* species appear to infect rice grown in the soil (Gyaneshwar et al, unpublished, Hurek et al, this volume). Endophytes usually infect rice roots through the epidermis or lateral root cracks (Cocking, Barraquio et al, and James et al, this volume, Reddy et al 1997, Reinhold-Hurek and Hurek 1998, Sevilla and Kennedy, this volume, Webster et al 1997). Endophytes such as *Serratia* and *Azoarcus* were aggressive colonizers, predominantly establishing in xylem vessels and aerenchyma (Gyaneshwar et al, unpublished, Hurek et al, this volume). The flavonone naringenin stimulated the colonization of *Azorhizobium caulinodans* and certain endophytes through lateral root cracks

(Webster et al 1997, Ladha et al, unpublished). Observations of *nif* gene expression in endophytes such as *Azoarcus* and *Serrutia* suggest that, in rice roots, conditions are conducive to nitrogen fixation (Egener et al 1998, Gyaneshwar et al, unpublished. Hurek et al, this volume). Recent studies also evidenced that rice harbors a diverse array of nonculturable endophytic diazotrophs that probably make a significant contribution to the nitrogen requirement of the plants (Hurek et al, this volume). Compelling evidence also shows that some endophytes, especially some rhizobia, promote plant growth other than by supplying N through nitrogen fixation (Dazzo et al, Mirza et al, and Phillips et al, this volume, Yanni et al 1997, Biswas et al, unpublished).

Diverse endophytic bacteria, including diazotrophs, colonize the interior of rice and other gramineous crops, thus suggesting that the endophytic bacterial colonization of the internal tissues of healthy plants is a universal phenomenon. In most instances, however, unlike in legume-*Rhizobium* symbiosis, the association between the plants and endophytes does not appear to be governed by any host and bacterial specificity. Nevertheless, the limited host range of diazotrophs such as *Acetobacter diazotrophicus* and *Herbaspirillum* spp. that grow endophytically in the stems and leaves of sugarcane and in the tubers of sweet potato (see Baldani et al 1997) and *Azoarcus* that inhabits the roots of Kallar grass (see Reinhold-Hurek and Hurek 1998) seems to indicate that some endophytes do exhibit host specificity. In rice, a wide range of endophytes colonize the internal tissues. Future studies should be directed toward determining whether any particular endophytic diazotroph species specifically colonizes rice tissues. It is also essential to determine the nitrogen-fixing potential of the endophytes and the limiting factors for *in planta* nitrogen fixation. Such information will be helpful in devising strategies to enhance N_2 fixation by endophytes.

Determining the genetic predisposition of rice for symbiosis with rhizobia

Legume-rhizobia1 interactions culminate in the formation of structures known as nodules, where rhizobia, insulated from microbial competition, fix nitrogen. This N then becomes directly available for plant growth. A long-standing goal of biological nitrogen fixation research has been to extend the nitrogen-fixing symbiosis to nonnodulated cereal plants such as rice (see Khush and Bennett 1992, Reddy and Ladha 1995). In the past, several researchers examined rice-rhizobia interactions and reported the induction of "nodule-like structures" or "hypertrophies," albeit at extremely low frequencies (in 0.1–0.2% of the plants), upon inoculation of rhizobia to either normal roots (Bender et al 1990, de Bruijn et al 1995, Jing et al 1990, 1992, Li et al 1991, Rolfe and Bender 1990) or enzyme-treated roots in the presence of polyethylene glycol and calcium chloride (AI-Mallah et al 1989). In our studies, we relied on the extensive knowledge of legume-rhizobia symbioses to formulate strategies for developing rice-rhizobia symbioses. Adopting a systematic approach, we initially addressed the following questions to evaluate the extent of predisposition of rice to form an intimate association with rhizobia:

- Do rice root exudates induce the expression of the nodulation *(nod)* genes in rhizobia?
- Do rhizobia elicit morphological and developmental responses in rice roots?
- Do rhizobia provoke a defense response in rice?
- Do rice roots perceive Nod signals and, if so, is the signal transduced appropriately?
- Does rice possess homologues of legume early nodulin genes and, if present. are their regulatory mechanisms similar to those in legumes?

Recent research has found that *Rhizobium* and rice roots interact and that many of the elements found in the legume symbioses are also present in rice. For example. for *Rhizobium* to infect the legume root. the bacterial *nod* genes must be induced by plant-produced flavonoids. Our studies indicated that the roots of some rice cultivars exude compounds that are able to induce. albeit to a low extent, transcription of the nod genes of *Rhizobium* sp. NGR234, a rhizobial species that has an extraordinarily large host range (Reddy et al and Rolfe et al, this volume). But neither rhizobia nor purified Nod factors could elicit root hair deformation or cortical cell divisions leading to true nodule development in rice (Reddy et al 1997).

Rhizobia primarily invade rice roots through cracks in the epidermis and fissures created during the emergence of lateral roots (Reddy et al 1997, this volume). This infection process, unlike in legumes, is nod-gene independent, and does not involve the formation of infection threads. Moreover, rhizobial invasion provokes a mild defense response localized in the vicinity of the colonization site. During legume nodulation, specific plant genes, known as early nodulin *(ENOD)* genes, are induced and are required for normal nodule development. Reddy et al (1999) showed that several legume *ENOD* genes hybridized to DNA from a variety of rice genotypes. This work was subsequently confirmed by isolating rice cDNAs showing considerable sequence homology to legume *ENOD93* (Reddy et al 1998a) and *ENOD40* (Kouchi et al 1999).

Legume nodulation also requires the ability of the legume to respond to the rhizobial Nod signal, and the consequent signal-induced expression of the *ENOD* genes necessary for nodulation. Studies revealed that rhizobial Nod factors can induce the expression of legume *ENOD12* promoter in rice (Reddy et al 1998b, Werthmuller et al 1998). Results with *ENOD12*-GUS transgenic plants are intriguing because they indicate that rice possesses not only the mechanism to recognize rhizobial Nod signals but also the signal transduction chain that links such recognition to *ENOD* gene transcription. In addition, similar to the situation in legumes, the expression of the legume *ENOD40* promoter is induced only in the vascular tissues in rice (Reddy et al this volume). Likewise, the expression of rice *ENOD40* in soybean nodules is also restricted to the vascular bundles (Kouchi et al 1999). These findings clearly suggest that legume and rice *ENOD40s* share a similar regulatory mechanism(s).

In legume-Rhizobium symbiosis, the host plant provides the genetic information for the development of nodules. The role of the rhizobia is to trigger the host-

plant genes to facilitate infection and help create a nodular niche for nitrogen fixation (see Denarie et al 1996, Long 1996). A monocot plant such as rice would unlikely possess the complete complement of genes or genetic programs involved in the nodule ontogeny. Rice, however, although it does not develop a symbiotic association with rhizobia, is able to enter into symbiotic associations with mycorrhizal fungi (see Khan and Belik 1995). Genetic links between the processes involved in nodulation and arbuscular mycorrhizae have been found in legumes (Albrecht et a1 1998, 1999, Gough and Denarie, this volume, Denarie and Gianinazzi-Pearson 1997, Gianinazzi-Pearson 1996). For example, studies on nodulation mutants of pea have demonstrated that the early nodulin genes *ENOD2, ENOD11, ENOD12,* and *ENOD40,* which control the initial steps of nodulation, also govern the early stages of mycorrhizal development (van Rhijn et al 1997). Thus, because rice is able to form symbiotic associations with mycorrhizal fungi, and because the formation of such an association of mycorrhizal fungi with legumes is mediated by *ENOD* genes, we can infer that at least some of the genetic mechanisms required to promote endosymbiosis with rhizobia likely exist and function in rice. The challenge then becomes to identify the plant traits that are unique to rhizobia1 nodulation so that they can be engineered into the rice plant.

Conclusions

Certain critical differences exist between the association of rice and rhizobia relative to the root-nodule symbiosis in legumes. Some of the molecular interactions that occur in these plant-microbe associations, however, may be similar. It is therefore essential that studies be extended at the cellular and molecular levels to identify why such responses do not occur fully in rice in order to contemplate genetically engineering this major cereal crop to form a more intimate endosymbiotic association with rhizobia.

The strategies of enabling rice to fix its own nitrogen are complex and long-term in nature. The experts attending the 1992 workshop opined that "much progress can be made by using molecular and cellular biology to enhance the level of nitrogen fixation during rice cultivation. This will probably be gained through an endophyte process. The great progress in our understanding of nitrogen-fixing symbiosis suggests that further exciting discoveries in plant science will assist in reaching the goal" (Khush and Bennett 1992). The experts stressed the need to support fundamental plant and microbial science, for discoveries in these areas will help solve the many problems involved in achieving symbiotic nitrogen fixation in rice. Several scientists from various developed and developing countries are assessing the feasibility of these novel propositions through rigorous and methodical approaches, and considerable progress has been made in the past five years. In the next five to ten years, we envisage even more progress in realizing the goal.

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Novel nitrogen-fixing bacteria associated with the root interior of rice

Thomas Hurek, Zhiyuan Tan, Natarajan Mathan, Tanja Egener, Michaela Engelhard, Prasad Gyaneshwar, Jagdish K. Ladha, and Barbara Reinhold-Hurek

We have for a long time been working with Kallar grass (*Leptochloa fusca* (L.) Kunth), a pioneer grass grown on salt-affected, flooded low-fertility soils in the Punjab of Pakistan, which gives high yields of hay without application of N fertilizer, as a model system for interactions between diazotrophs and grasses. A new genus of proteobacteria of the beta subgroup, *Azoarcus* spp., occurs in high numbers in the root interior of this grass. Some members of this genus share with other diazotrophs such as *Acetobacter diazotrophicus* a rather novel type of interaction with the plant: as "endophytes," they proliferate within the tissue without causing symptoms of plant disease, but they do not form an endosymbiosis inside living plant cells. *Azoarcus* spp. show similar colonization patterns in Kallar grass and—in laboratory cultures—in rice seedlings: they are capable of infecting roots, spreading inside systemically, and multiplying within the aerenchyma and the stele. Therefore, we extended our studies to rice.

Our lab focused on several different aspects of interactions of diazotrophs with graminaceous plants such as rice. First, for taxonomy and diversity of rice endophytes, we screened for abundant diazotrophs in cultivated and wild rice species, and detection and identification of abundant noncultured diazotrophs in rice by DNA- and RNA-based methods. Second, we studied the regulation of *nif*-gene expression in *Azoarcus* sp.: under certain culture conditions, the bacteria are "hyperinduced," that is, they fix nitrogen more efficiently and actively. Therefore, we are now unraveling the regulatory cascade for activation of *nif*-genes in *Azoarcus* sp. Third, we analyzed bacterial gene regulation in association with rice roots: a way to analyze functions of bacteria inside roots is to study which specific genes are induced in their microhabitat, the root interior. We demonstrated recently, by using a reporter gene approach, that the interior of rice roots may provide suitable conditions for the expression of nitrogenase genes by *Azoarcus* sp. (low concentrations of O_2 and combined N), thus representing a putative niche for nitrogen fixation. Fourth, we identified new endophytes of rice: taxonomy of a novel *Serratia* group associated with rice, and taxonomy of novel seed-associated putative endophytes of rice.

The plant root system offers various microhabitats for bacterial growth, such as the rhizosphere soil, the rhizoplane, or the endorhizosphere. When N_2-fixing bacteria were isolated in large numbers from the root interior of grasses (Olivares et al 1996, Reinhold et al 1986), they attracted attention because they were able to interact more closely with the plant and were better protected from environmental changes than rhizosphere soil bacteria. Moreover, some graminaceous crops. such as Brazilian sugarcane varieties grown with low inputs of nitrogen fertilizer. can derive a substantial percentage of their nitrogen requirements from nitrogen fixation (Lima et al 1987, Urquiaga et al 1992). Significant N_2 fixation has also been demonstrated for some rice varieties grown under waterlogged conditions (Boddey et al 1995, Wu et al 1995). These results have stimulated research on mechanisms of interactions between N_2-fixing microorganisms, especially endophytes, and graminaceous plants, in order to provide the basic knowledge for exploiting N_2 fixation in agriculturally important crops.

Endophytic growth, that is, the colonization of inner plant tissues of graminaceous plants, has been demonstrated for several diazotrophs with appropriate controls, including immuno-microscopical or reporter gene studies such as *Acetobacter diazotrophicus* in sugarcane (James and Olivares 1998, James et al 1994), *Herbaspirillum seropedicae* in sorghum and sugarcane (James and Olivares 1998, James et al 1997, Olivares et al 1997), and *Azoarcus* sp. in Kallar grass and rice (Hurek et al 1994). These diazotrophs show similar patterns of infection and colonization (Reinhold-Hurek and Hurek 1998a,b). Sites of ingress are often the points of emergence of lateral roots and the zone of elongation and differentiation above the meristem of root tips. As sites of root colonization, intercellular spaces and dead plant cells of the outer cell layers, the root cortex region, and, more rarely, the stele were observed. An occasional colonization of xylem vessels may result in systemic, vertical spreading of bacteria into the shoot (Hurek et al 1994, James and Olivares 1998). In waterlogged plants such as Kallar grass and rice, the root cortex is disrupted for gas transport in mature plants, forming aerenchymatic tissue that is also a site for bacterial colonization.

Roots of rice seedlings are infected in gnotobiotic culture by endophytic diazotrophs isolated from other host plants such as *Azoarcus* sp. from Kallar grass (Hurek et al 1994) or *Rhizobium leguminosarum* from rice (Yanni et al 1997). Therefore, rice also appears to be a plant that has the potential to harbor endophytic bacteria. An intensive screening for endophytic diazotrophs in rice and an assessment of their capacities is thus urgently required.

Rice roots as microhabitats for endophytic expression of nitrogenase genes

Transcriptional reporter gene fusions to quantify and visualize gene expression

To study the capacities of diazotrophs in host plants, one approach is to localize the expression of bacterial genes of interest inside the host plant. Visualization of gene expression can be achieved by fusion of target genes to suitable reporter genes. Re-

porter genes that are widely used because they have low or no background activity in plant tissue are the b-glucuronidase of *E. coli* (Jefferson et aL 1987) or the jellyfish green fluorescent protein (GFP) (Chalfie et al 1994).

One disadvantage of b-glucuronidase as a reporter gene is that blue GUS staining is not always restricted to the bacterial cells, but may diffuse into bacteria-free plant material (Hurek et al 1994, Reinhold-Hurek and Hurek 1998a). After cleavage of the substrate X-Gluc (5-bromo-4-chloro-3-indolyl-b-D-glucuronide), a dye monomer is released that will only precipitate as insoluble blue dimer in the presence of O_2. If immediate dimerization is lacking, precipitation may occur at distant loci after diffusion of the monomers. Thus, the presence of stained bacteria has to be reconfirmed by high resolution microscopy (S. Saxena, J.K. Ladha, P. Gyaneshwar, B. Reinhold-Hurek, R.J. Hernandez-Oane, and J.C. Biswas, in preparation). This disadvantage is overcome by application of GFP, which does not require staining because the protein is autofluorescent.

We have constructed two cassettes suitable for generating transcriptional fusions of target genes to *gusA* or *gfp* by cloning (Egener et al 1998, 1999). They carry translational stop codons in all three reading frames upstream of the reporter gene in order to avoid translational fusions of the target and reporter gene. To allow long-term studies without loss of the reporter gene, the contructs are integrated into the chromosome by homologous recombination.

Environmental factors affecting nitrogenase gene expression in *Azoarcus* sp.

To study the influence of environmental factors on nitrogenase gene expression in *Azoarcus* sp. BH72, an endophyte of Kallar grass, a transcriptional fusion between *nifH,* the gene for the iron protein of nitrogenase, and *gusA* was constructed (Egener et al 1999). In N_2-fixing batch cultures of the reporter strain, GUS activity correlated well with acetylene reduction activity (not shown). As in other free-living aerobic bacteria fixing N_2 microaerobically, *Azoarcus* sp. *nif* gene expression was also re-

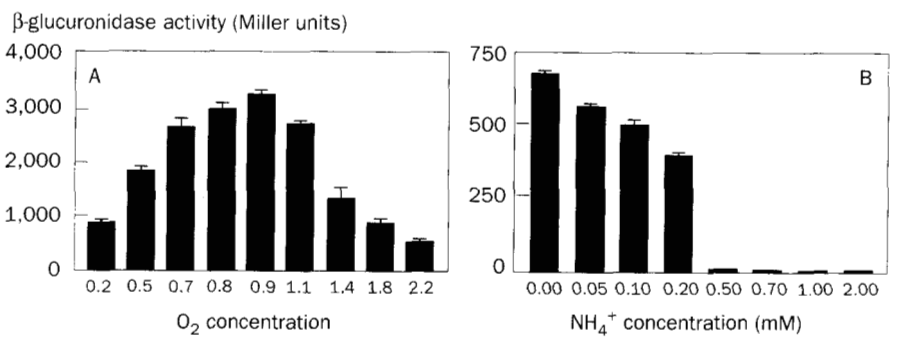

Fig. 1. Expression of a *nifH::gusA* fusion in a reporter strain of *Azoarcus* sp. BH72. Effects on ß-glucuronidase activity of different O_2 concentrations in the headspace (A) or different concentrations of NH_4^+ in the medium (B) after 4 or 2 h of incubation, respectively. From Egener et al (1999).

pressed by high O_2 concentrations and combined nitrogen sources. *nifH::gusA* expression was obtained up to 2% O_2 (Fig. 1A) or 0.2 mM NH_4Cl (Fig. 1B) (Egener et al 1999). Nitrate and several amino acids (L-glutamine, L-arginine, L-cysteine, L-histidine, L-asparagine, at 10 mM) also repressed nitrogenase gene expression (data not shown).

Expression of *nif* genes in the apoplast of the rice root

Do rice roots provide suitable environmental conditions for endophytic nitrogen fixation? This is one of the most crucial questions to be addressed when an endophytic nitrogen-fixing association is considered for rice plants. We addressed this question using the Kallar grass endophyte *Azoarcus* sp. BH72 as a model organism because it is capable of invading rice. Ten days after inoculating *Oryza sativa* L. cv. Nipponbare seedlings with the *nifH::gusA* reporter strain, GUS staining was visible on roots of 42% of the seedlings (Egener et al 1999) when negligible amounts of carbon source (5 mg L^{-1}) were added to the plant medium to enhance survival of the inoculum. The degree of colonization and staining was very variable, however; in only 6.3 ± 7.3% of the plants, roots were well colonized by aim-expressing bacteria (50% of the root system stained). In such well-stained roots, *nifH::gusA* expression occurred mainly at the points of emergence of lateral roots and at the zone of elongation and differentiation. Transversal semithin sections of resin-embedded roots revealed large microcolonies in the aerenchymatic tissue of the main roots, which were immunogold-silver stained with antibodies specific for *Azoarcus* sp. (Fig. 2A). These microcolonies showed blue color, single stained bacteria being visible after a short GUS staining time (0.5–2 h of incubation) (Fig. 2B) carried out before embedding into LR White resin (Egener et al 1999). Strong nitrogenase expression inside the rice roots was also confirmed using a reporter strain of *Azoarcus* sp. carrying a *nifH::gfp* fusion (Egener et al 1999).

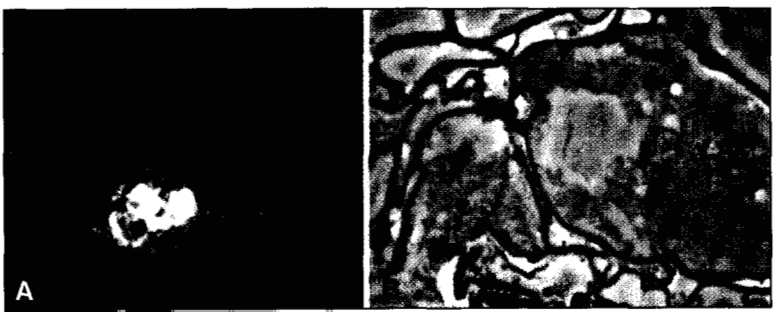

Fig. 2. Location of bacterial *nifH::gusA* expression inside rice roots. Microcolonies of a reporter strain of *Azoarcus* sp. BH72 were detected in the aerenchymatic air spaces of a rice seedling 10 d after inoculation (A). Micrograph of a transversal semithin section at dark field illumination, immunogold-silver stained with antibodies against *Azoarcus* sp. BH72. In a closeup of a successive section (B), bacterial cells in the microcolonies showed blue color when inspected at bright field illumination, indicating *nifH::gusA* activity. Incubation with X-Gluc had been carried out for 0.5–2 h before embedding into LR White resin. Micrographs from Egener et al(1999).

Thus, rice roots may provide a suitable microenvironment for bacterial nitrogenase gene expression. The concentrations of oxygen and combined nitrogen appear to be sufficiently low to allow highly active *nif* gene expression. Surprisingly, the expression is apoplastic: the bacteria were located between plant cell walls but not in living plant cells. Apparently, apoplastic nutrient flow in the aerenchyma was high enough to sustain bacterial growth and *nif* gene expression.

Novel putative endophytes of rice

Azoarcus spp. colonize preferentially roots of wild rice and landraces of *O. sativa*

In a search for putative diazotrophic endophytes in rice, a promising approach is to screen for naturally occurring *Azoarcus* spp. because these bacteria are known as endophytes of Kallar grass and are capable of nitrogenase gene expression in gnotobiotic culture inside rice. Moreover, molecular data suggested the natural occurrence of *Azoarcus* sp. in association with rice roots. In DNA retrieved from field-grown Japanese rice, a *nifH* gene fragment had been detected (Ueda et al 1995b), which clustered with *nifH* sequences of Azoarcus spp. according to phylogenetic sequence analysis (Hurek et a1 1997).

Therefore, we screened rice for culturable *Azoarcus* spp. as natural putative endophytes. Cultivation and breeding of rice have considerably influenced the morphology and physiology of roots (Matsuo and Hoshikawa 1993) and may have thus affected interactions with endophytes as well. Because Azoarcus spp. were known to be associated with an undomesticated grass, our screening included wild *Oryza* species in addition to traditional and modern cultivars, mostly from Nepal.

Isolations from serial dilutions of surface-sterilized roots revealed the presence of three different *Azoarcus* species (Engelhard et al 1999). *Azoarcus indigens* and *Azoarcus* sp. group C were detected only rarely, whereas *Azoarcus* sp. group D occurred frequently in samples of flooded plants: in 75% of wild rice, in 67% of landraces of 0. sativa from Nepal, and in 60% of modern rice cultivars from Nepal and Italy, albeit in lower numbers or not endophytically. *Azoarcus* spp. were not detected in nonflooded samples, where numbers of diazotrophs were usually lower. Lower numbers were observed by others, too (Barraquio et al 1997), indicating that flooded rice may provide more suitable conditions for diazotrophs than dryland rice. The endophytic populations from flooded samples differed by rice genotype, with the landraces having an intermediate position between modern races and wild species (Fig, 3). In *O. minuta* from Nepal and the Philippines and *O. officinalis* from Nepal, *Azoarcus* sp. group D was the predominant putative endophyte in roots. In contrast, its number was significantly lower in modern cultivars of *O. sativa* (Fig. 3), where in most samples other diazotrophs predominated, such as *Klebsiella* sp., *Sphingomonas paucimobilis, Burkholderia* sp., *Azorhizobium* sp., and *Azospirillum* spp. (Engelhard et al 1999). Surprisingly, the diversity of putative endophytes, that is, the number of diazotrophic isolates that were obtained per root sample, was significantly higher in modern cultivars (Fig. 3).

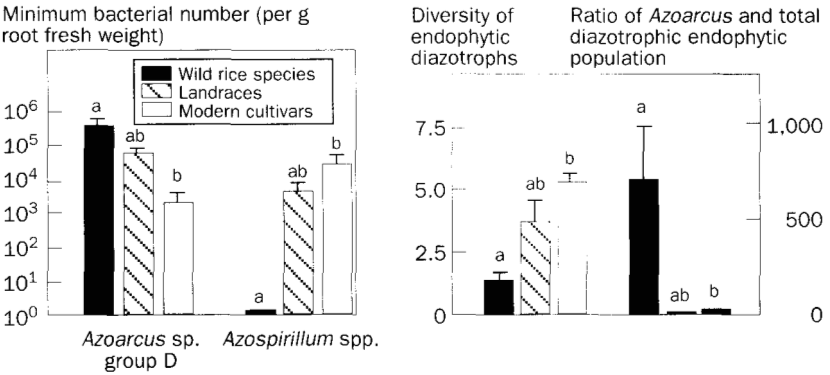

Fig. 3. Differences in populations of diazotrophs associated with the root interior of wild rice species and landraces or modern cultivars of *O. sativa.* The calculation of minimum bacterial numbers was based on the dilutions in which the representative bacteria were detected. The diversity of diazotrophic endophytes was calculated only for surface-sterilized root material. For the ratio of *Azoarcus* sp. group D and the total diazotrophic endophytic population, the highest dilutions from which *Azoarcus* sp. or other endophytes had been isolated were divided by each other. Bars above columns give standard deviations. Columns that are significantly different from each other in the Mann-Whitney test ($P<0.05$) are headed by different letters. From Engelhard et al (1999).

Thus, we detected *Azoarcus* sp. group D as a novel putative endophytic diazotroph in rice, with the potential of these bacteria to be studied in the future. The surprising difference in diversity of culturable diazotrophs in rice of different levels of domestication indicates that the plant genotype may have a profound effect on the diazotrophic population, wild species possibly having more specific interactions with endophytes than modern races.

A novel subgroup of *Serratia marcescens* associated with rice roots

At IRRI, we screened several modern rice cultivars for predominant diazotrophic bacteria. Several isolates sharing cell and colony morphological features were putatively characterized as enterobacteria at IRRI. Taxonomic identification in our laboratory revealed that six isolates (designated Cy) belong to the genus *Serratia,* which are Enterobacteriaceae of the gamma subgroup of proteobacteria. Analysis of an almost complete fragment of the 16S rRNA gene revealed that the sequence was almost identical to the type strain of *S. marcescem* (99.7% identity). The capacity to fix nitrogen has not yet been described in this genus. Acetylene reduction activity was also not detected in the *Serratia* isolates from rice in the laboratory of Marburg.

To investigate the species affiliation of the new isolates, we used a polyphasic taxonomic approach (Z. Tan, P. Gyaneshwar, J.K. Ladha, and B. Reinhold-Hurek, manuscript in preparation). SDS-polyacrylamide gel patterns of SDS-soluble cell proteins were used to group the strains (data not shown). All six isolates were clearly highly related, showing patterns very similar to those of *S. marcescens* reference strains. Because of a few minor bands, however, the isolates fell into two subgroups. Because bacteria showing almost identical SDS-PAGE patterns are known to have similar

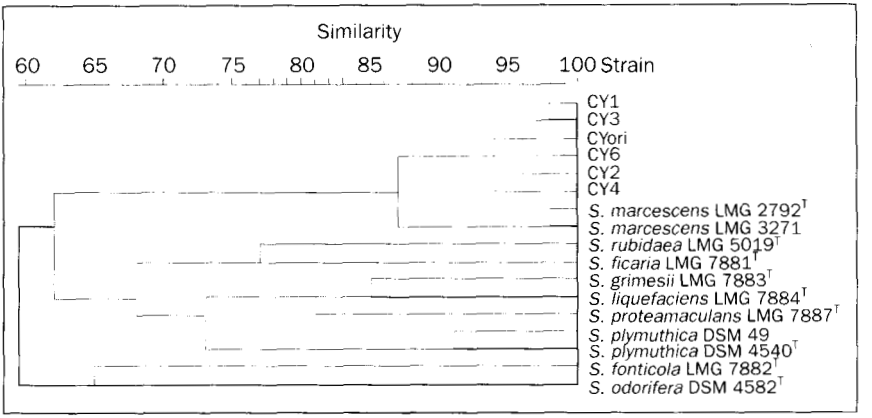

Fig. 4. Numerical taxonomy of members of the genus *Serratia*. Isolates from rice (CY) were compared with reference strains in their carbon-use patterns and biochemical tests. The dendrogram shows the degree of similarity.

genomes and are likely to belong to the same species (Kersters 1985). and because members of both subgroups had identical 16S rRNA sequences, all six isolates can be regarded as members of the same species. The isolates appeared to be a very homogeneous group also in carbon source use patterns. One hundred different carbon sources. as well as biochemical reactions typically applied for differentiation of Enterobacteriaceae. were used for numerical taxonomic analysis of the new species and reference strains of known *Serratia* species. The isolates were clearly located in one cluster and were highly related to *S. marcescens* (Fig. 4).

The crucial parameter for species affiliation is the degree of DNA-DNA homology of two bacteria. In the most stringent definition, bacteria are regarded as different species when they show less than 70% DNA-DNA binding in reassociation assays. Two of the isolates were tested and showed 100% DNA-DNA binding to each other and to the type strain of *S. marcescens* (data not shown). Thus, the new isolates clearly belong to the species *S. marcescens*. albeit as a separate subgroup. Members of *S. marcescens* are often opportunistic pathogens of hospitalized patients, or insect pathogens, or occur in water (Grimont and Grimont 1992). *S. marcescens* has also been reported to be associated with various plants (Ordentlich et al 1987) and has been isolated as a putative endophyte from cotton and sweet corn (McInroy and Kloeppcr 1995). It has been used as a biocontrol agent because of its chitinase activity (McInroy and Kloepper 1995, Sirit et al 1993, Rosales et al 1993) and can also induce systemic resistance in plants (Press et al 1997). *S. marcescens* was also isolated earlier from the rice rhizosphere in IRRI fields (Rosales et al 1993), and *S. plymuthica* was isolated from surface-sterilized seeds of rice (Mukhopadhyay et al 1996). The novel diazotrophic putative endophytes may thus have novel features, and their capacities for endophytic growth and plant growth promotion are currently being studied at IRRI. It is of special interest that they belong to a species of human and insect pathogenic bacteria because this will enable us to compare molecular mechanisms of bacterial invasion and pathogenicity in different eukaryotic hosts.

Isolates intimately associated with rice seeds form novel phylogenetic groups

Microorganisms that are intimately associated with a plant and infect it systemically might be expected to be present on or in seeds. A transfer of bacteria to the next plant generation is also desirable from the viewpoint of biotechnological application because seed transmission can result in a simple, farmer-friendly inoculation technology. Therefore, at IRRI we screened different rice varieties for seed-associated putative endophytes. Seeds were mildly surface-disinfected and germinated on a complex medium. Seedlings free of bacterial contaminants (at the surface) were aseptically transferred to a sterile plant growth medium. After several days of incubation, seedlings were harvested and shoots, roots, and seeds macerated and streaked on a complex medium to screen for the presence of bacteria. In this way, the screening was not restricted to diazotrophic bacteria. Isolates were then compared according to colony morphology and genomic fingerprints.

From several rice varieties, bacteria that had escaped surface sterilization of seeds and were probably tightly adhering in crevices or present in deeper layers of the seeds were isolated. These seed-associated bacteria did not appear to be a homogeneous group. From the broad range of different isolates, those occurring more frequently according to colony morphology were selected for a detailed analysis in Marburg (N. Mathan, T. Hurek, J.K. Ladha, and B. Reinhold-Hurek, manuscript in preparation). Grouping of strains according to electrophorograms of SDS-soluble proteins supported the observation that there was a high diversity of seed-associated bacteria. Only a few strains appeared to be highly related at the species level, showing almost identical protein patterns (data not shown). To identify representative strains, phylogenetic sequence analysis was carried out (Fig. 5). Most isolates were novel strains for which a corresponding identical 16S rDNA sequence was not available in the database. Some strains were highly related to *Klebsiella* sp., *Agrobacterium* spp., *Pseudomonas* sp., and *Rhizobium* sp., genera which are known to colonize plants. Some isolates, however, were only distantly related to known taxa: two novel lineages were localized in the genera *Paenibacillus* and *Bacillus* (both genera harbor plant-associated strains), one deeply branching lineage in the Gram-positive bacteria, and two lineages related to *Stenotrophomonas* (opportunistic human pathogens and rhizosphere bacteria) or *Frateuria* (Fig. 5). Interestingly, in some cases isolates from different rice varieties clustered together, for example, in a new lineage within *Alcaligenes* (soil/rhizosphere bacteria), in a new lineage within *Pseudomonas* (soil/rhizosphere bacteria), and in the *Paenibacillus* cluster. These groups thus appear to be more widespread on and in rice seeds. Surprisingly, the novel *Alcaligenes* cluster was clearly diazotrophic, a novel feature in this genus.

In conclusion, the approach to screen for bacteria tightly associated with seed was very successful. Most isolates were novel, demonstrating that the analysis of a new habitat, the rice seed, may reveal new types of bacteria adapted to this microenvironment. Therefore, these strains can be expected to have some unique features for interactions with rice, and are promising bacteria for studies on inoculation by seed transmission.

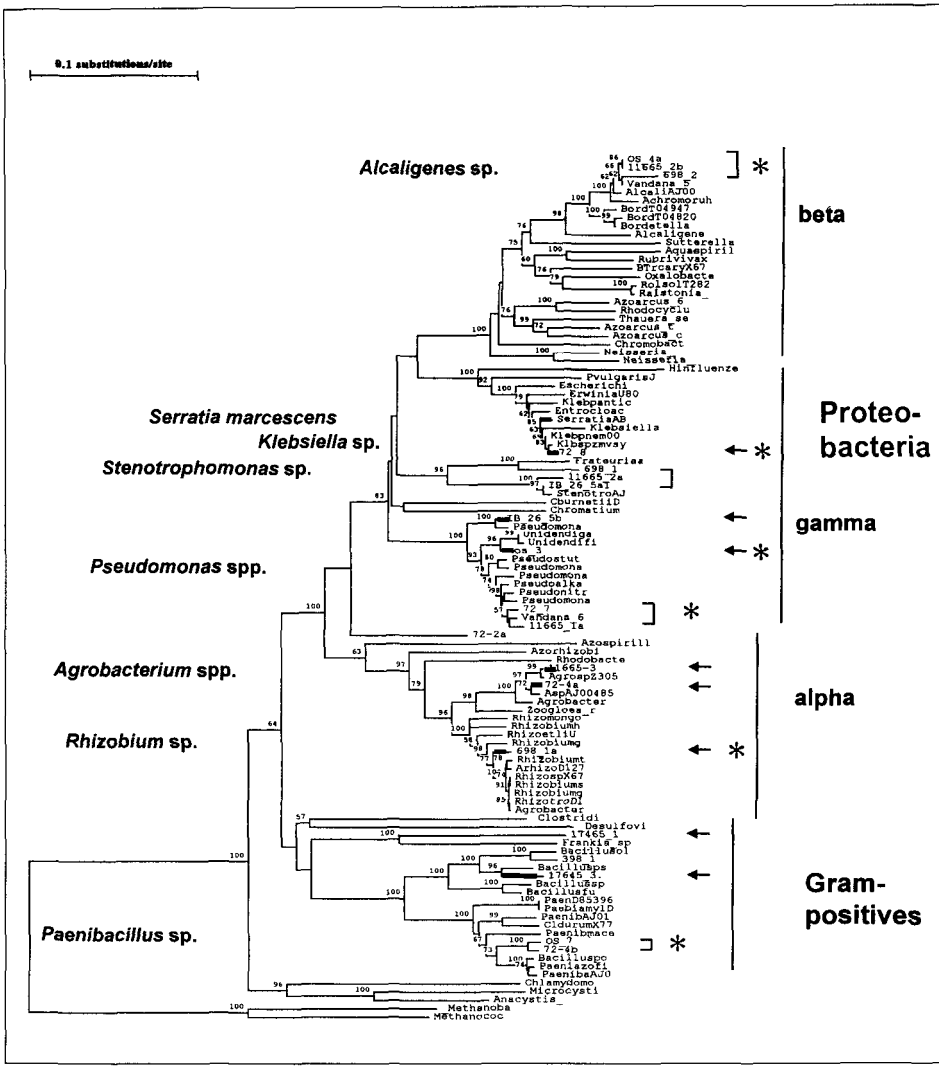

Fig. 5. Phylogenetic relationships of rice-seed-associated bacteria and reference strains. The phylogenetic tree is based on almost complete 16S rDNA sequences and was constructed using the neighbor-joining algorithm with Poisson correction and 100 bootstrap replicates in the TREECON program. Values at the nodes represent the bootstrap confidence estimates. Asterisks indicate diazotrophy, arrows and brackets single or groups of isolates, respectively.

As yet uncultured diazotrophs are common in rice roots

Development of methods for molecular detection of diazotrophs

In the past, knowledge about the diversity of microorganisms has been restricted to those that were cultivated in laboratories. Because of the development of molecular-phylogenetic techniques, such as those based on 16S rDNA sequences, an increasingly robust map of evolutionary diversification has been compiled (Olsen and Woese 1993). The application of molecular-phylogenetic methods to study natural microbial ecosystems without the traditional requirement for cultivation has resulted in the discovery of many unexpected evolutionary lineages (Pace 1997). Studies on several types of environments estimate that more than 99% of organisms that are visible microscopically are not cultivated yet by routine techniques (Amann et al 1995). Therefore, the question is whether we can cultivate the most important diazotrophs in a given ecosystem, such as rice roots.

An experimental approach to molecular detection of diazotrophs is the phylogenetic analysis of cloned nitrogenase *(nifH)* genes after amplification from environmental DNA by polymerase chain reaction (PCR). Because the iron protein of nitrogenase is highly conserved, its phylogeny largely follows the phylogenetic tree of organisms based on 16S rDNA sequences (Hurek et a1 1997, Ueda et al 199%). Therefore, sequence analysis *nifH* genes can be applied to identify diazotrophs present in an environment. A study on Japanese rice roots by Ueda et al (1995a,b) has revealed a surprising diversity of diazotrophs.

The first crucial step in the experimental protocol is an unbiased disruption of all types of bacteria and *Archaea* with a different cell wall structure. Especially when the protocol is adopted to isolate unstable bacterial mRNA for studies on bacterial activity, the cell lysis needs to be not only complete but also fast. Our initial standard protocols using lysozyme and SDS were not satisfactory in speed and retrieval of all cell types. A protocol including a fast mechanical disintegration of the cells using the FastPrep cell disruptor (Bio 101, USA), however, met both requirements. Compared with the standard technique applied to the same root sample, a higher diversity of nitrogenase genes was detected (data not shown) from Gram-positive diazotrophs, which have a more rigid cell wall than Gram-negative bacteria and are thus more difficult to lyse. Moreover, we were able to retrieve mRNA for nitrogenase genes from soil-grown rice roots, to amplify it by reverse-transcriptase (RT) PCR. and to verify the identity of *nifH* by cloning and sequencing. Thus, the methods for molecular detection of diazotrophs in the environment have been optimized.

Increased diazotrophic diversity in roots of modern rice versus wild species

The molecular methods for detecting diazotrophs in the environment were applied to compare diazotrophic diversity in two rice genotypes. Rice root samples from Nepal had shown differences in the diversity of putatively endophytic diazotrophs by traditional techniques of cultivation (see above). Root homogenates of the same samples studied previously, one wild rice species (g 1 = *Oryza minuta*) and one modern variety

of *O. sativa* (15 = *O. sativa* R2Nov2 B4448 from the National Agriculture Research Council, Khumaltar), were also compared using molecular techniques. A clone library was constructed from nitrogenase (*nifH*) genes amplified from total DNA extracted by the FastPrep method (see above) and 16 clones were randomly picked and subjected to sequence analysis. Phylogenetic tree inference (Fig. 6) showed that the cultivation-independent method supported the observation that diazotrophic diversity is lower in wild rice species (Engelhard et al 1999). Out of 16 clones of the gl series, 11 fell into clusters of three, three. or five identical sequences. In total. only seven different lineages were detected (Fig. 6). In contrast, 13 different lineages were found in the modern rice variety (Fig. 6). For example. none of the sequences from wild rice were located in the alpha-proteobacterial group, whereas three sequences from *O. sativa* belonged to this group. This shows on the one hand that modern rice and wild rice species appear to harbor diazotrophic populations of different diversity. On the other hand, it is evident that, by traditional methods of cultivation, bacterial diversity in roots is largely underestimated.

Most rice root-associated diazotrophs are as yet uncultured

A sequence analysis of environmental *nifH* sequences from rice roots (Fig. 6) showed that most sequences retrieved did not match with sequences from known diazotrophic microorganisms from databases. Deeply branching new lineages were detected in the nitrogenases from Gram-negatives (15(108)), from the gamma-subgroup of proteobacteria (e.g., gl(102), g1(114)), from the delta subgroup of proteobacteria (e.g., gl(%)). and from the Gram-positives (e.g., 15(82)) (Fig. 6). Also. within the *Azoarcus nifH* cluster, several up to now uncultured lineages were detected. This demonstrates that traditional cultivation techniques may not only underestimate bacterial diversity but may also miss important members of the diazotrophic community in roots.

Methods for detecting inoculated strains in field experiments

Assessing the benefit of endophytic plant growth-promoting bacteria requires field trials. One important aspect is to determine whether the inoculum has established in or on field-grown plants, or whether soil-borne strains were more competitive in colonization. To identify the inoculated strains. serological techniques may not be sufficient if similar strains are present in the soil. Therefore, we attempted to develop techniques that would allow us to identify the inoculum and that would avoid genetic engineering.

Three rice growth-promoting strains from IRRI were identified in our lab in Marburg as *Bradyrhizobium japonicum* (two strains) and *Agrobacterium tumefaciens* a group of bacteria that also contains rhizobia when the strains harbor *sym* plasmids instead of Ti plasmids (Wang et al 1998, Young and Haukka 1996). To identify these strains in field experiments, a highly variable genomic region may be useful as a target for their molecular detection. A region known to be highly variable in bacteria

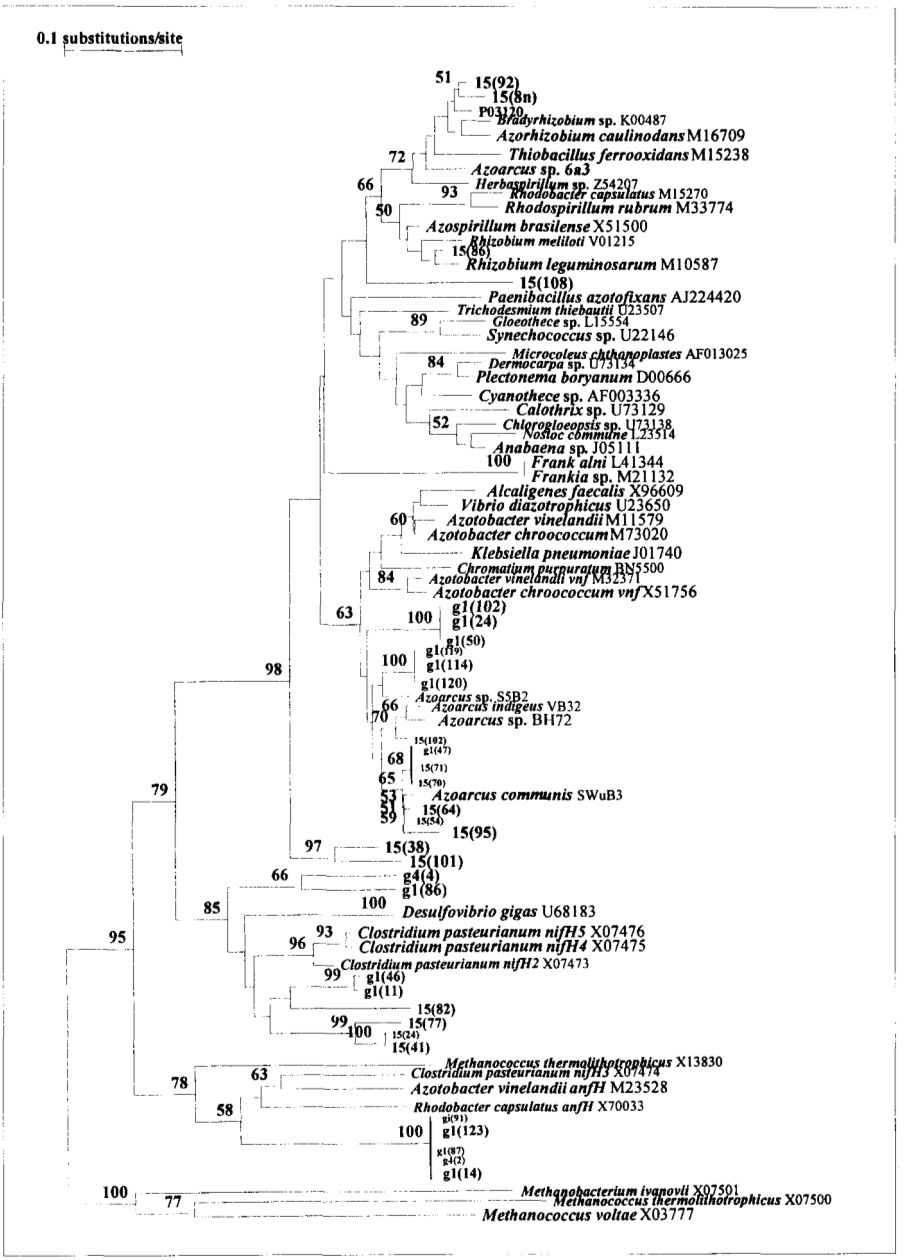

Fig. 6. Phylogenetic relationships among *nifH* gene products of reference strains and DNA clones retrieved from surface-sterilized roots of wild rice (g1(...), *O. minuta*) *and* a modern rice variety (15(...), *O. sativa* R_2Nov_2 B4448) from Nepal. Tree inference was by phylogenetic distance analysis with a neighbor-joining algorithm and Poisson correction including 100 bootstrap replicates with the TREECON program. Accession numbers are given after the names of the organisms. From Engelhard et al (1999).

is the intergenic spacer (IGS) between the 16S and 23S rRNA genes (Otten and Ruffray 1996). A strategy for PCR amplification of this region and successive direct sequencing of the PCR product was developed in our laboratory. High homology to reference strains was only in the areas from which ^{Ile}tRNA and ^{Ala}tRNA are transcribed, which are located between the 16S and 23S rRNA genes (Kundig et al 1995, Otten and Ruffray 1996). The spacer regions in between showed multiple insertions or deletions and sequence variations. A more detailed analysis including several highly related rhizobia1 strains will prove whether this region is suitable for designing PCR primers for a strain-specific nested PCR assay.

Conclusions

For the engineering of a beneficial rice-endophyte association in the future, several lines of research provided promising results:

1. The interior of rice seedlings, especially the aerenchyma, provides a microenvironment suitable for nitrogenase gene expression by endophytes (*Azoarcus* sp.).
2. Putative endophytes of rice roots have been isolated that have not been described previously in rice (diazotrophic *Azoarcus* sp. group D and *S. marcescens*).
3. Diazotrophic and nondiazotrophic bacteria were found to occur in tight association with rice seeds and are capable of systemically colonizing rice seedlings after germination. Many strains are novel, representing separate 16S rDNA lineages; some are widespread, occurring in seeds of different rice varieties, such as a novel nitrogen-fixing *Alcaligenes* group.
4. As yet uncultured lineages of diazotrophs were detected by molecular methods in association with rice roots; thus, the diversity of rice-associated bacteria is higher than expected.

The detection of novel strains may provide a pool of bacteria that can be expected to have some unique features for interactions with rice.

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Evaluating diazotrophy, diversity, and endophytic colonization ability of bacteria isolated from surface-sterilized rice

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Information about the nitrogen-fixing potential, diversity, and sites of colonization of endophytic bacteria from rice is needed to expand our understanding of microbial ecology and plant-microbe interactions in general and more specifically to lay the foundations for future studies aimed at using biologically fixed nitrogen to replace nitrogen fertilizers. Therefore, a collection of 142 bacteria isolated from mechanically abraded, surface-sterilized rice roots was studied. Polymerase chain reaction (PCR)-mediated gene amplification using degenerate primers derived from highly conserved regions of the nitrogenase *nifD* gene revealed 20 isolates harboring *nifD* gene sequences. Southern hybridization analysis confirmed the presence of *nif* genes in 19 of these isolates. The diazotrophic nature of these 19 isolates was confirmed using acetylene reduction assays (ARA). Examination of genetic diversity using amplified ribosomal DNA restriction analysis (ARDRA) and rep-PCR genomic fingerprinting in combination with computer-assisted pattern analysis revealed 56 unique ARDRA and 71 unique rep-PCR genomic fingerprints. Clusters of similar combined fingerprints consisting of 37, 15, 12, and 9 nondiazotrophic bacteria, as well as two clusters each containing 4 diazotrophic bacteria, were found. Analysis of partial small subunit (SSU) ribosomal RNA (rRNA) gene sequences revealed the presence of isolates with similarity to strains from the alpha, beta, and gamma subdivisions of the Proteobacteria and to members of the Bacillaceae and Microbacteriaceae. Many of the ARDRA fingerprints and/or SSU rRNA gene sequences of these bacteria were highly similar to those of other bacteria previously isolated from the rhizosphere of rice. Two isolates from the collection and *Sinorhizobium meliloti,* a control, were tagged with the biomarker *gus* or *gfp,* and the recolonization of rice tissue was examined. Visualization *in situ* of colonization of 3-wk-old inoculated rice seedlings revealed no significant endophytic colonization of rice tissue by these bacteria. Clumps of bacteria, as well as individual cells, however, could be visualized on the surface of the roots. On very rare occasions, an isolated epidermal cell filled with bacteria was observed.

Increased rice (*Oryza sativa* L.) production is clearly essential to meet rising global food demand (Hossain and Fischer 1995. Peoples et al 1995). To meet this demand in a sustainable manner, the amount of industrially produced fertilizer nitrogen used needs to be reduced (Bohlool et al 1992, Ladha et al 1997), One method of achieving this goal would be to use diazotrophic bacteria that make biologically fixed nitrogen available to rice for plant growth. The recent isolation of endophytic nitrogen-fixing bacteria from Brazilian sugarcane varieties capable of high sustainable yields without input of nitrogen fertilizer has suggested that endophytic diazotrophic bacteria may indeed be an efficient means for supplying graminaceous crops with biologically fixed nitrogen (Ladha et al 1997, James and Olivares 1998).

It is not clear, however. to what extent endophytic bacteria colonize crop plants such as rice. A considerable amount of information is available about the nature of diazotrophic bacteria found closely, but not necessarily endophytically, associated with rice. In the early 1970s, acetylene reduction assays (ARA) were used to demonstrate the occurrence and activity of nitrogen-fixing organisms in flooded rice fields (Yoshida and Ancajas 1971, 1973a.b. Dommergues et al 1973). Many of these early studies lacked proper controls or were performed using imprecise techniques, causing considerable disagreement about the actual amount of nitrogen fixation taking place and its impact on rice growth (Hirota et al 1978).

The first diazotrophic bacterium isolated from the rhizosphere in the mid- 1970s was called *"Spirillum"* and was common in the rhizosphere of grasses, including rice (Day and Döbereiner 1976, Döbereiner et al 1976, Lakshmi Kumari et al 1976). The ability of this bacterium (renamed *Azospirillum*) to supply nitrogen to plants has been of considerable interest, and its association with plants has been studied intensively (see Fendrik et al 1995, Vande Broek and Vanderleyden 1995).

Continued isolation efforts discovered additional diazotrophic bacteria associated with rice plants. For example, Watanabe et al (1979) examined different media for isolation of nitrogen-fixing bacteria from rice and found numerous distinct nitrogen-fixing Gram-negative rod-shaped bacteria. suggesting that *Azospirillum* might not be the most common diazotroph associated with rice. They studied the percentage of diazotrophic bacteria isolated from different plant tissues and suggested the lower stem as a possible site for nitrogen fixation (Watanabe and Barraquio 1979). In their study, 81% of bacterial isolates from roots that had been shaken with glass beads to remove exogenous bacteria were diazotrophic.

Other isolation experiments, using a semisolid medium made with exudates from germinating rice seedling as the carbon source, revealed members of the family Enterobacteriaceae as the most common nitrogen-fixing rhizosphere bacteria, followed by *Azospirillum* and *Pseudomonas* spp. (Bally et al 1983). Nitrogen-fixing *Pseudomonas diazotrophicus, Enterobacter cloacae.* and *Klebsiella planticola* strains were also isolated from the rhizosphere of Philippine rice (Ladha et al 1983, Watanabe et al 1987). Moreover, in an extensive study. Oyaizu-Masuchi and Komagata (1988) reported the isolation of >1,000 nitrogen-fixing bacteria from rice roots. Seventy-four of the diazotrophs with the highest acerylene-reducing activity were classified

using a variety of biochemical and morphological tests and shown to include *Xanthobacter autotrophicus, Azospirillum* spp., Enterobucteriaceae spp., *Alcaligenes* spp., *Protomonas*-like bacteria, and *Azotobacter*-like bacteria. However, 52 of the 74 isolates could not be assigned to any known gelno-species group and 30 were not similar to any of the reference genera. These 53 bacteria were morphologically and phenotypically diverse. falling into seven distinct groups (Oyaizu-Masuchi and Komagata 1988).

Bacteria isolated from natural environrnents using current culture techniques represent only 21 small portion of the total number of bacteria observed by microscopy (Amann et al 1995). Therefore, in addition to the culturable bacteria, it is likely that uncultured nitrogen-fixing bacteria contribute to the diversity of diarotrophs in the rice rhizosphere or endorhirosphere. To determine the diversity of nonculturable diazotrophs, phylogenetic studies of nitrogen-fixing bacteria associated with rice roots using PCR primers designed to amplify fragments from *nif* genes have been carried out (Ueda et al 199Sa,b). The DNA sequences of 16 *nifD* and 23 *nifH* PCR products thus generated were found to be distinct from sequences of nitrogen-fixing genes from culturable bacteria. and were found to form separate branches in a phylogenetic tree (Ueda et al 1995a,b). This provides further evidence for the notion that many of the nitrogen-fixing bacterin from the rice rhizosphere remain to be characterized.

The studies summarized above revealed a diversity of diazotrophic bacteria associated with rice roots. but provided little insight into the diversity of bacteria that colonize internal rice tissues. To identify endorhizosphere-related bacteria. Watanabe and Barraquio (1979) attempted to remove rhizoplane bacteria by vigorously shaking the rice tissue with glass beads prior to isolation. While enriching for bacteria from the "inner rhizoplane." this isolation method undoubtedly did not exclude bacteria that adhere tightly to the surface of the root. Other isolation protocols made no effort to distinguish between bacteria from the interior of the root and bacteria from the rhizoplane and rhizosphere or to evaluate their colonization sites. The lack of information about the diversity of bacteria specifically isolated from internal tissues (e.g., endophytes) leaves a gap in our understanding of an important niche in the microbial ecology of grasses such as rice. Information on endophytic bacteria that occupy this niche is needed not only for basic scientific purposes but also in applied contexts because such bacteria may present a vehicle to increase the supply of biologically fixed nitrogen to rice (de Bruijn et al 1995. Stoltzfus et al 1997).

Disagreements exist about the definition of "endophytic" bacteria. Quispel (1992) restricts his definition of endophytic organisms to "nonpathogenic microorganisms that spend a considerable part of their life cycle within the plant host, either in inter- or intracellular locations." Other definitions of endophytes also exclude pathogens (Misaghi and Donndelinger 1990, Frommel et al 1991, Fisher et al 1992). Kloepper et al (1992), however, do not exclude pathogens, nor will we in this article, because of the difficulty of distinguishing between pathogenic and nonpathogenic interactions. especially when condering "quiescent" pathogens and variations in plant reactions to an "endophyte" depending on plant genotype (James and Olivares 1998). Kloepper

et al (1992) excluded bacteria colonizing only the epidermis. We will also exclude this type of bacteria from our definition because it is likely that many opportunistic rhizosphere/rhizoplane bacteria occupy the epidermal niche.

Baldani et al (1997) define obligate endophytes as those that survive only inside the plant host, whereas facultative endophytes can also survive in the soil. Distinguishing between these two classes in a robust manner is difficult. This distinction may be useful, however, for identifying bacteria most suited for future use in crop inoculations because many "obligate" endophytes form more intimate associations with their host plant. Here, endophytic bacteria will be defined as bacteria capable of survival and multiplication inter- or intracellularly, in plant tissues internal to the epidermis.

Bacterial endophytes fitting this description have been isolated from healthy plants, including onion and potato (Frommel et a1 1991), maize (Fisher et al 1992, McInroy and Kloepper 1995, Palus et a1 1996). cotton (Misaghi and Donndelinger 1990, McInroy and Kloepper 1995). Kallar grass (Reinhold-Hurek and Hurek 1998a,b). sugarcane (James and Olivares 1998), C_4 grasses (Kirchhof et al 1997), and rice (Barraquio et al 1997; see below). In terms of their colonization sites, a nonfluorescent *Pseudomonas* sp. has been shown to colonize the vascular tissues of potato (Frommel et al 1991). *Acetobacter diazotrophicus* and *Herbaspirillum* spp. to colonize the vascular tissues of sugarcane (James and Olivares 1998), and *Azoarcus* to colonize the cortex and vascular tissue of rice and Kallar grass (Hurek et al 1994). Little is known about the sites of colonization of other natural bacterial endophytes, especially those associated with rice plants.

Using surface sterilization protocols and most probable number estimates, Barraquio et a1 (1997) calculated the population of putative endophytic bacteria in rice tissues to be between 10^5 and 10^8 CFUs g^{-1} dry weight. The population size of endophytic bacteria varied with the rice genotype examined. Less than 10% of the putative endophytic bacteria isolated were shown to be diazotrophic. The physical presence of diverse endophytic bacteria in rice tissue has been supported by electron microscopic analysis of field-grown rice plants revealing the presence of bacteria with diverse morphologies in the plant root interior (Yanni et al 1997).

The issue of establishing Koch's postulate has been very complex in most cases. Laboratory studies have suggested that certain bacteria isolated from rice tissues can reinfect rice tissues endophytically. For example, Barraquio et al (1 997) used the *gus* marker gene to visualize subepidermal colonization of rice roots by *Herbaspirillum seropedicae,* a diazotroph found associated with many graminaceous crops (Baldani et al 1986). You and Zhou (1988) described the colonization of cortical rice root cells by *Alcaligenes faecalis* A15 bacteria using both light and electron microscopy. *A. faecalis* A15 cells, tagged with the *gus* marker gene, however, were only found in epidermal cells of rice roots (Vermeiren et al 1998). *A. faecalis* cells containing the gus gene under the control of the *nifH* promoter colonizing epidermal root cells of rice were found to have *gus* activity, suggesting that the bacteria were fixing nitrogen (Vermeiren et al 1998). Moreover, a diazotrophic bacterium belonging to the genus *Azoarcus,* isolated from the roots of Kallar grass. has been shown to be able to invade

the cortex and vascular system of rice roots and occupy dead plant cells (Hurek et al 1994). *Azoarcus* cells expressing the *gfp* gene under the control of the *nifH* promoter have also been found in the intercellular spaces of the cortex and in dead epidermal cells, suggesting that the proper physiological environment for the derepression of nitrogen fixation genes can be found in such tissues (Egener et al 1998. Reinhold-Hurek and Hurck 1998b).

Evidence also exists that selected rhizobia can colonize rice tissue. Cocking et al (1995) have reported the presence of rhizobia in the vicinity of short. thick lateral roots, and in intercellular infection pockets. and have reported nitrogen fixation activity following inoculation of rice or wheat seedlings with *Azorhizobium caulinodans* ORS571 or *Rhizobium* ORS310. *A. caulinodans* and *Rhizobium* ORS310 are capable of forming root and stem nodules on *Sesbania rostrata* and *Aeschynomene indica,* respectively. The ability of *A. caulinodans* and other rhizobia to stimulate the formation of short, thick lateral roots. to colonize intercellular spaces between the epidermis and cortex at the point of lateral root emergence, and to occupy apparently dead cortical cells has subsequently been confirmed (Reddy et al 1997, Websteret al 1997). The claim that these bacteria supply fixed nitrogen for rice plant growth. however, has not been substantiated. Another rhizobial strain, *Rhizobium leguminosarum* bv. *trifolii,* has been isolated from field-grown rice plants rotated with Egyptian berseem clover, the legume host of *R. leguminosarum* bv. *trifolii.* These bacteria were shown to be able to colonize the upper portion of the rice root (Yanni et al 1997). Colonization of rice by these bacteria stimulates plant growth. As reported above for other bacteria, however, the observed plant growth stimulation is most likely not due to nitrogen fixation (Yanni et al 1997).

The studies highlighted above confirm that diazotrophic bacteria do interact intimately with rice roots. and in some cases display endophytic colonization patterns. The mechanisms underlying the ability of these bacteria to colonize rice roots in an endophytic manner, however, are poorly understood, and in none of these cases has it been shown that the rice plant benefits from nitrogen fixed by the bacteria. Intracellular colonization of living plant cells by bacteria has also not been demonstrated conclusively.

While progress has been made in understanding the interactions of specific nitrogen-fixing bacteria with cereals and their potential contribution to crop yield. more information is needed about the bacteria capable of invading rice roots, including the diversity of such bacterial endophytes, modes of bacterial invasion, sites of bacterial colonization, competitiveness of endophytes on and in roots, endophytic population sizes, and the ability of the bacteria to fix nitrogen and transfer it to the plant. Previous studies have primarily concentrated on identifying diazotrophic bacteria from the rhizosphere of rice roots or examined the ability of a particular type of bacterium to colonize rice and supply it with nitrogen. A comprehensive study of the diversity of rice endophytes and the sites of endophytic colonization has not been undertaken.

In this study, we attempted to examine the genetic diversity of endophytic bacteria associated with rice and the sites they colonize. Putative rice endophytes were

isolated from mechanically abraded, surface-sterilized rice tissues and a preliminary characterization of their genetic diversity and diazotrophic capacity was carried out (see also Stoltzfus et al 1997). *nifD*-specific PCR primers were developed as part of the latter study and used to identify diazotrophic bacteria in a collection of putative rice endophytes. The genetic diversity of 142 putative enclophytic bacteria was evaluated using amplified ribosomal DNA restriction analysis (ARDRA), rep-PCR genomic fingerprinting, and small subunit (SSU) ribosomal RNA (rRNA) gene DNA sequencing. Moreover, two biomarkers, GUS and GFP. were employed to study the sites and levels of rice colonization of two putative rice endophytes and one *Rhizobium* control strain. These data provide insight into the diversity of bacteria isolated from surface-sterilized rice tissue and the utility of biomarkers for observing colonization of rice tissue by tagged bacteria, and will be useful in the planning of future experiments to study the potential of endophytic bacteria to supply rice with biologically fixed nitrogen.

Phylogenetic diversity of bacteria isolated from surface-sterilized rice plants

We focused on refining the genetic diversity of a collection of 142 bacteria described previously (Stoltzfus et al 1997) using combined ARDRA and rep-PCR fingerprinting analysis (Vinuesa et al 1998, Rademaker et al 1998). This analysis revealed both the presence of clusters of similar bacteria and a variety of bacteria with unique fingerprints. Gelcompar analysis, in conjunction with visual inspection of original fingerprints ofthe 142 rice isolates in this collection, revealed 56 unique combined *Rsa*I, *Msp*I, and *Taq*I ARDRA fingerprints and 7 I unique combined ERIC-. REP-. and BOX-PCR genomic fingerprints. Eight clusters (A to H) were identified that contained four or more isolates with combined ARDRA and rep-PCR fingerprints more similar to each other than the combined fingerprints of replicates of the control strain *A. caulinodans* (Table 1).

Cluster analysis of combined ARDRA and rep-PCR fingerprints using Gelcompar software revealed the presence of groups of similar bacteria that were isolated from different rice varieties grown in different soil types. The clusters of bacteria with fingerprints with a higher similarity level than the replicate fingerprints of *A. caulinodans* are shown in Table I. which also summarizes the number of soil types. rice varieties, and soil type/rice variety combinations from which the strains were isolated. Groups A, B, E, and G exemplify isolates with similar ARDRA fingerprints that have a single dominant rep-PCR genomic fingerprint pattern. For bacteria in these groups, the genotype ofthe strain may be important for interactions with rice. Groups C, D, F, and H exemplify isolates with similar ARDRA fingerprints but distinct rep-PCR genomic fingerprints. For these strains. the genotype of the strain may not be as important in interactions with rice.

Gelcompar analysis of the combined ARDRA and rep-PCR fingerprints revealed only two groups (containing more than two isolates) of diazotrophic bacteria with identical ARDRA fingerprints. The first group of diazotrophic isolates (Group E) contained four strains isolated from two soil types and two rice varieties, representing

Table 1. Clusters of isolates with similar combined fingerprints. This table contains information on the eight clusters containing four or more isolates with combined fingerprints more similar than the replicate fingerprints of *Azorhizobium caulinodans*. The columns contain the following information: group, the letter designation given to each group; strains, the number of strains in each group; soil types, the number of soil types in which plants containing these strains were grown; rice var., the number of rice varieties from which these strains were isolated; comb., the number of soil type/rice variety combinations from which these strains were isolated; ARDRA, the number of unique ARDRA fingerprints present in the cluster; rep-PCR, the number of unique rep-PCR genomic fingerprints present in the cluster. The numbers in parentheses indicate the number of strains within each cluster displaying unique fingerprints.

Group	Strains	Soil types	Rice var.	Comb.	ARDRA	rep-PCR
A	37	5	6	11	1	3(32,4,1)
B	15	3	3	4	2(13,2)	3(10,3,2)
C	12	4	5	9	3(10,1 × 2)	11(2.1 × 10)
D	9	5	3	7	2(5,4)	3(3,3,3)
E	4	2	2	2	1	1
F	4	3	3	4	1	3(2,1 × 2)
G	4	2	1	2	1	1
H	4	3	4	4	1	4(1 × 4)

two soil type/rice variety combinations (Table 1). All bacteria in this group reduced acetylene and harbored *nif* genes using PCR and Southern blot analysis. The combined ARDRA and rep-PCR finger prints of these bacteria were identical (Fig. 1A). The partial DNA sequences of the SSU *rRNA* gene of isolates in this group were most similar to the SSU rRNA gene DNA sequence of genus *Burkholderia.* The ARDRA fingerprints for these isolates share many fragments with the ARDRA fingerprint for M 130, a putative endophytic *Burkholderia* sp. isolated from rice in Brazil (Boddey et al 1995, Hartmann et al 1995). This suggests that the bacteria in this cluster indeed belong to the genus *Burkholderia.*

The second group of diazotrophic bacteria (Group F) contained four bacteria isolated from three soil types and three rice varieties. representing four soil type/rice variety combinations (Table 1). All bacteria in this group reduced acetylene and harbored *nif* genes using PCR and Southern blot analysis. These bacteria had ARDRA fingerprints identical to those of *A. caulinodans* (Fig. 1B). The complete DNA sequence of the R94(7) SSU rRNA gene was determined and was more than 99% identical to the DNA sequence of the *A. caulinodans* SSU *rRNA* gene. *A. caulinodans* forms root and stem nodules on the tropical legume *Sesbania rostrata.* Interestingly. however, isolates R81 (90), R94(17), and R99(65) were unable to nodulate *S. rostrata.* The relationship between isolates in Group F and *A. caulinodans* was further investigated using restriction fragment length polymorphism (RFLP) analysis. No polymorphisms were found between R94(17) and *A. caulinodans* genomic DNA digested with *BamHI, HindIII.* or *EcoRI* and hybridized with the *A. caulinodans nifD* gene. Like-

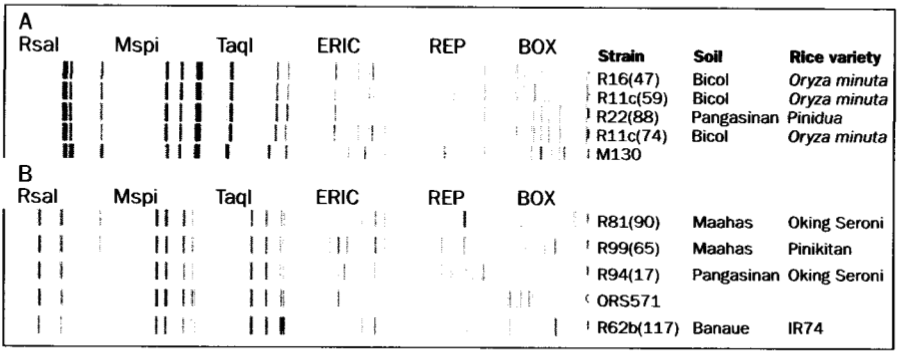

Fig. 1. Fingerprints from clusters of nitrogen-fixing bacteria. Shown here are the combined ARDRA and rep-PCR fingerprints of the isolates in the two clusters of nitrogen-fixing bacteria. The strain designation, soil type, and rice variety from which the strain was isolated are indicated in the three vertical columns following the fingerprints. (A) Fingerprints from isolates similar to *Burkholderia,* (B) fingerprints from isolates similar to *Azorhizobium caulinodans* (ORS571).

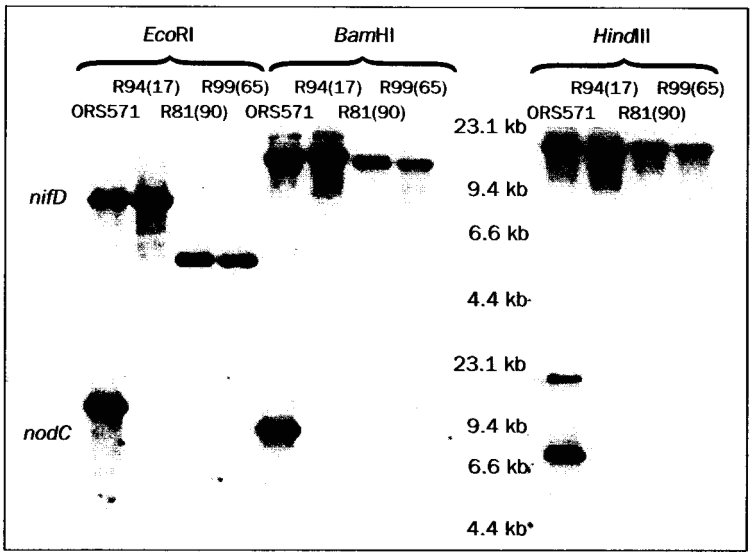

Fig. 2. Southern blots of genomic DNA from three isolates similar to *Azorhizobium caulinodans* (ORS571). The genomic DNA was digested with the restriction enzyme *Eco*RI, *Bam*HI, or *Hind*III and hybridized with a *nifD* or *nodC* gene probe.

wise, genomic DNA from strains R90(8) and R99(65) digested with *Bam*HI and *Hind*III and hybridized with the *A. caulinodans nifD* gene showed RFXP patterns similar to those of *A. caulinodans.* Polymorphisms between these two strains and *A. caulinodans* were detected, however, when genomic DNA was digested with *Eco*RI and hybridized with the *A. cadinodans nifD* gene (Fig. 2). Interestingly, no hybridization signal was detected with the *A. caulinodans nodC* gene in Southern hybridization analysis

of genomic DNA from R81(90). R94(17), and R99(65) (Fig. 2), providing an explanation for the observed inability of these isolates to nodulate *S. rostrata.* This suggests that the bacterial isolates in this group are very closely related to, but clearly distinct from, *A. caulinodans*

Studies on rice root colonization by selected bacteria

As pointed out above, the long-term goal of our experiments is to isolate and characterize stable "endophytic" bacteria from rice plants. Our expectation was that the isolation of bacteria from mechanically abraded, surface-sterilized rice plant tissue (Stoltzfus et al 1997) would enrich for endophytic bacteria. It was also recognized, however, that some nonendophytic bacteria would "escape" surface sterilization and be isolated along with the endophytic bacteria. Therefore, the ability of these bacteria to recolonize plant tissue endophytically had to be verified. To accomplish this, we focused on reinfection of gnotobiotically grown rice seedlings with selected bacterial isolates and *in planta* microscopic localization of bacteria harboring different biomarkers.

First, reisolation experiments were carried out to determine whether bacteria previously isolated from surface-sterilized rice tissue were able to recolonize rice seedlings. Fourteen bacterial isolates were selected for this analysis ("test collection"; see Stoltzfus et al 1997). The number of bacteria isolated from different seedlings reinoculated with the same isolate showed considerable variability. No bacteria could be reisolated from seedlings inoculated with *Escherichia coli, Azoarcus indigens, Herbaspirillum seropedicae,* or strain R90(8). In the case of all other bacterial strains, bacteria could be reisolated from at least one test seedling.

In principle, it should be possible to visualize the colonization of rice by putative endophytes using light microscopy, fluorescence microscopy, and electron mcroscopy (You and Zhou 1988, Hurek et al 1994, Yanni et al 1997, Reddy et al 1997). Unless the location of colonization can be predicted, however, it is difficult to locate the bacteria by random scanning of tissues. Moreover, unless the plants are grown under gnotobiotic conditions or highly specific antibodies are available, the identity of the bacteria observed will remain questionable.

Recently, however, different types of biomarkers have become available that facilitate tracking bacteria in the environment, including plants (Wilson et al 1995, Unge et al 1998, Jansson and de Bruijn 1999). The use of biomarkers facilitates the localization and unambiguous identification of endophytes in infected plant tissues (Hurek et al 1994, Barraquio et al 1997, Reddy et al 1997, Webster et al 1997, Vermeiren et al 1998). In the studies cited above, the biomarker ß-galactosidase (LacZ) or ß-glucuronidase (GUS) was employed. We chose to use the green fluorescent protein (GFP) and GUS biomarkers because they offer distinct advantages such as low background and easy detection (Jansson and de Bruijn 1999, Stoltzfus et al 1999). Both of these markers have been used successfully to detect tagged bacteria in (legume) plant tissues, such as those resulting from infection with rhizobia (Wilson et al 1995, Gage et al 1996).

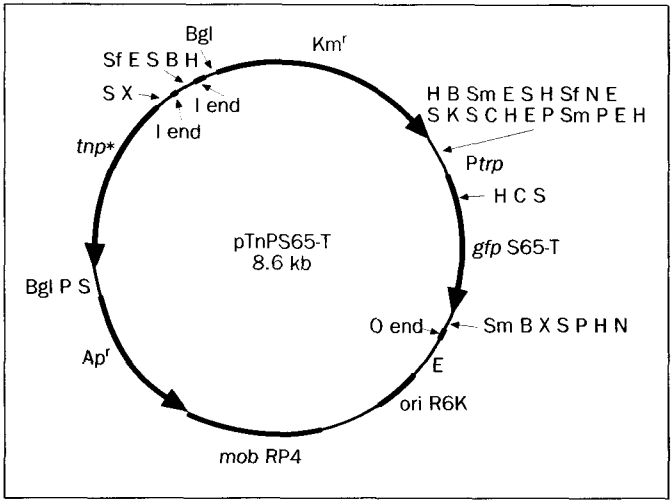

Fig. 3. Map of pTnPS65-T. Structure of plasmid pTnPS65-T, constructed for the delivery of a mini-Tn5 transposon containing a kanamycin resistance cassette and the gene for the S65T mutant of GFP controlled by the Ptrp promoter. Restriction sites: B, *Bam*HI; Bgl, *Bgl*I; C, ClaI; E, *Eco*RI; H, *Hind*III; K, *Kpnl;* N, Nod; P, *Pst*I; S, *Sal*I; Sf, *Sfi*I; Sm, *Sma*I; X, *Xba*I.

Multiple attempts were made to obtain green fluorescent derivatives of bacteria of the "test collection" (Stoltzfus et al 1997) by introducing *gfp* containing transposons from pRL765gfp (Tombolini et a1 1997) or pTnPS65-T (Fig. 3) into 14 rice isolates and 9 previously described rice rhizosphere/endophytic reference strains (Stoltzfus et a1 1997). In selected cases, *E. coli* cells. harboring the conjugation helper plasmid pRK2013, were added to the mating mixture to boost the transfer efficiency. Mating mixtures of each strain were spread on plates containing different selective media. Kanamycin-resistant transconjugants were obtained in approximately 40% of the cases. Detectable green fluorescence was observed for only six of the kanamycin-resistant transconjugants. Three transconjugant bacteria. *Entobacter cloacae, Klebsiella planticola,* and R66(40), showed intense green fluorescence following the introduction of the transposon from pRL765gfp. The fluorescence was rapidly lost, however. when the transconjugants were removed from kanamycin selection. Only three bacterial strains, *S. meliloti::gfp,* R33:: *gfp* and R 100:: *gfp,* displayed stable, detectable green fluorescence levels following the introduction of transposon TnPS65-T (Fig. 4).

Transposon Tn121TH1 (Stoltzfus et a1 1999) was subsequently introduced into *S. meliloti,* R33(120). and R100(64) to obtain *S. meliloti::gus.* R33:: *gus,* and R100:: *gus* marked derivatives. all of which had GUS activity (data not shown).

Both test tube-grown rice seedlings as well as seedlings grown in the "pillow system" described by Szczyglowski et al (1998) inoculated with the tagged strains were examined for colonization patterns. The root systems of rice seedlings inoculated with *S. meliloti::gfp,* R33:: *gfp,* and R100:: *gfp* were observed using fluorescence microscopy (12 seedlings per strain). Likewise. the root systems of rice seedlings

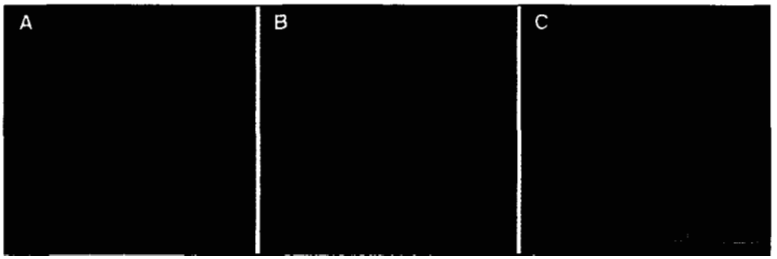

Fig. 4. Bacteria marked with GFP. Fluorescent micrographs of three bacterial strains expressing GFP are shown. These strains were used for visualization of colonization of rice seedlings. (A) *Sinorhizobium meliloti::gfp.* used as a control. (B) R33::*gfp.* and (C) R100::*gfp,* isolated from surface-sterilized rice. The bacteria were tagged with the transposon TnPS65-T. Bacteria were grown in liquid TY media and placed on glass slides in 0.8% agarose. The micrographs were taken using a Kodak Digital Science DC120 digital camera attached to a Zeiss Axiophot microscope equipped with a standard FITC filter set.

inoculated with *S. meliloti::gus.* R33::*gus.* and R100::*gus* were examined following incubation with X-Gluc by eye and via light microscopy (four rice seedlings per strain).

When bacteria harboring the GUS biomarker were used to infect rice seedlings. blue staining. indicating the presence of large numbers of bacteria, could generally be observed without the aid of a microscope (Fig. 5). The staining of plants grown in test tubes was more pronounced in all cases. The degree of staining varied between seedlings inoculated with the same tagged bacterial isolate. In general, however, differences in the degree and distribution of GUS staining in rice seedlings inoculated with the tagged bacterial isolates were not observed. GUS staining was most pronounced in primary and secondary roots proximal to the seed. Infrequently, staining of root tips was also observed (Fig. 5). Seeds were darkly stained in most cases (Fig. 5). These observations suggest that bacteria colonize the root system primarily on the upper portions of the primary and secondary roots.

GFP-mediated bacterial fluorescence could not be detected on rice roots using an ultraviolet transilluminator or a CCD camera equipped with proper filters for GFP excitation and emission detection. Fluorescence could only be detected using a 25x or greater immersion lens and an epifluorescent microscope. Therefore. the search for areas with a high degree of colonization required scanning the entire root system using an epifluorescent microscope, which proved to be a time-consuming process. More fluorescent bacteria were observed on the upper portion of the primary and secondary roots of plants inoculated with strains expressing GFP, supporting the observations made using the tagged bacteria expressing GUS (data not shown).

Clumps of bacteria and individual bacteria cells, tagged with either GUS or GFP, could be observed on the root surface (Fig. 6). Roots colonized by bacteria expressing GFP were further examined using laser-scanning confocal microscopy, which allows optical sectioning (Fig. 7). A careful determination of spatial relationships between bacteria and plant tissues was made using software that creates three-dimensional images from optical sections (Fig. 8). Observations of the relationship

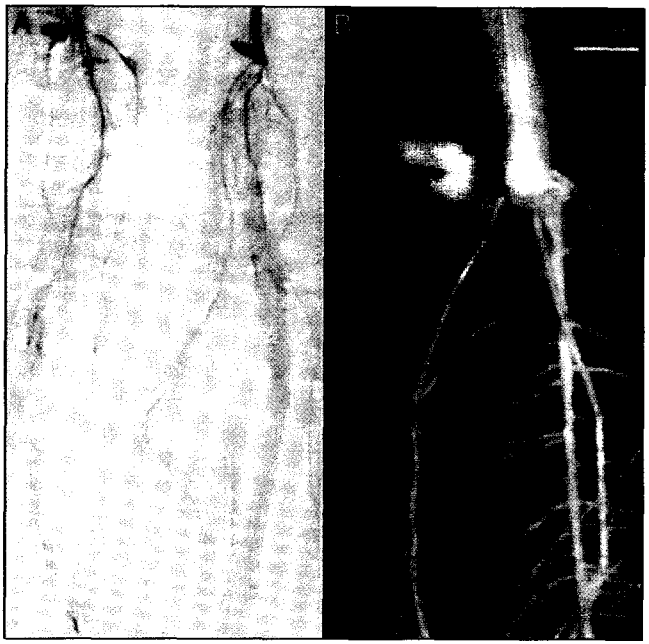

Fig. 5. GUS staining of rice plants. Pictures of rice seedlings inoculated with bacteria expressing the GUS biomarker incubated in buffer containing X-Gluc are shown. The blue color is indicative of bacterial GUS activity. (A) Colonization patterns of R100::gus bacteria on 2-wk-old rice roots, (B) seed colonization pattern of plants inoculated with R33::gus bacteria.

between the plant and bacteria marked with GFP were facilitated by staining the plant with a fluorescent dye such as propidium iodide (Gage et al 1996, Figs. 6–8),which causes the edges of plant cells to emit red fluorescence when excited with blue light.

No significant endophytic colonization by GFP-tagged *S. meliloti,* R33(120), or R100(64) bacteria was observed. Bacteria colonized the rice root surface in clumps, often at the base of root hairs (Figs. 6 and 8). Individual GFP-tagged cells were also observed associated with root hairs and along the junctions of epidermal cells (Fig. 6). No qualitative differences in colonization efficiency were observed between the three different tagged strains. On rare occasions (four cells in 21 root systems examined), an epidermal cell harboring a large number of R 100::*gfp* bacteria was observed (Figs. 7 and 8).

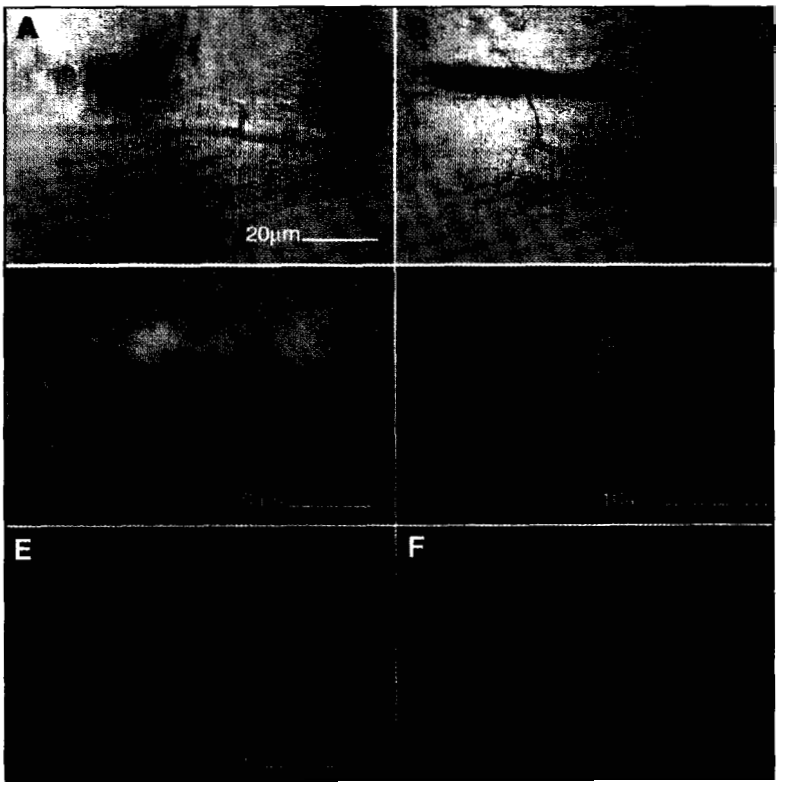

Fig. 6. Microscopic observation of colonization. Micrographs of plants inoculated with bacteria expressing the biomarker GUS (A and B) or GW (C-F) are shown. Transmitted micrographs taken using a Leitz microscope of (A) R100::*gus* bacteria (blue color) in a clump on the surface of a rice root and (B) colonizing an epidermal cell. Overlay of a transmitted and fluorescent micrograph of (C) *S. meliloti::gfp* bacteria (green color) in a clump on the surface of a rice root and (D) individual bacterial cells at the junction between epidermal cells. fluorescent micrographs of rice roots stained with propidium iodide (red color) and colonized by (E) R33::*gfp* bacteria (green color) at the junctions of epidermal cells and (F) R100::gfp bacteria (green color) in clumps on the surface of the root.

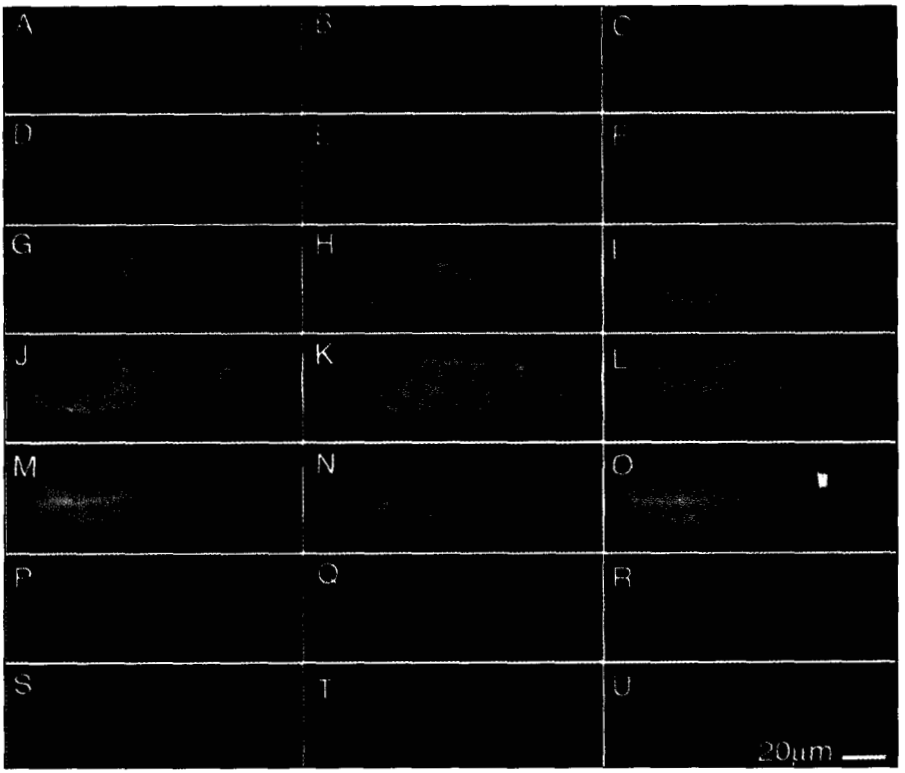

Fig. 7. Optical sections of a rice cell colonized by R100(64) expressing GFP. Optical sections of an epidermal cell colonized by R100::*gfp* bacteria (green color). Each section is 0.3 μm deeper into the sample. The rice root tissue was stained with propidium iodide (red color). Section one starts at the surface of the rice root and section 80 is 24 μm below the surface of the root. (A-E) Individual optical sections 1 through 5, (F) overlay of sections 1-5. (G-N) overlays containing ten optical sections, starting with sections 1 through 10 and ending with sections 71 through 80, (0) overlay of all 80 optical sections, (P-T) individual optical sections 76 through 80, (U) overlay of optical sections 76 through 80.

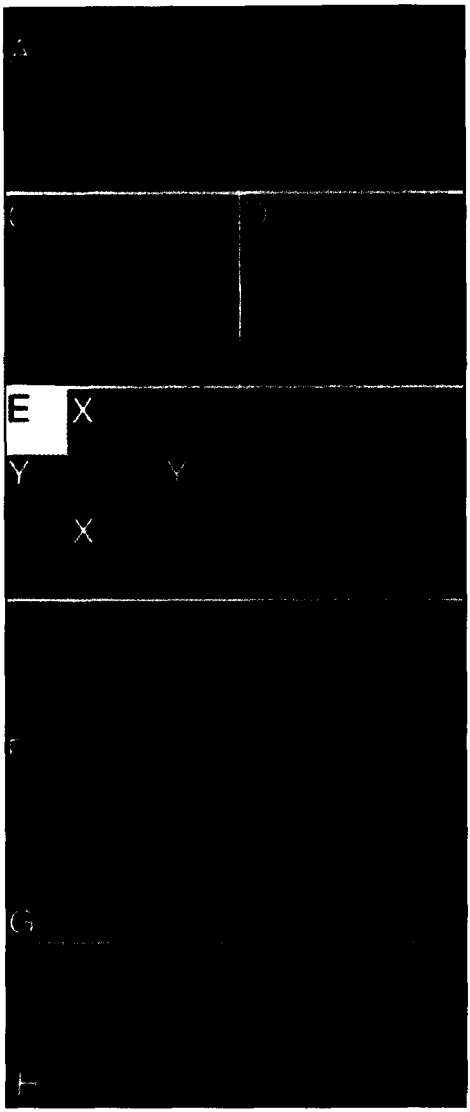

Fig. 8. Three-dimensional computer reconstructions of rice root colonization by bacteria expressing the GFP biomarker. These renderings are constructed from optical sections made using a laser-scanning confocal microscope. (A and B) Two views of an epidermal root cell and attached root hair filled with R100:: *gfp* bacteria (green color), (C and D) two views of R33:: *gfp* bacteria (green color) colonizing the surface of a rice root (red color). Artificial colorization is based on fluorescent intensity. A low level of yellow autofluorescence from the root tissue is depicted in red. The intense green fluorescence from the tagged bacteria is depicted in green. (E-H) Images created using dual-channel detection of R100:: *gfp* bacteria colonizing an epidermal cell on a rice root. The red fluorescence is from propidium iodide-stained root tissue. The green fluorescence is from R100:: *gfp* bacteria. (E) Phi Z sections along the X and Y axis. (F) Oblique view of the 3D reconstruction showing only the root tissue (red channel), (G) only the bacteria (green channel), and (H) the root tissue and bacteria (red and green channels) combined.

Genetic diversity of putative endophytic bacteria

Bacteria isolated from mechanically abraded, surface-sterilized rice roots displayed diverse ARDRA and rep-PCR fingerprints. Seventy-one unique rep-PCR genomic and 56 unique ARDRA fingerprints were observed among the 142 isolates studied. Both Gram-positive and Gram-negative bacteria were represented in the collection of rice isolates analyzed. The genetic diversity of the isolates analyzed can be explained in several equally plausible ways. It is possible that these bacteria are present in numerous unique ecological or physiological "niches" associated with rice plants and the genetic diversity of bacteria simply reflects niche diversity. It is also possible that these bacteria are present in a few unique niches, and the genetic diversity of the bacteria indicates that a variety of bacteria can colonize a few distinct niches.

The importance of plant and bacterial genotypes in plant colonization provided us with limited insight into the specificity of these interactions. While the data from our study do not contain the numbers required for statistical analyses, it appears that in many cases the interactions we observed between rice plants and putative endophytes are nonspecific. The rice plant genotype does not appear essential for colonization by specific bacteria in our collection. Every cluster containing four or more bacteria harbored isolates from several rice varieties and soil types (Table 1), indicating that each type of bacteria isolated is capable of interacting with a broad variety of rice genotypes. Likewise, in some cases, the bacterial genotype does not appear essential for colonization of a specific rice genotype because bacterial isolates with a similar SSU *rRNA* gene structure, but diverse genomic fingerprints, colonized the same rice variety. Groups C and D both contained bacteria that were isolated from the same rice variety grown in the same soil type and displayed identical ARDRA- but diverse rep-PCR fingerprint patterns (Table 1).

In selected cases, however, the bacterial genotype may be important for interactions with rice plants. Groups A, B, E, and G all contain bacteria with a single prominent rep-PCR genomic fingerprint. This could indicate that for some types of bacteria a specific genotype confers an advantage when interacting with the rice plants.

Partial SSU *rRNA* gene DNA sequences indicated that selected isolates from this collection are related to endophytes previously isolated from rice, or bacteria previously isolated from the rhizosphere of rice and legume symbionts.

For example, diazotrophs were found to be isolates from group E and have SSU *rRNA* genes similar to those of *Burkholderia vietnamiensis* and M 130. *B. vietnamiensis* was isolated from rice tissue and is the only nitrogen-fixing species of Burkholderia (Gillis et al 1995). M130 is a putative endophytic *Burkholderia* strain isolated from rice in Brazil (Boddey et al 1995, Hartmann et al 1995). The diazotrophic strain R48b has SSU rRNA genes similar to those of *Azoarcus* sp6a3, a diazotrophic endophyte of Kallar grass (Hurek et al 1997b). Some strains of *Azoarchus* are able to colonize rice endophytically (Hurek et al 1994, this volume). In addition, a *nifH* DNA sequence derived from DNA isolated from rice roots in Japan (Ueda et al 1995b) was similar to *Azoarcus nifH* sequences (Hurek et al 1997a). Our isolation of *Burkholderia*- and *Azoarcus* -like bacteria, in conjunction with previous observations, clearly suggests

that these bacteria may colonize rice endophytically and should be considered as targets for further studies.

Interestingly, no bacteria with SSU *rRNA* genes similar to those of *Herbaspirillum* spp. were found in our collection. Herbaspirillurn has been reported as a common diazotrophic endophyte of graminaceous plants, including rice (Baldani et al 1986, James and Olivares 1998).

Selected diazotrophic bacteria in our collection had SSU *rRNA* genes similar to those of *Xanthomona flavus, Pseudomonas,* and *Azospirillum,* diazotrophic bacteria commonly isolated from the rhizosphere of rice (Ladha et al 1982, Bally et al 1983, Watanabe et al 1987, Oyaizu-Masuchi and Komagata 1988, Reding et al 1991). It is unclear whether these bacteria are truly endophytic strains or simply rhizosphere bacteria that "escaped' surface sterilization.

The isolates in group F had SSU rRNA gene sequences similar to those of *A. caulinodans,* the unusual stem- and root-nodulating symbiont of the tropical legume *Sesbania rostrata* (Dreyfus and Dommergues 1981). They clearly differ from *A. caulinodans,* however, because they do not harbor the *nodC* gene and are unable to form nitrogen-fixing nodules on *S. rostrata.* To our knowledge, this is the first report of the isolation of *Azorhizobium*-like bacteria from field-grown rice. Previous studies, however, have shown that *A. caulinodans* is capable of colonizing rice under laboratory conditions (Christiansen-Weniger 1996. Webster et al 1997, Khokhar and Qureshi 1998) and field conditions (Ladha et al 1989). Moreover, *A. caulinodans* is thought to be a promising candidate for supplying rice with biologically fixed nitrogen because, unlike other rhizobia, it is able to fix nitrogen independent of its legume host (Gebhard et al 1984) and can tolerate relatively high levels of O_2 during nitrogen fixation (Ratet et al 1989). Therefore, the isolates in group Fare also excellent candidates for further studies on the use of bacteria to supply biologically fixed nitrogen to rice.

In addition to the isolates sharing similarities with *Azorhibium,* ten isolates in this collection had SSU *rRNA* genes similar to those of *Rhizobium, Bradyrhizobium,* and *Agrobacterium* species. Five of these isolates are diazotrophic, including two with SSU *rRNA* genes similar to those from *Agrobacterium. Agrobacterium* is closely related to *Rhizobium,* and when *nif* and *nod* genes from *Rhizobium* are introduced into *Agrobacterium* it can interact with legumes and fix nitrogen (Hirsch et al 1985, Martinez et al 1987, Novikova and Safronova 1992). As discussed below, the interactions between these bacteria and rice plants also deserve further investigation.

Several authors have suggested extending nodulation to rice as a method of supplying rice with biologically fixed nitrogen (Ladha et al 1997, Reddy et al 1997, Kennedy et al 1997). When examining this possibility, the ability of *Rhizobium* and related species to interact with rice has been of keen interest. For example, *R. leguminosarum* has been isolated from surface-sterilized rice grown in Egypt and shown to colonize rice grown under gnotobiotic conditions (Yanni et al 1997). The production of nitrogen-fixing nodules on legumes, however, is a complex process requiring the development of specialized structures by the plant host (van Rhijn and Vanderleyden 1995, Long 1989), a process that clearly does not take place in the

interaction between rhizobia and rice (de Bruijn et al 1995, Reddy et al 1997, Webster et al 1997). Therefore, further investigations of the interactions between putative rhizobia1 endophytes from rice, which may be well adapted for rice colonization, are clearly needed and may provide information useful for future attempts to extend nodulation to rice and other cereal crops.

As pointed out in the introduction, it has been proposed that endophytic bacteria should be divided into obligate and facultative endophytes (Baldani et al 1997). Facultative endophytes can survive in the soil and on the plant surface as well as in the interior of the plant, *Azospirillum* strains capable of colonizing the interior of the plant could be considered facultative endophytes because they are also commonly found in the rhizosphere (Baldani et al 1997). Bacteria such as *Herbaspirillum* spp. and *Burkholderia* spp., however, seem to be found only inside plant tissues and could be considered to be obligate endophytes. Assuming that bacteria isolated from mechanically abraded, surface-sterilized rice plants are capable of colonizing the interior of the plant to some extent, the bacteria in this collection can also be divided into obligate and facultative endophytes.

Bacteria in Groups A and B, the largest groups in the collection, as well as isolates with partial SSU *rRNA* sequences similar to those of *Azospirillum* spp. and *Xanthobacter* spp., should be tentatively classified as facultative endophytes because Pseudomonads, Enterobacteriaceae, *Azospirillum* spp., and *Xanthobacter* spp. are commonly isolated from the rhizosphere of rice (Bally et al 1983, Ladha et al 1983. Watanabe et al 1987, Oyaizu-Masuchi and Komagata 1988, Reding et al 1991).

Bacteria in Groups C. D, and E, and those with SSU *rRNA* genes similar to those of A:onrcus spp. should be tentatively classified as obligate endophytes because *Microbacterium* spp., *Alcaligenes* spp., *Burkholderia* spp., and *Azoarcus* spp. have not been isolated as common rhizosphere bacteria from rice. Many of the common nitrogen-fixing isolates from the rhizosphere of rice, however, have not been classified (Bally et al 1983, Oyaizu-Masuchi and Komagata 1988). Additional evidence that some of these isolates may be obligate endophytes comes from the observations that *Azoarcus* spp. have never been isolated from soil (Reinhold-Hurek and Hurek 1998a) and that *Burkholderia* spp. appear to require a living plant host for survival (Baldani et al 1997).

Bacteria from Group F and those with partial SSU *rRNA* gene sequences similar to those of rhizobia and *Agrobacterium* spp. could be classified as either facultative or obligate endophytes. These bacteria have not been reported as common isolates from the rhizosphere of rice. It is known, however, that rhizobia and *Agrobacterium* spp. can survive well in the soil environment.

As pointed out above, the difference between pathogen and endophyte is often quite subtle (James and Olivares 1998). It is possible for two very similar bacteria to have drastically different interactions with plants. Different subspecies of *Clavibacter xyli,* distinct species of *Herbaspirillum,* and *Agrobacterium* spp./*Rhizobium* spp. are examples of bacteria having endophyticlsymbiotic interactions with plants in some cases and pathogenic interactions in others. In some cases, the type of interaction may vary depending on the plant genotype or environmental conditions. Because of this.

great care must be taken when proposing the use of endophytic bacteria as inoculum for supplying crops with biologically fixed nitrogen. Bacteria such as *Azoarrcus* spp. or *Azorhizobium* spp., which have no close relatives known to cause plant disease. may be more suitable for these approaches than *Burkholderia* spp. or *Herbaspirillium* spp., which include phytopathogenic isolates.

In conclusion, the phylogenetic part of this study shows that genetically diverse bacteria can be isolated from mechanically abraded, surface-sterilized rice tissue. It reveals that many of these bacteria are similar to bacteria commonly isolated from the rhizosphere of rice, but others are not. It also demonstrates that a variety of nitrogen-fixing bacteria can be isolated from this niche. Some of these nitrogen-fixing bacteria, specifically those similar to *Azoarcus* spp.. and *Azorhizobium* spp., are sometimes found in the rhizosphere of rice (Hurek et al 1994. Ladha et al 1989), can colonize rice under laboratory conditions, are not closely related to known plant pathogens, and have been isolated from surface-sterilized rice tissue at multiple sites. Further study of these bacteria will increase our understanding of plant-microbe interactions and may provide new insights into the possibility of using nitrogen-fixing endophytes to supply rice with biologically fixed nitrogen.

Use of *gfp* and *gus* biomarker genes to track tagged bacteria *in planta*

Isolation of bacteria from mechanically abraded, surface-sterilized plant tissue has been proposed to lead to the isolation of endophytic bacteria that engage in intimate interactions with plants. To examine this proposal, we selected several putative rice endophytes isolated using this approach and observed their ability to recolonize rice tissues. Most of these putative endophytes could be reisolated following inoculation and growth on gnotobiotic seedlings. No bacteria (colony-forming units, CFUs). however, could be reisolated from seedlings inoculated with control bacteria, including *E. coli.* suggesting that bacteria not normally found to be endophytes of rice do not generally survive the surface sterilization protocol used.

Interestingly, two putative rice endophytes, *Herbaspirillum seropedicae* and *Azoarcus indigens,* could not be reisolated after inoculation on gnotobiotically grown seedlings. This suggests that these bacteria do not colonize 3-wk-old Lemont rice seedlings endophytically, or that the surface sterilization protocol used was too harsh. Other putative endophytic bacteria from our collection, however, were able to survive this same surface sterilization protocol, suggesting that these bacteria are better protected from the sterilizing agent than *H. seropidicae* or *A. indigens.* This protection may be the result of more aggressive endophytic colonization. A large variation was observed in the number of reisolated bacteria from different seedlings inoculated with the same strain, however, making quantitative interpretation of the data impossible.

As pointed out above, a variety of molecular markers are now available that allow the detection and monitoring of bacteria in the environment (Akkermans et al 1995, 1997, 1998, Jansson and de Bruijn 1999). The GUS and GFP molecular markers were selected to facilitate the visualization of bacteria colonizing rice seedlings in

our study. Our initial studies revealed that each marker has its advantages. For example, colonization of plant tissue by bacteria expressing GUS could easily be detected at the macroscopic level by an intense blue color (Fig. 4). This allowed the examination of colonization patterns using entire infected root systems (Fig. 4). It was difficult. however. to determine three-dimensional spatial location of GUS-tagged bacteria in relation to the surface of the root at the microscopic level. In addition, diffusion of the cleaved GUS enzyme substrate prior to dimerization caused the appearance of blue color in areas not directly colonized by bacteria.

Visualization of bacteria expressing GFP overcame both of these problems. Because bacteria expressing the gene encoding GFP could be detected using fluorescent microscopy (Fig. 4), laser-scanning confocal microscopy could be used to generate optical sections of infected tissue and to create images that depicted three-dimensional spatial relationships (Figs. 7 and 8). In addition, GFP does not diffuse, rendering localization quite precise and reproducible. In our experience, however, bacteria expressing GFP in root systems were detectable only with the aid of high-power objectives in conjunction with epifluorescence or laser-scanning microscopes. This feature rendered GFP less suitable than GUS as a biomarker for examining macroscopic colonization patterns.

Moreover, although GFP has been used successfully as a biomarker/bioreporter in a wide variety of bacteria (Stoltzfus et al 1999), in our hands repeated attempts using several protocols and two different GFP transposons resulted in the tagging of only three bacteria that displayed stable, detectable levels of green fluorescence. There are several possible explanations for the failure to tag most of the bacterial isolates in the "test collection'' with GFP.

First, it is possible that the *gfp* gene is not expressed at high enough levels to allow detection of the GFP protein. For example, in the case of pTnPS65-T (Fig. 3), it is possible that the *Ptrp* promoter is not active in some of the rice isolates. The *Ptrp* promoter is active in *S. meliloti* 1021 (Gage et a1 1996: Fig. 4). *E. coli* cells harboring pTnPS65-T, however, are not fluorescent. It is possible that *E. coli* and other bacteria do not express genes controlled by this promoter properly, explaining why nonfluorescent kanamycin-resistant transconjugants were found. Second, the *gfp* gene and the *npt* antibiotic resistance gene contained on pRL765gfp are both under the control of the *PpsbA* promoter. Therefore, kanamycin-resistant transconjugants would be expected to also express GFP. None of the stable kanamycin-resistant transconjugants obtained in this study, however, displayed observable levels of green fluorescence or, at best, they showed faint fluorescence that faded rapidly. This suggests that, although the *gfp* gene is expressed at low levels, the fluorescence emitted from the GFP protein produced was insufficient for detection.

Alternatively, the GFP protein may not be processed correctly and/or sequestered in nonfluorescent inclusion bodies (Heim et al 1994). Difficulties in obtaining stable green fluorescent transconjugants may also be due to minor toxic effects produced by GFP. This hypothesis is supported by other evidence, including the difficulty in constructing plasmids with the *gfp,* gene under the control of strong constitu-

tive promoters (Stoltzfus and de Bruijn, unpublished data: Herman Spaink, personal communication).

Finally, it is also possible that the transposons were not properly integrated into the genomes of these bacteria. Plasmid pTnPS65-T (Fig. 3) carries the origin of replication of R6K and therefore requires the Pir protein for replication (de Lorenzo et al 1990). This plasmid should not replicate in most bacteria. Plasmid pRL765gfp carries the pRK2 origin of replication, which replicates in *E. coli* and other Enterobacteriaceae. Three bacteria, *Enterobacter cloacae, Klebsiella planticola,* and strain R66(40), showed intense green fluorescence following the introduction of plasmid pRL765gfp. When removed from kanamycin selection, however, the green fluorescence was quickly lost. The most logical explanation is that the pRL765gfp plasmid was replicating autonomously in these bacteria and that the transposon never integrated into the genome. Because of the rapid loss of fluorescence in the absence of a selectable marker, these bacteria could not be used for *in situ* colonization studies.

Derivatives of three types of bacteria. *S. meliloti* 1021, R33(120), and R 100(64), were obtained that did express GFP and GUS. No observable differences in colonization patterns were observed between different transconjugants of the same isolates. Nor were major differences in colonization patterns observed when comparing the same isolate expressing GFP or GUS. Bloemberg et al (1997) observed no difference in growth and motility of *Pseudomonas* strains harboring the *gfp* gene on a plasmid, suggesting that GFP activity did not constitute an excessive metabolic burden in this Gram-negative bacterium. Therefore, the integration of only two genes into the genome of these isolates would not be expected to add significantly to metabolic load and/or alter colonization patterns.

Two to 3 wk after inoculation of R33(120) or R100(64) to Lemont rice seedlings grown in the pillow system, endophytic colonization could not be observed. Colonization was limited to the surface and, on rare occasions, to isolated epidermal cells (Figs. 7 and 8). This does not necessarily mean that these bacteria are not endophytic in nature. One possible explanation is that they were isolated from Philippine rice varieties at the heading stage. It is therefore possible that endophytic colonization does not take place until later in rice plant development. It is also possible that Lemont is not amenable to colonization by these isolates or that colonization does not take place because of a shortcoming of the pillow system. For example, colonization may require the mechanical wounding that takes place when a root pushes its way through field soil. Some endophytes may also use fungi as aids for endophytic establishment (Paula et al 1991). If colonization required the partnership between the tagged bacterial isolate and a fungus or other bacteria, colonization would not be expected to occur in the gnotobiotic pillow system.

Bacteria expressing GFP could be detected using the laser-scanning confocal microscope. This also allowed optical sectioning (Fig. 7) and three-dimensional reconstruction of the images (Fig. 8). Without endogenous fluorescence of rice tissue/cells, it was not possible to gain information on the spatial relationship between the bacteria and the plant tissues. In selected cases, the autofluorescence of plant tissue

could be used to orient the viewer (Fig. 8). In our experience, however, the yellow fluorescence from rice roots was quite variable and unsuitable for reliable three-dimensional reconstruction. Propidium iodide can be used to fluorescently stain plant tissues (Gage et al 1996). With the proper instrumentation, this adaptation of the protocol allowed real color optical sectioning and three-dimensional reconstruction of the images (Figs. 7 and 8).

The fluorescence observed using propidium iodide was very bright and tended to mask weak GFP signals, even after short staining times and long periods of destaining. This may not be a problem for digital imaging because the relative intensities of the different fluorescence could be altered; however, it created problems for scanning infected tissues using the oculars of a standard epifluorescent microscope. In addition, the staining of the plant tissue was variable, with intense staining in some areas and virtually no staining in others. Therefore, development of better stains or staining techniques would greatly enhance the use of GFP as a biomarker for bacterial colonization of plant tissues.

In conclusion, the reinfection and microscopy section of this study establishes that GFP and GUS are promising tools for visualizing endophytic colonization of rice and other plants. Better vectors with suitable promoters and new versions of GFP, however, need to be developed for this biomarker to reach its full potential. GFP is useful for observing microscopic colonization by individual bacterial cells, or clumps of cells, and can be used in conjunction with laser-scanning confocal microscopy to determine spatial relationships between bacteria and plant tissues. GUS is more suited for visualization of macroscopic colonization patterns.

The use of GFP and GUS as biomarkers revealed the absence of a significant level of endophytic colonization of rice seedlings by two isolates, R100(64) and R33(120). Because of the limited scope of this study, however, definitive conclusions on the endophytic nature of these bacteria cannot be made. A more detailed investigation should be carried out, including the study of endophytic colonization by other strains, and using different rice varieties, specifically Philippine varieties from which these bacteria were originally isolated. In addition, older plants should also be examined, as endophytic colonization may not occur in young seedlings.

More general considerations about the potential of using endophytic bacteria to actually provide nitrogen to rice plants for growth

For bacteria to effectively supply crop plants with nitrogen, they must have an adequate carbon supply to sustain the energy-intensive process of nitrogen fixation. In addition, the intracellular oxygen tension must be high enough for efficient nitrogen fixation without inactivating the oxygen-sensitive nitrogenase enzyme, and the nitrogen fixed by the bacteria must be transferred from the bacteria to the plant (Kennedy and Tchan 1992, de Bruijn et al 1995, Kennedy et al 1997). There must also be enough bacteria to produce the nitrogen needed, which will be affected by the three conditions mentioned above, as well as by the total nitrogen demand of the crop plant.

Problems with nutrient transfer between (endo)-rhizosphere bacteria and plants are illustrated by the interactions between *Azospirillum* and various cereal crops. Colonization of wheat by *A. brasilense* is limited to the surface of the root, with occasional intercellular colonization of the cortex (Schank et al 1979, Levanony et al 1989, Bashan and Levanony 1989. Kennedy et al 1998). Plants gain little nitrogen from bacterial nitrogen fixation when inoculated with *Azospirillum* (Nayak et al 1986, Christiansen-Weniger and van Veen 1991, Kennedy et al 1998). Nitrogen supplied to the plant could potentially be increased by the use of ammonium-excreting mutants of *Azospirillum* (Christiansen-Weniger and van Veen 1991, Christiansen-Weniger 1992): however. these bacteria compete poorly in field soil. This indicates that in the rhizosphere the bacteria use most of the nitrogen they fix for their own growth, rather than supplying nitrogen to the plant. The same scenario may apply to the type of endophytic diazotrophs described in our study.

The intimate association between plant and endophyte may provide more suitable conditions for nutrient transfer between bacteria and their host, however, than the association between predominantly rhizosphere (versus endorhizosphere) bacteria and plants. *Acetobacter diazotrophicus,* an endophytic diazotroph isolated from sugarcane, excretes fixed nitrogen (Cojho et al 1993), does not survive well in soils (James and Olivares 1998), colonizes the interior of sugarcane, and appears to supply the host with nitrogen (James and Olivares 1998). This provides an example of a plant host providing protection from rhizosphere competition, while simultaneously allowing an ammonium-excreting bacterium to flourish and provide nitrogen to the host. Based on this example, it is conceivable that genetically modified ammoniun-excreting endophytic bacteria could effectively supply the host plant with nitrogen.

The ambient oxygen tension required for efficient nitrogen fixation varies among diazotrophs, with optimal oxygen tension ranging from 0.3% to 6.5% (Vande Broek et al 1996). For effective nitrogen fixation, bacteria require enough oxygen for efficient respiration, but low concentrations of free oxygen to protect the oxygen-sensitive nitrogenase enzymes. Sites in the interior of plant tissue, especially in flooded rice, may provide the environment required for efficient nitrogen fixation. The apparently high rates of nitrogen fixation by endophytic diazotrophs in sugarcane (Urquiaga et a1 1992) and the derepression of *nif* genes by *Azoarcus* (Egener et al 1998) and *Alcaligenes faecalis* (Vermeiren et al 1998) following internal colonization of rice roots suggest that conditions favorable for nitrogen fixation, including low oxygen tension, are likely to exist inside the plant tissue.

The number of bacteria needed to provide enough biologically fixed nitrogen to reduce or eliminate fertilizer inputs will depend heavily on the efficiency of nitrogen fixation and nutrient transfer. In sugarcane, estimated population sizes for *A. diazotrophicus* range from 10^3 to 10^7 bacteria per gram fresh weight of roots (Boddey 1995). Studies of BNF in Brazilian sugarcane cropping systems suggest contributions of 39 kg N ha^{-1} yr^{-1} to more than 170 kg N ha^{-1} yr^{-1} (Boddey 1995). It is unclear whether this nitrogen is fixed by *A. diazotrophicus* alone or in conjunction with other endophytic diazotrophs. It has been suggested, however, that BNF by endophytes

contributes enough nitrogen for maximum yields, while requiring minimal inputs of chemical nitrogen fertilizer (Boddey et al 1995).

Our knowledge of the complex interaction between endophytes and rice is now limited and much of the information needed to use diazotrophic endophytic bacteria to replace chemical nitrogen fertilizer is still lacking (Quispel 1992). The diversity of rice endophytes and the niches they colonize have not been determined. Therefore, more studies that focus on determining the types of endophytic bacteria that associate with rice. the nature of the interactions between host and endophyte, and the sites of endophytic colonization are clearly needed.

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Diazotrophic enterobacteria: What is their role in the rhizosphere of rice?

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The search for more interesting and potentially beneficial diazotrophic enterobacterial endophytes continues to be carried out as part of the "New Frontier Project on Nitrogen Fixation in Rice." Diverse diazotrophic enterobacteria, such as *Enterobacter cloacae, Erwinia herbicola/Enterobacter* agglomerans (Pantoea agglomerans), *Klebsiella planticola, K. oxytoca,* and *Serratia marcecens* inhabit the rhizosphere of rice plants growing in both tropical and temperate climates. The ubiquity of enterobacteria can perhaps be attributed to their great metabolic diversity and fast growth rates. Indeed, the widespread occurrence in the rhizosphere of high populations of r-strategist bacteria, such as free-living *Klebsiella* and endophytic *Enterobacter* and *Serratia* species, indicates that there is sufficient availability of organic carbon in and around roots to meet their metabolic needs. The possession by enterobacters of diverse traits, such as diazotrophy in the presence and absence of O_2 and tolerance for heavy metals, probably also contributes enormously to their ability to survive and grow in diverse habitats, including the rhizosphere. In addition, many enterobacters have the ability to produce gums, siderophores, indole acetic acid, and metabolites active against phytopathogens, and they may also have phosphate-solubilizing, chitinase, cellulase, and pectinase activities. The potential role of these diazotrophs in plant growth is now being increasingly understood.

Diazotrophic bacteria that produce acid and gas from glucose fermentation are usually facultative anaerobes. These include members of Gram-positive endospore-forming *Bacillus* and Gram-negative short-rods in the families Vibrionaceae and Enterobacteriaceae (Krieg and Holt 1984). Of the three groups of diazotrophic facultative anaerobes, members of the Enterobacteriaceae are those most commonly encountered in association with plants, both in tropical and cold-temperate habitats. They are Proteobacteria under the g subdivision (Young 1992). Since the first reports of a diazotrophic enterobacterium in 19.58 (Jensen 19.58. Wilson 19.58), similar isolations have been made from a wide variety of sources. including animals, freshwater and marine sites, soils, and the phylloplanes and rhizospheres of various plants. With the

exception of *Klebsiella pneumoniae* M5A1 that has been used as a workhorse for nitrogen fixation genetics (Drummond 1984). however. relatively few studies have been made on diazotrophic enterobacteria, especially for their interactions with plants. This is surprising, as both diazotrophic and nondiazotrophic enterobacteria are considered to be important root colonists. especially of the endorhizosphere (Kleeberger et al 1983. Lynch 1988, Ruppel et al 1992. Hallmann et al 1997). Indeed, several diazotrophic enterobacters are actually regarded as being facultative endophytes (Baldani et al 1997), although they are rarely given much attention in most reviews of this subject (Kennedy and Tchan 1992. Rocldey et al 1995. Baldani et al 1997, James and Olivares 1998, Reinhold-Hurek and Hurek 1998).

Enterobacters are of great metabolic diversity, with fast growth rates. They prefer environments rich in organic substrates, indicating that the sites from which they are isolated contain organic carbon compounds (including plant metabolites) that are readily usable by them. These properties, as well as the ease of isolating, detecting, and identifying diazotrophic enterobacteria (Rennie 1980, Krieg and Holt 1984), have resulted in increasing numbers ofreports on their incidence within various plants. as well as in their rhizospheres. Indeed. an earlier review by Vose (1983) highlighted the nitrogen-fixing Enterobacteriaceae as being of importance in virtually all plant-associative systems, and hence suggested that they warranted the same sort of intensive effort that has been applied to *Azospirillum.*

This chapter reviews the occurrence of diazotrophic enterobacteria in the rhizosphere of monocots and dicots. In particular, it focuses on the potential roles of these bacteria in the rhizosphere of rice *(Oryza sativa)* as there are strong indications that they play a significant role in the root zone and hence deserve at least attention equal to that given to other bacteria in any program designed to improve rice-diazotroph associations (Ladha and Reddy 1995).

Diazotrophic enterobacters

Diazotrophic members of the Enterobacteriaceae have been isolated endophytically or epiphytically from a wide variety of sources both clinical and nonclinical (Table I), indicating no clear relationship between habitat and diazotrophy. Diazotrophic enterobacteria include *Klebsiella oxytoca, K. pneumoniae, K. planticola, K. terrigena, Enterobacter cloacae, Enterrobacter agglomerans, Erwinia herbicola, Erwinia carotovora, Citrobacter freundii, C. intermedius, Rahnella aquatilis,* and *Serratia marcescens.* The genetics of nitrogen fixation by *K. pneumoniae* M5A1 has been among the most documented of any diazotroph (Drummond 1984). The nif genes of *K. oxytoca* NG 13 and K. *pneumonie* M5A1 are chromosomally located (Barraquio et al 1981. Uozumi et al 1982, Drummond 1984), indicating some stability in their ability to fix N_2. In contrast, the *nif* genes of *E. cloacae* and *E. agglomerans* are located in a plasmid (Singh et al 1981. Kreutzer et al 1991, Steibl et al 1995). The whole *nif* genome of *K. oxytoca* and *K. pneumoniae* can easily be transferred to foreign backgrounds such as *Esherichia coli, Salmonella typhimurium, Azospirillum lipoferum, Pseudomonas putida,* and *Alcaligenes faecalis,* and also be expressed therein

Table 1. Diazotrophic enterobacteria and their source.

Organism	Isolation source	Reference
Citrobacter freundii, C. intermedius	Animals, grassland soil, paper mill process waters	Line & Loutit 1971, Neilson & Sparell 1976, Haahtela et al 1983, Neilson & Allard 1985
Enterobacter cloacae, E. aerogenes, unidentified strains	Decaying tree, xylem, rhizosphere, soil, seeds, inside stem, roots: wetland and dryland rice, forage grasses *(Cynodon dactylon, Panicum coloratum),* maize, *Zea luxurians,* sugarcane, wheat, sorghum, Kallar grass, millet, radish, pea, cucumber, wheat, grape, white fir tree, *Astragalus cicer, Atriplex* spp., *Alysicarpus orali, Indigofera hirsuta, Saccharum spontaneum, Elmyus angustus, Elmyus junceus, Melilotus officmales, Typha latifolia, Andropogon gerardii, Panicum virgatum, Panfcum maximum, Schizachir/um scoparium, Juncus baltms, Agrostis tenus, Chloris divaricata, Elytrigia repens*	Mahl et al 1965, Line & Loutit 1971, Raju et al 1972, Aho et al 1974, Koch & Oya 1974, Nelson et al 1976, Nellson & Sparell 1976, Kaputska & Rice 1976, Pedersen et al 1978, Rennie 1980, Wright & Weaver 1981, Thomas-Bauzon et al 1982, Rennie 1981, Rennie et al 1982, Garcia et al 1983, Ladha et al 1983, Haahtela et al 1983, Fujii et al 1987, Omar et al 1987, Oyalru-Masuchi & Komagata 1988, Berge et al 1991b, Bilal et al 1993, Bell et al 1995, Hinton & Bacon 1995, Palus et al 1996, Roberts et al 1996a & b, Mukhopadhyay et al 1996, Raicevic et al 1997, Viña 1998, Banta 1998. Barraquio et al 1999
Erwinia herbicola or *Enterobacter agglomerans (Pantoea agglomerans)*	Paper mill process water, compost, human, animal, and plant sources (rhizosphere, phyllosphere, soil, inside stem, surface-sterilized and non-surface-sterilized roots, seeds, decaying tree): wetland and deepwater rice, sugarcane, wheat, sorghum, millet, white fir tree, alfalfa, buckwheat, kallar, olive, *Elmyus angustus, Elmyus junceus, Agropyron dasystachyum. Agropyron elongatum, Atriplex* spp., *Melilotus officinales, Astragalus cicer, Achillea millefolfium, Phalans arundinacea, Calamagrostis arundinacea, Phleum pratense, Dactylis glonlerata, Elytrlgia repens, Festuca rubra, Poa pratenss*	Aho et al 1974, Potrikus & Breznak 1977, Pedersen et al 1978, Papen &Werner 1979, Nellson 1979, Rennie 1980, 1981, Haahtela et al 1981, 1983. Rennie et al 1982, Singh et al 1983, Kleeberger et al 1983, Lindberg & Granhall 1984, Haahtela & Korhonen 1985, Lindberg et al 1985, Oyairu-Masuchi & Komagata 1988. Mahk & Bilal 1989, Ercolani 1991, Berge et al 1991b. Lindh et al 1991, Kreutrer et al 1991, Ruppel et al 1992, Sterbl et al 1995. limura & Hosono 1996, Mukhopadhyay et al 1996, Raicevic et al 1997, Moline & Kuhk 1997, Kim et al 1998, this chapter
Erwinfa carotovora	Potato tubers	Pishchik et al 1998
Klebsiella oxytoca. K. pneunloniae, K. planticola, K. terrigena, unidentified strams	Pulp and paper mill effluents, lakes, rivers, drainage and sewage systems, surface of nodules, rhizosphere, phyllosphere. soil. clinical samples, inside roots, stem: wetland and dryland rice, sorghum, wheat, sugarcane, maize, millet, cucumber, white fir tree, forage grasses *(Cynodon dactylon. Panicum coloraturn), Saccharum spontaneum, Pennisetum purpureum Agrostis stolonifera, Carex pallescens, Phalaris arundinacea, Poa pratensis, Zea luxurians, Quercus petraea*	Mahl et al 1965. Line & Loutit 1971, Evans et al 1972, Knowles et al 1974, Aho et al 1974. Chambers & Silver 1977. Pedersen et al 1978, Elmerich 1979, Cakmakci et al 1981, Haahtela et al 1981, Bagley et al 1981, Wright & Weaver 1981, Rennle 1980, 1981, Uozumi et al 1982, Thomas-Bauzon et al 1982, Rennie et al 1982, Haahtela et al 1983, Ladha et al 1983, Haahtela 1985, Haahtela & Korhonen 1985. Yoo et al 1986, Fujii et al 1987, Omar et al 1987, Oyalzu-Masuch) & Komagata 1988, Qureshi et al 1988, Ercolani 1991, Berge et al 1991a, Boonjawat et al 1991, Espina 1994, Emtsev 1994, Ghulam et al 1996, Cornista & Barraqulo 1999, Barraquio et al 1999
Rahnella aquatilis	Maize and wheat rhizosphere, buckwheat seeds	Berge et al 1991b. Heulin et al 1994, Selenska-Pobell et al 1995, limura & Hosono 1996
Serratia marcescens	Surface-sterilized rice roots, olive leaves	Ercolani 1991, Rosales et al 1993, Gyaneshwar et al, unpublished, Hurek et al and James et al, this volume

Table 2. Specific nitrogenase activity[a], specific growth rate (μ)[b], and generation time (g)[b] of some diazotrophs.

Organism	nmol C_2H_4 mg^{-1} protein h^{-1}	g(h)	μ (h^{-1})
Acetobacter diazotrophicus Pal5	1,340 (641)	2.41	0.29
A. diazotrophicus CFNE	252 (119)	1.88	0.36
Azorhizobium caulinodans ORS571	420 (445)	2.17	0.32
Azospirillum brasilense Sp 60	ND	0.51	1.35
A. lipoferum 34H	610 (27)	0.94	0.74
A. lipoferum FS	758 (119)	1.07	0.65
Enterobacter agglomerans	59 (18)	0.54	1.29
Escherichia coli ATCC 1175	ND	0.49	1.40
Herbaspirillurn seropedicae 267	741 (327)	1.20	0.57
Klebsiella oxytoca NG13	2,472 (3,513)	0.53	1.31
K. pneumoniae M5A1	26,327 (2,576)	0.51	1.36

[a]Grown in semisolid N-deflclent medium (LGIP lor Acetobacter, Nfb lor *Azospirillum,* JNFb for *Herbaspirillum,* and Rennie's medium for enterics). The value presented is the highest activity from 6-h acetylene reduction assay of any two cultures (2 and 4 d). Values in parentheses are standard deviations of the mean of 5 replicates. ND = not determined.[b]Grown in glucose-glutamic acid-peptone-yeast extract (GGPYE) medium (James et al 1994) at 30 °C and shaken at 200 rpm. Absorbance was measured at 500 nm. Initial protein was 2–6 μg mL^{-1}. *Escherichia coli* does not fix nitrogen. [c]Two replicates.

(Dixon and Postgate 1972, Postgate and Krishnapillai 1977. Chen and Ye 1983. Wang et al 1985, Postgate and Kent 1987). *K. oxytoca* can also accommodate and express other *nif* genes (Inoue et al 1996). These facts and their close genetic relationship to *Escherichia coli* indicate the apparent ease of manipulating genes in enterobacteria.

The specific nitrogenase activity of enterobacteria in pure culture is almost equal to that of azospirilla (51-1.000 nmol C_2H_4 mg^{-1} protein h^{-1}) (Table 2, Christiansen-Weniger and van Veen 1991, Gonzalez 1999). In our study, the activity was even higher than that of azospirilla and other diazotrophs (Table 2). For example, the activity of enterobacteria recently isolated from sugarcane ranged from 1.9 to 4.5 x 10^4 nmol C_2H_4 mg^{-1} protein h^{-1} (Gonzalez 1999). The reported activity from other enterobacteria ranges from 240 to 4,500 nmol C_2H_4 mg^{-1} protein h^{-1} (Line and Loutit 1971, Neilson 1979), assuming 50% cell protein. Under natural conditions. however. activity may be lower because of the many environmental factors that could affect nitrogenase expression and activity.

Enterobacters in the rhizosphere

Enterobacteria, including diazotrophs, have been isolated from a considerable range of plants. especially members of the *Gramineae* but also from various dicots and even trees (Table 1). Although many of the newer reports (Table I and some that

follow) did not mention whether the strains were diazotrophic or nondiazotrophic. new diazotrophic enterobacters have been discovered recently, such as an endophytic strain of *K. terrigorena* that was isolated from maize, along with *Enterobacter agglomerans* (Fisher et al 1992). It is worth noting that *E. agglomerans* and *Erwinia herbicola* are considered to be essentially the same and are now placed in the genus *Pantoea* as *Pantoea agglomerans* (Gavini et al 1989). Most recently, diazotrophic (and avirulent) strains of bacteria related to *Erwinia carotovora* and *K. mobilis* were found within potato tubers by Pishchik et al (1998). Some of these strains had antagonistic effects against phytopathogens, such as *E. carotova.* and also produced phytohormones (Pishchik et al 1998).

For rice. diazotrophic enterobacteria have been isolated from many different varieties grown under wetland, dryland, and deepwater conditions. They can be isolated from all parts of the plant, including the rhizosphere, spermosphere, leafsheath. stem, phyllosphere, and seeds, as well as the inner and surface portions of surface- and non-surface-sterilized roots (Tables 3 and 4). So far, all enterobacters isolated from rice belong to the genera *Enterobacter, Klebsiella,* and *Serratia.* Interestingly, diazotrophic enterobacteria were also detected in *Monochoria vaginalis,* a wetland plant frequently associated with paddy fields (Barraquio and Watanabe 1981). These data show that diazotrophic enterobacters are ubiquitous in rice soil. and that they possess the ability to compete in a setting full of other microorganisms. They also suggest the likelihood of a close relationship between diazotrophic enterobacteria and rice. The density of the enterobacterial population in the rhizosphere of rice typically ranges from 10^6 to 10_8 bacteria g^{-1} dry weight of soil (Balandreau et a1 1975. Thomas-Bauzon et al 1982. Garcia et al 1983. Omar et al 1989). Our best estimates. however, put their density at approximately 10^4 to 10^6 g^{-1} dry weight of root or soil, which is similar to that of *Azospirillum* (Table 4. Watanabe and Barraquio 1979, Watanabe et al 1979, Barraquio and Watanabe 1981, Ladha et al 1983, Barraquio et al 1997).

Diazotrophic enterobacteria are easily detected and isolated by incubating the sample in either semisolid N-deficient glucose-yeast extract (100 mg L^{-1}) medium (Watanabe's medium; Watanabe and Barraquio 1979, Omar et al 1989) or in combined carbon source medium (Rennie's medium; Rennie 1981, Knowles and Barraquio 1994). The cultures are then subjected to the acetylene reduction assay (ARA) to determine if they contain diazotrophs. In both these media, the organisms produce acid and gas, and repeated subculturing of the semisolid cultures onto MacConkey agar should yield easily distinguishable colonies of enterobacteria.

Table 3. Summarized data on the isolation or detection of diazotrophic enterobacteria in rice.

Rice cultivar or related grasses from where enterobacteria were isolated	Remarks	Organism	Reference
10 wetland varieties, 2 varieties grown in both dryland and wetl and, 2 wetland wild rice gentotypes, *Monochoria vaginalis* (IRRI, Philippines)	Roots, leaf sheath, culm, and rhizosphere soil: plants grown fertilized or unfertilized in IRRl flooded field, with pesticide application	*Klebsiella planticola, Enterobacter cloacae,* unidentified enterobacteria	Watanabe and Barraquio 1979, Watanabe et al 1979, 1981, Barraquio and Watanabe 1981. Barraquio et al 1982, Ladha et al 1983
Delta (France), Cigalon	Rhizosphere, spermosphere: plants grown fertilized	*Enterobacter cloacae, Klebsiella oxytoca*	Thomas-Bauzon et al 1982. Bally et al 1983. Omar et al 1989
Deepwater rice (DW6255) (IRRI)	Roots, culms, leaf sheath: plants unfertilized in deep water	Unidentified enterobacteria	Kulasooriya et al 1981
Unknown variety (India)	Roots and phyllosphere	Unidentified enterobacteria	QUI et al 1981, Sengupta et al 1981
20 rice cultivars (Senegal)	Surface-sterilized roots	Enterobacter cloacae	Balandreau et al 1975, Garcia et al 1983
Giza(Egypt)	Rhizosphere: plants grown in flooded condition	*Enterobacter cloacae, Klebsiella planticola*	Omar et al 1987
C5444 and T65 (Japan)	Paddy soil and roots: plants grown in pots, flooded, fertilized	*Erwinia herbicola, Enterobacter cloacae, Klebsiella oxytoca*	Oyaizu-Masuchl & Komagata 1988
IR42 and BKNFR (deepwater)	Surface-sterilized seeds	*Enterobacter cloacae,*[a] *E. agglomerans, E. intermedius, Yersinia enterocolitica*	Mukhopadhyay et al 1996
25 cultivated and wild rice (IRRI)	Surface-sterilized roots and culms: plants grown in pots, unfertilized and flooded	Unidentified enterobacterta	Barraquio et al 1997
Jaisurya (deepwater) (India)	Surface-sterilized roots: field	*Enterobacter agglomerans*	Verma et al 1999
6 or so wetland varieties (Philippines)	Surface-sterilized roots: plants grown in flooded condition, fertilized	*Serratia marcescens*	Rosales et al 1993, Gyaneshwar et al, unpublished, Hurek et al and James et al, this volume
Oryza minuta (Philippines)	Surface-sterilized roots and culms: plants growing naturally in canal, unfertilized and no pesticide applied	Unidentified enterobacterla	This chapter
California (Philippines)	Roots: plants grown in Banaue rice terrace field, unfertilized, without pesticide applied, flooded	Unidentified enterobacteria	This chapter

Table 4. Most probable number (MPN) of diazotrophic enterobacteria in surface-sterilized root and culm of *Oryza minuta* growing naturally on a canal.

Source/sample[a]	MPN g^{1} dry weight
Root	
Total bacteria	1.8×10^7
Bacteria in GYE medium	1.1×10^6
ARA-positive enterics in GYE	4.8×10^5
ARA-positive in MYE medium	2.7×10^6
Culm	
Total bacteria	5.2×10^6
Bacteria in GYE medium	9.5×10^4
ARA-positive enterics in GYE	9.5×10^4
ARA-positive in MYE medium	2.7×10^5

[a]GYE = semisolid N deficient glucose + 100 mg L^{-1} yeast extract: MYE = semtsolid N-deficient malate + 100 mg L^{-1} yeast extract (Watanabe et al 1979). ARA = acetylene reduction activity.

Carbon and other nutrients available to enterobacters in the rhizosphere

In pure culture. enterobacteria have a wide spectrum for carbon use (Krieg and Holt 1984, see earlier, and the references cited in Table 1). All of them, however, can use glucose, most of them sucrose, and, with the exception of *S. marcescens,* arabinose. So far, *K. pneumoniae, Erwinia carotovora* (Krieg and Holt 1984), and *Enterobacter agglomerans* (this study) are the only ones reported to have pectate-degrading activity, and only *Erwinia herbicola* does not produce gas from glucose. The genera containing diazotrophic enterobacters can be further distinguished from each other quite easily via various other phenotypic characteristics. For example, *E. cloacae* EcCT501 grows on amino acids, peptides, and a variety of mono- and oligosaccharides when colonizing the spermosphere of cucumber and pea plants (Roberts et al 1992. I996a. b). Other enterobacteria, including *Klebsiella* spp., can grow on fructo-oligosaccharides (Hartemink et al 1997) and on hardwood hydrolysate (Mende et al 1993). K. *planticola* and *Enterobacter cloacae* can metabolize 1-chloro-2,4-dinitrobenzene (CDNB) and the herbicide alachlor (Zablotowicz et al 1995). *Klebsiella* and *Enterobacter* spp. can use internal endogenous reserves that may aid in their survival when competing with other bacteria (Sjogren and Gibson 1981). Nitrogen-fixing enterobacteria have been isolated from pulp and paper mill effluents. industrial waste waters, and biological treatment systems (Knowles et al 1973, Neilson and Allard 1985), and they presumably also tolerate heavy metals (Lee at al 1996, Hernandez et al 1998) within the eftluents, Chitinase, cellulase, and pectinase activities were all found in *Entorebacter asburiae* JM22, *E. agglomerans, Erwinia carotovora* and *S. marcescens* (Fig. 1, Krieg and Holt 1984, Chapman and Lynch 1985, Sirit et al 1991, Chemin et al 1997, Quadt-Hallmann et al 1997a,b).

Glucose as a component of the semisolid media (e.g., Watanabe's and Rennie's) used to isolate rhizosphere diazotrophs is logical. In rice, glucose (followed by malate)

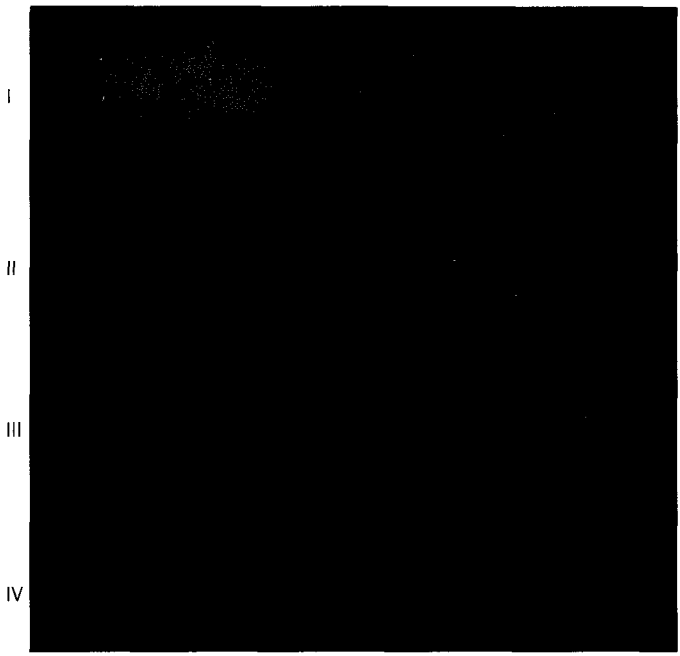

Fig. 1. Cellulase (A) and pectinase (B) activities of diazotrophic bacteria from deepwater rice (Jalsurya). I, *Enterobacter agglomerans* SBW1; II, SBW2; III, SBW3; and IV, SBW4. Cellulase activity was determined according to Reinhold-Hurek et al (1993), and pectinase activity by the method of Mateos (1992).

was the single most important carbon source in the rice root exudate (MacRae and Castro 1967, Boreau 1977), although sucrose and amino acids were also abundant in rice phloem sap (Fukumorita and Chino 1982). Other than glucose and sucrose, root exudates or rhizodeposition (Lynch and Whipps 1990) from aseptically grown rice seedlings contain raffinose, fructose, arabinose, xylose, ribose, organic acids (malate, acetate, pyruvate), two oligosaccharides, alkyl resorcinols, and 10 amino acids (lysine, aspartic acid, threonine, serine, glutamic acid, glycine, alanine, isoleucine, leucine, methionine) (MacRae and Castro 1966, 1967, Waschutza et al 1992, Bouillant et a1 1994). No carbon flow data are available for field-grown rice (see later), but we could imagine that the occurrence of several organic carbon compounds in the root zone of rice would be expected to allow many types of heterotrophs to thrive and, indeed, this is the case. A huge number and variety of diazotrophic chemoorganotrophs, such as enterobacteria, azospirilla, herbaspirilla, pseudomonads, azotobacters, burkholderias, agromonads, xanthobacters, alcaligenes, azoarcus, and serratias and photoorganotroph rhodopseudomonads, have been isolated from the rhizosphere of rice (Balandreau et al 1975, Ladha et al 1982, Thomas-Bauzon et al 1982, Barraquio et al 1983, 1986, Ohta and Hattori 1983, Bally et al 1983, Ladha et al 1983. Baldani et al 1986, Watanabe et al 1987, Fujii et al 1987, Oyaizu-Masuchi and Komagata 1988, You and Zhou

1988, You et al 1991, 1995, Reding et al 1991. Santos et al 1991, Chan et al 1994, Gillis et al 1995, Gyaneshwar et al, unpublished, Hurck et al and James et al. this volume). Analyses *nifH* and *nifD* gene sequences and rep-PCR-generated genomic fingerprints from bacteria in rice roots have recently, confirmed this remarkarble N_2-fixing bacterial diversity (Ueda et al 1995a,b, Stoltzfus et al 1997).

Why do diazotrophic enterobacters predominate in the rhizosphere of rice?

The rhizosphere can be viewed as an ecosystem that favors the existence of organisms with one of two growth types: the *r*-strategists and the *K*-strategists (Andrews and Harris 1986), *r*-strategists are those that rely on high reproductive rates for continued survival within the commnunity and they rapidly take over and dominate situations in which resources are temporarily abundant (Atlas and Bartha 1993). *K*-strategists, on the other hand, are microorganisms that thrive under very low organic carbon levels (Ohta and Hattori 1983). Table 2 gives the specific growth rates (μ) of some diazotrophs, and we can see that *Klebsiella* spp. and *E. agglomerans* are among the highest. Therefore, as high growth rates are likely to be important in successful rhizosphere colonization (Rovira et al 1983. Schroth and Weinhold 1986), it may not be surprising that diazotrophic enterobacters also appear to be very well adapted to the rice rhizosphere, be it dryland or wetland (Table 3). This hypothesis, however, is not in agreement with De Leij et al (1994) or Balandreau and Roger (1996), who suggested that *K*-strategist should dominate. A study comparing the effect of bacterization with an *r*-strategist (copiotroph, Atlas and Bartha 1993) with that of a *K*-strategist (oligotroph, Ohta and Hattori 1983) under both gnotobiotic and natural conditions could shed some light on this apparent contradiction. It is also interesting to look at the dynamics of copiotrophic and oligotrophic diazotrophs in the rice rhizosphere in relation to soluble carbon flux as has been done with wheat (Semenov, et al 1999). All the organisms shown in Table 5 could be classified as *r*-strategists. which correlates with the alternate betting and drying of the soil and the diurnal cycle during rice culture. When resource become scarce or conditions turn unfavorable, their populations decrease rapidly, satisfying the "boom-crash" growth pattern. A consequence of this is that after the "crash" the cell degradation products then become available to plants and other organisms in the rhizosphere. Not one organism in Table 2 classifies as a *K*-strategist, and the only diazotroph so far isolated from rice that could be described as such is *Agromonas oligotrophica* (Ohta and Hattori 1983).

The apparent ubiquity of Enterobacteriaceae in the rhizosphere of rice (Table 3) may suggest that. in a batch-fed fashion (Barber and Lynch 1977), there is a high flux of organic carbon compounds from within the roots, especially under waterlogged conditions. Indeed, organisms in the rhizosphere can exist because of the carbon flow through the plant and enterobacteria in particular probably depend heavily on rhizodeposition, or carbon loss, from the roots (Whipps and Lynch 1985). When plants are stressed by flooding, they usually deposit more carbon around their roots because the stress causes root cell membranes to become leaky (Lynch 1988). Based on continuous ^{14}C-labeling experiments on young cereals (Barber and Lynch 1977. Whipps

Table 5. Beneficial traits of putative endophytic diazotrophic enterobacteria from *Oryza minuta* growing naturally on a canal.

Isolate[a]	Tissue, medium. and dilution used to Isolate[b]	ARA in GYE/ MYE/Nfb	Siderophore production[c]	PO_4^{3-} solubilizing activity[d]	IAA production[e]
Om3, 4, 10, 11, 8	Stem, MYE $5^{-4 \text{ to } -6}$	+	+	+	+
Om25, 26. 28, 29. 33, 36, 37, 38, 39, 46	Roots, MYE $5^{-4 \text{ to } -6}$	+	+	+	+
Om49, 52, 53, 54. 59	Stem, GYE $5^{-4 \text{ to } -5}$	+	+	+	+
Om61, 62, 63, 64, 65	Roots, GYE $5^{-4 \text{ to } -5}$	+	+	+	+
Om70, 98, 99	Stem, RSE $5^{-4 \text{ to } -6}$	+	+	+	+
Om80, 81, 88, 94	Roots, RSE $5^{-4 \text{ to } -5}$	+	+	+	+
Enterobacter aglomerans SWB1[f]	Roots and seeds, Nfb medium	+	ND	ND	+

[a]Isolates Om70, 80, 88, 63, 49, 39 showed acetylene reduction activity (ARAJ only in GYE; Isolate Om26 was positive only in MYE. The rest of the isolates showed positive ARA in both media. [b]GYE = semisolid N-deficient glucose + 100 mg yeast extract L^{-1}: MYE = sernisolid N-deficient malate + 100 mg yeast extract L^{-1}; RSE = semisolid rice stein extract (1 extract:2 water); Nfb = semisolid N-deficient malate: ND = not determined. [c]Alexander and Zuberer (1990) method. [d]Goldstein (1986) method. [e]Gordon & Weber (1951) method; IAA = indole-acetic acid. [f]Identifled by Verma et al (3999)

and Lynch 1983), there is probably a very large substrate made available by the roots of rice growing under tropical wetland conditions to the microbial population of the rhizosphere. In higher plants. 30–60% on average of the net photosynthetic carbon is allocated to the roots and a substantial proportion of this carbon is subsequently released in the form of organic compounds into the rhizosphere (Marschner 1996). Estimates of the amount of carbon fixed from photosynthesis that is subsequently translocated to the belowground roots have been made for various temperate cereal crops and grasses, but not for rice. Up to 30% of total fixed carbon was translocated in wheat, barley, and maize (Barber and Martin 1976). and as much as 40% of the carbon that enters seedlings of wheat, maize, tomatoes, and peas is lost through the roots (Lynch 1988). In hydroponically cultured rice grown with 0.5, 5, and 10 ppm P, root exudation ranges from 0.5 to 36.7 mmol C $plant^{-1}$ h^{-1} (Lu et al 1999). Therefore, we can envisage a situation in the rice rhizosphere where organisms compete for this carbon (e.g.. the glucose), and under such conditions enterobacteria may dominate because of their rapid growth rates. On the other hand, it must be remembered that the above studies are generally performed under gnotobiotic conditions and hence will not be representative of a field situation. For example. the roots of cereals may release about twice as much of their photosynthetically fixed carbon into unsterilized soil as they do when grown under sterile conditions (Barber and Martin 1976).

Survival in the rhizosphere: motility, attachment, O_2, and respiration

Because all enterobacters, except for *Klebsiella* and *Rahnella,* are motile (Krieg and Holt 1984), they should be able to move through nutrient and O_2 gradients in the root zone. The ability of bacteria to move along the root is important tor successful rhizosphere colonization (Hozore and Alexander 1991). Some enterobacteria also possess fimbriae for attachment to nutrient-containing surfaces (Hanhtela and Korhonen 1985. Haahtela et al 1986. 1988b), and Achouak et al (1994) have shown that an *Enterobacter. agglomerans* strain isolated from the rhizosphere of rice may actually have some specificity in its attachment to its "host" plant via symplasmata. N_2-fixing enterobacters grow best and exhibit nitrogenase activity in the presence of decreasingly low levels of O_2 (e.g.. growth in N-deficient semisolid media). These conditions probably exist in the rhizosphere, allowing the bacteria to generate considerable energy through respiration for nitrogen fixation and other anabolic processes (Klucas 1972. Hill 1975. 1976a,b, Postgate 1982). In the absence of O_2. because they are facultative anaerobes they can still generate energy for N_2 fixation through fermentation. They can also grow anaerobically by respiring with nitrate as an electron acceptor (virtually all enterobacters can reduce nitrate: Krieg and Holt 1984). When they produce acid during fermentation, CO_2 and H_2 can become electron acceptors and donors, respectively, for hydrogen oxidation in the rhizosphere of rice (Watanabe et al 1982. Gowda and Watanabe 1985a,b), as well as for other processes, such as chemolithotrophic N_2 fixation (Barraquio and Knowles 1989), methanogenesis. acetogenesis, and sulfate reduction in submerged soil (Yoshida 1975. Knowles 1978). For example, when cocultured with non-glucose-using *Azospirillum brasilense* in a semisolid medium containing peptone-yeast extract-glucose, *E. cloacae* consumed more than half the glucose without fixing N_2, but provided the fermentation products as substrates for the *A. brasilense* to fix N_2 (Kaiser 1995). Some enterobacteria can produce gums and other biopolymers (Meade et al 1994. Na and Lee 1997. Dlamini and Peiris 1997). and these may be useful in protecting nitrogenase against oxygen damage as well as assisting in survival under desiccated conditions.

Endophytic enterobacters

We have shown that enterobacteria are very common in the rhizosphere, especially in that of rice (Tables 1, 3-4). More specifically, besides being epiphytic and hence colonizing the rhizosphere and plant surfaces, some of these bacteria may also be *endo*phytic. Indeed, there has been much recent interest in "endophytic diazotrophs" associated with grasses because they may be partially responsible for the substantial N_2 fixation observed in, for example, sugarcane, Kallar grass, and rice (Ladha and Reddy 1995, James and Olivares 1998, Reinhold-Hurek and Hurek 1998, James et al. this volume). The endophytic occurrence (after surface disinfestation) of various enterobacteria has been reported in the roots, stems, leaves, leaf nodules, seeds, and fruits of cotton, maize, cucumber, rough lemon, grapevine, sugarbeet, red clover, and potato and other plant species such as cereals, vegetables, and woody plants (Table 1,

Samish et a1 1961, 1963, Centifanto and Silver 1964, Mundt and Hinkle 1976, Gardner et al 1982, Jacobs et al 1985, Gagné et al 1989, Misaghi and Donndelinger 1990, Fisher et a1 1992, Bell et al 1995, Mclnroy and Kloepper 1995a,b, Palus et al 1996, Sturz et al 1998). Surface steri1ization, however, does not completely eliminate the possibility that some of the bacteria were isolated from outside the tissue and so it is now believed that the best methods of determining whether bacteria are endophytic are those using microscopy of fixed and ernbedded material. Such studies also have the advantage of giving a precise location of the bacteria within the tissue. When examining such material, it is also essential to use some form of recognition, such as an antibody or a molecular marker, to confirm the identity of the bacteria in the micrographs (Hallmann et al 1997, Reinhold-Hurek and Hurek 1998, James et al. this volume).

Several recent studies have employed microscopical techniques to determine the exact location of enterobacters within roots and other tissues. For example, using optical and transmission electron microscopy (TEM) in combination with immunogold labeling, *Enterobacter asburiae* JM22, an endophyte of cotton, cucumber, and *Phaseolus vulgaris* (Quadt-Hallmann and Kloepper 1996), was shown to actively penetrate cotton seedlings via the hydrolysis of wall-bound cellulose, and subsequently colonize intercellular spaces in the roots and leaves (Quadt-Hallmann and Kloepper 1996, Mahaffee et a1 1997, Quadt-Hallmann et a1 1997a,b). Similarly, diazotrophic strains of *S. marcescens* and *E. agglomerans* have recently been confirmed as endophytes of rice (Gyaneshwar et al, unpublished, James et al. this volume). The initial entry of *S. marcescens* cella into rice roots probably occurs via cracks at lateral root junctions or via root tips, and at 2 d after root colonization these cells were observed colonizing aerenchyma and xylem vessels in the stems and leaves (Gyaneshuar et al. unpublished, James et al, this volume). The TEM study of Ruppel et al (1992) showed that *Enterobacter (Pantoea) agglomerans* colonized intercellular spaces and xylem vessels in the roots and leaves of wheat, confirming earlier observations of Lindberg et a1 (1985).

Effect of inoculation on plant growth

So far, no pot or field inoculation studies in rice involving diazotrophic enterobacteria, in either pure or mixed culture. have been reported. Enterobacteria (free-living and endophytic) may promote the growth of plants in various ways, such as via phosphate-solubilizing activity (Table 5, Kim et a1 1998) or by enhancing hyphal growth and mycorrhizal colonization (Azcon et a1 1976. Will and Sylvia 1990). The production of siderophores and indole-acetic acid (IAA) may also be important (Table 5, Zimmer et al 1994, Costa and Loper 1994. Brandl and Lindow 1996, Cornista and Barraquio 1999), Haahtela et al (1990) detected IAA in 88% of enterobacterial isolates from *Poa pratensis.* Enterobacters also produce metabolites active against phytopathogens and are thus potential biocontrol agents (Hadar et al 1983, Sneh et a1 1981, Nelson et al 1986, Wilson et al 1987. Howell et al 1988. Fravel et a1 1990. Hebbar et al 1992, Briffaerts et al 1996, Chernin et al 1996. Kearns and Hale 1996.

Montesinos et al 1996, Mukhopadhyay et al 1996, Thompson et al 1996, Burkhead et al 1998). *S. mercescens* as a biocontrol agent induces systemic resistance in cucumber, tobacco, and tomato against several fungal pathops (van Loon et al 1998).

When evaluating inoculation experiments, we should be aware of whether the work was undertaken gnotobiotically or in the field because the physiological, chemical, and biological conditions that exist under the two setups differ tremendously. Success in the test tube does not necessarily, get replicated in the field and vice versa. For example, the inoculation of axenic cultures of wheat plants with *K. oxytoca* ZMK-2 or in combination with *Azotobacter vinelandii* produced no significant increase in plant growth based on plant dry weight and N content (Cakmakci et al 1981). The development of gnotobiotically raised wheat seedlings was not positively affected by inoculation with *E. agglomerans* possibly because of the slight degree of pathogenicity shown by the endophytic bacterin (Lindberg et al 1985). In studies involving mixed cultures, the growth of *Azospirillum brasilense* JM82 in the rhizosphere of axenically grown rice was inhibited by the presence of *Enterobacter* sp. QH7 and *E. agglomerans* AX I2 (Bilal et al 1993). On the other hand, *K. pneumoniae K. terrigena E. agglomerans, E. aerogenes,* and *E. cloacae* grown with *Poa pratensis* in tubes showed high nitrogenase activity (acetylene reduction), whereas no activity was found in control plants inoculated with autoclaved bacteria (Haahtela et al 1988a. 1990). Further studies with *P. pratensis* in Leonard jars showed that shoot dry matter and total N content increased when *P. pratensis* was inoculated together with *K. pneumoniae* Pp, *K. terrigena* Cp, and *E. agglomerans* Am. Furthermore, the number of root hairs and lateral roots increased significantly and the length of the zone of elongation decreased.

It has been suggested that bacterial-induced physiological changes in plant roots may be more important than the amount of nitrogen transferred to the plant. Inoculation of soil-grown forage grasses (*Cynodon dactylon* and *Panicum coloratum*) with *E. cloacae* and *K. pneumoniae* showed that 25% of plant-soil systems were active in acetylene reduction and that this activity was high enough to indicate that agronomically significant quantities of N_2 were being fixed (Wright and Weaver 1982). Plant systems extrapolated to fix more than 8 kg N ha^{-1}, however, contained less nitrogen and accumulated less dry matter than plants less active in acetylene reduction. Inocula could not be reisolated from healthy grass roots. That the beneficial effects of enterobacteria could be seen when in combination with other organisms tells us of the need to look at the consortium approach. Indeed, when attempting to establish an efficient N_2-fixing association between rice and heterotrophic diarotrophs (Ladha and Reddy 1995, James et al, this volume), it should be borne in mind that the remarkable diversity in bacteria in the rhizosphere of rice shows that N_2 fixation is extremely unlikely to be monopolized by only a few groups of diazotrophs. Therefore, little success will likely arise from experiments in which only pure culture inoculations are used.

Finally, it is evident that in most inoculation studies there is an urgent need to monitor the inoculated bacteria to evaluate the extent of colonization, and also to recover the original inoculum from the plants to fulfill Koch's postulates. The use of

reporter genes and other molecular assays (Wilson et al 1995) will greatly assist in this.

Conclusions and further work

The following points conclude this chapter and suggest work that could be done.

- Enterobacteria (epiphytic or endophytic) are common inhabitants of the rhizosphere of many monocot and dicot plant species grown in tropical and temperate environments.
- The rhizosphere of rice teems with diverse types of chemoorganotrophic diazotrophs, oligotrophic and copiotrophic, and epiphytic and endophytic.
- The number and incidence of culturable diazotrophic bacteria differ according to rice genotype, and the rhizosphere of rice grown in various geographical sites and soils yields diverse genetic types of diazotrophs.
- Among culturable diazotrophs, enterobacteria are those most frequently found in the rhizosphere of rice grown in various soils.
- Rice root exudate is a mixture of organic carbon compounds suitable for heterotrophic N_2 fixation, but no studies have looked at the relationship between root exudation and colonization of the rhizosphere by diazotrophs.
- Diazotrophic enterobacteria associated with rice roots possess desirable traits besides N_2 fixation.
- Inoculation with diazotrophic enterobacters can promote plant growth. but. when they are inoculated alongside other types of bacteria, they may act synergistically to give effects greater than single-strain inoculations.

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Endophytic diazotrophs associated with rice

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Wetland rice receives a significant proportion of its nitrogen requirements from biological nitrogen fixation (BNF). This is partly provided by free-living photosynthetic diazotrophs, especially cyanobacteria, that live in the soil and floodwater. Another significant source of fixed nitrogen are the abundant heterotrophic bacteria in the rhizosphere, particularly species of *Azospirillum, Burkholderia,* and *Pseudomonas,* and bacteria belonging to the Enterobacteriaceae. Some rice varieties can obtain more fixed nitrogen from heterotrophic BNF than others, suggesting that they have a more "intimate" association with diazotrophs. In support of this, a wide range of diazotrophs appear to live within the tissues of the plants and it has been suggested that these endophytic diazotrophs are actually responsible for much of the N_2 fixation. Some of these bacteria, such as *Azoarcus* spp., *Herbaspirillum seropedicae, Pseudomonas stutzeri* A15 (formerly *Alcaligenes faecalis* A15), *Rhizobium leguminosarum* bv. *trifolii,* and *Serratia marcescens,* have been confirmed to be endophytes by using microscopy allied with marker genes and/or immunogold labeling. Endophytic diazotrophs usually live within the root apoplast, that is, the intercellular spaces and/or the xylem vessels, and may enter the plants via the root tips or epidermal cracks at lateral root junctions. Some also colonize the aerial parts of the plants, as well as the seeds. No obvious "symbiotic" organs are present, and the mechanism by which these relatively unstructured associations operate in the plant has yet to be established. The challenge that now faces research on heterotrophic BNF by rice is to find the optimum combination(s) of plant genotypes and endophytic diazotrophs, and to establish beyond a doubt that they can fix significant quantities of N_2.

Rice is arguably the most important cereal crop in the world, feeding well in excess of 2 billion people, particularly in Asia, Africa, and Latin America (Ladha et al 1997). The populations of these regions are growing at a rapid rate, however, and rice yields will therefore need to be enhanced to match the increased demand. Tropical lowland rice agriculture is now responsible for 86% of the total world rice crop, and yields are

typically in the range of 2–3.5t ha^{-1}. By 2025, these yields will have to increase by at least 50% to feed the projected increase in population. Because nitrogen (after water) is the most critical factor for rice productivity, this will necessitate at least a doubling of the current rate of nitrogen fertilizer use of 10 million t yr^{-1} (Ladha and Reddy 1995). This increased use of chemical fertilizer is undesirable, however, because (1) its production is an energetically costly process, and most of the energy is provided by the consumption of nonrenewable fossil fuels, and (2) considerable pollution is caused through both the production and use of mineral N fertilizers, and this is exacerbated by the relatively low efficiency of their uptake by the plants (Ladha and Reddy 1995, Ladha et al 1997).

An alternative to the increased use of mineral nitrogen fertilizers is to use more N derived from biological nitrogen fixation (BNF). Indeed, at present, the low-input systems typical of much tropical rice agriculture add little or no mineral N, subsisting mainly on nitrogen provided by BNF from free-living and plant-associated diazotrophs. A variety of organisms are responsible for this BNF, ranging from cyanobacteria and photosynthetic bacteria in the floodwater and soil surface to heterotrophic bacteria within the soil and root zone (Roger and Ladha 1992, Ladha and Reddy 1995, Boddey et al 1995). Estimates using data obtained from N-balance studies have suggested that indigenous cyanobacteria may contribute as much as 80 kg N ha^{-1} $crop^{-1}$, averaging 30 kg N ha^{-1} $crop^{-1}$, and, when inoculated into the rhizosphere, they can increase rice yields by an average of 337 kg ha^{-1} $crop^{-1}$ (App et al 1980, Watanabe 1986, Roger and Ladha 1992). In addition, mutant cyanobacteria that have been engineered to release much of the ammonia that they fix, such as SA-1, an *Anabaena variabilis* strain that is nitrogenase-derepressed, may have some potential as a biofertilizer, contributing in pot experiments the equivalent of an application of 73 kg N ha^{-1} of $(NH_4)_2SO_4$ (Kamuru et al 1998).

Heterotrophic rhizosphere bacteria appear to contribute less BNF than the phototrophs. Various N-balance studies in which growth and N_2 fixation by cyanobacteria and other phototrophs were prevented by shading the soil/floodwater (Chalk 1991, Boddey et al 1995) suggest that heterotrophic BNF ranges between 1 and 70 kg N ha^{-1}, depending on the variety (App et al 1980, 1986, Watanabe 1986, Roger and Ladha 1992), and typically averages only 7 kg N ha^{-1} (App et al 1986). This is still a significant and potentially important input, however, and, together with possible contributions from endophytic bacteria (see later), free-living heterotrophs are the likely source of the N_2 fixation reported by numerous studies using the acetylene reduction assay (ARA) (Barraquio et al 1986, Watanabe 1986, Roger and Ladha 1992, Boddey et al 1995, 1998). Unfortunately, although ARAs can give direct evidence of N_2 fixation at the time they are performed, the actual amount fixed by a crop over a growth season is not easily quantified (van Berkum and Bohlool1980, Boddey et al 1995, 1998) and so it is now generally accepted that the ^{15}N isotope dilution assay is the best means of estimating BNF associated with Gramineae (Chalk 1991, Boddey 1987, Boddey et al 1995, 1998). Using the latter assay, recent work with several varieties of wetland rice has shown that, over a growth season, heterotrophic BNF may contribute from 0 to 35.9% of N derived from air (Ndfa), depending on the

variety, the soil, and the availability of soil N (Wu et a1 1995, Shrestha and Ladha 1996, Malarvizhi and Ladha 1999).

Rhizospheric diazotrophs

With the exception of those studies that have used inoculations with specific bacteria (e.g., Yoo et a1 1986, Fujii et a1 1987), there is no direct evidence as to the organisms responsible for the heterotrophic BNF reported for rice. The rhizosphere diazotrophs potentially responsible, however, are great in number and variety. For example, using conventional culturing techniques, it has been shown that the rhizosphere of rice is particularly abundant in species of *Azospirillum* and *Pseudomonas* and in members of the *Enterobacteriaceae* (Ladha et a1 1982, 1983, Thomas-Bauzon et al 1982, Barraquio et a1 1983, this volume, Bally et a1 1983, 1990, Fujii et a1 1987, Watanabe et a1 1987a, Oyaizu-Masuchi and Komagata 1988, Omar et a1 1989). In addition, various members of the genera *Agromonas, Alcaligenes, Azotobacter; Burkholderia, Clostridium, Flavobacterium, Xanthobacter;* and *Zoogloea* have also been isolated from paddy field soil or wetland rice (Bally et a1 1983. Ohta and Hattori 1983, Fujii et a1 1987, Oyaizu-Masuchi and Komagata 1988, Omar et a1 1989, Reding et a1 1991, Gillis et a1 1995, Malik et a1 1997). Furthermore, recent data obtained from analyzing *nifD* and *nifH* gene sequences of bacterial DNA extracted from washed roots of wetland rice in Japan have shown that a large and highly diverse population of so far unculturable diazotrophs is also associated with rice (Ueda et a1 1995a,b).

Unfortunately, there seems to be little scope to substantially increase the N supply from free-living diazotrophs, both phototrophic and heterotrophic, as the nitrogen that they fix is outside the plant and therefore subject to losses (Ladha and Reddy 1995). Moreover, cyanobacterial N_2 fixation is closely linked with their growth and the fixed nitrogen is not made available to the plants until the organisms die and their organic nitrogen is mineralized. This is probably also the case with most free-living heterotrophs, such as *Azospirillum* (Eskew et a1 1981, Nayak et a1 1986, James 2000). In addition, although inoculations with various diazotrophs, especially cyanobacteria (see earlier) and *Azospirillum* spp. (Sumner 1990, Chalk 1991), have shown significant increases in BNF and/or rice growth, the results are variable, and often not significantly higher than those obtained with indigenous populations of diazotrophs (Nayak et a1 1986, Ladha and Reddy 1995). This may be due to several factors, such as the inoculated organisms not being able to compete with the native microbial population (Kamuru et a1 1998). The problems involved in inoculation of rice and other grasses with free-living diazotrophs are discussed in detail by Watanabe (1986), Sumner (1990), and Roger and Ladha (1992).

An alternative approach to inoculation with free-living diazotrophs is the use of N_2-fixing green manures, such as symbiotic *Azolla, Aeschynomene,* and *Sesbania.* These can undoubtedly make a high N input into lowland rice soils (Ladha et a1 1992, Ladha and Reddy 1995). Unfortunately, farmers are reluctant to use them, as there is no economic advantage over mineral N fertilizer (Ladha and Reddy 1995, Ladha et a1 1997).

Endophytic diazotrophs

There is a clear need to go beyond the "conventional" BNF systems used in rice agriculture if sustainable increases in yield are to be obtained. The best way to achieve this is to increase the ability of rice to fix its own N_2. Recently, Ladha et al (1997) put forward several strategies for enhancing BNF in rice:

1. Identify and enhance associations between rice and endophytic diazotrophs.
2. Engineer rice to allow it to nodulate with rhizobia and then fix N_2 endosymbiotically.
3. Transfer *nif* genes to the rice genome.
4. Improve CO_2 fixation and N-use efficiency.

For the first strategy, there are indications that it is already being used successfully by other grasses. For example, using the ^{15}N isotope dilution and ^{15}N natural abundance techniques, significant BNF has been shown by Brazilian varieties of sugarcane (*Saccharum* sp.) (Urquiaga et al 1992) and by the salt-tolerant forage species, Kallar grass (*Leptochloa fusca*) (Malik et al 1997). With sugarcane, the BNF may contribute up to 70% of the plant's needs, depending on the variety. The organisms responsible for the BNF are generally held to be the N_2-fixing bacteria *Acetobacter diazotrophicus, Herbaspirillum seropedicae,* and *H. rubrisubalbicans.* These bacteria are all found in high numbers (up to 10^7) within Brazilian sugarcane and, as they do not survive in the soil, they have been termed "endophytic diazotrophs" (Dobereiner et al 1995). It is thought that by inhabiting the interior of the plants these bacteria can (1) avoid competition with rhizosphere bacteria and (2) derive nutrients directly from the host plants (Boddey et al 1995, Dobereiner et al 1995, Baldani et al 1997, James and Olivares 1998). In return, as the plant interior may provide an environment conducive to N_2 fixation by being low in O_2 and relatively high in carbon, the bacteria can fix N_2 more efficiently than rhizosphere diazotrophs and can also transfer the fixed N products more efficiently to the host (Sprent and James 1995, James and Olivares 1998). In the case of Kallar grass, the most likely organism responsible for the BNF is *Azoarcus* (see reviews by Reinhold-Hurek and Hurek 1998a,b), although it should be noted that several other bacteria, such as *Azospirillum* spp., can also be isolated from the interior of Kallar grass roots (Malik et al 1997).

If endophytic diazotrophs really are partly or wholly responsible for BNF in sugarcane and Kallar grass, it is possible that rice varieties that show significant heterotrophic BNF (App et al 1986, Wu et al 1995, Shrestha and Ladha 1996, Malarvizhi and Ladha 1999) may also be obtaining their fixed N from bacteria living within their tissues, in addition to N_2-fixing heterotrophs in the rhizosphere (see earlier). Indeed, numerous diazotrophs can be isolated from surface-sterilized rice plants using N-deficient media (e.g., Barraquio et al 1997, Stoltzfus et al 1997, Stoltzfus and de Bruijn, this volume). It remains to be seen, preferably by using microscopical methods, however, whether any of these "putative endophytes" really live within rice tissues. To accurately determine the exact location of putative endophytes, Reinhold-Hurek and Hurek (1998a,b), James and Olivares (1998), and James (2000) advocated using fixed and resin-embedded material and/or scanning confocal laser microscopy

(SCLM) with intact specimens. These methods should prevent artefacts caused by movement of the bacteria during processing and sectioning. Moreover, given the great diversity of bacteria within the rhizosphere of rice, a means of identifying specific bacteria *in situ* should also be employed. This could be via immunolocalization using antibodies raised against particular diazotrophs, or else the plants could be inoculated with bacteria that have been tagged with marker genes, such as b-glucuronidase *(gusA),* β-galactosidase *(lacZ),* or green fluorescent protein *(gfp).*

In this chapter, we summarize data published so far on the endophytic and putatively endophytic diazotrophs that live naturally within rice. The criteria that we use to define "endophytic" are based on Hallmann et al (1997), that is, that the organisms be isolated from surface-sterilized plants and that they be shown, using microscopy, to be located within the plant tissues. Table 1 summarizes our knowledge about confirmed endophytic diazotrophs within rice.

Alcaligenes

Diazotrophic members of this genus have been consistently isolated from the rhizosphere of wetland rice (Vermeiren et al 1999), and the dominant strain appears to be *A. faecalis* A15 (You and Zhou 1989, You et al 1991, 1995). On the basis of its small subunit ribosomal RNA (16s rRNA) sequence, however, Vermeiren et al (1999) recently reclassified this strain as *Pseudomonas stutzeri.* You and Zhou (1989) claimed that A15 was an endophyte on evidence from micrographs of fixed and embedded material that putatively showed bacteria both outside and within the root cortex cells. In a later paper, You et al (1991) supported this by presenting scanning electron micrographs (SEMs) showing bacteria within intercellular spaces in the root cortex, and within cells close to the xylem. On the other hand, as neither of these studies used immunolabeling or molecular markers to confirm the identity of the bacteria, it cannot be said with certainty that the bacteria in the micrographs were really A15. Moreover, the claims of intracellular bacteria made by You and Zhou (1989) and You et al (1991) are also controversial as, so far, intact bacteria residing within living host cells have not been observed in any association between grasses and diazotrophic bacteria (Sprent and James 1995, Reinhold-Hurek and Hurek 1998a, James and Olivares 1998).

Indeed, it is easy to mistake organelles for bacteria in micrographs of plant cells (Hallmann et al 1997, Reinhold-Hurek and Hurek 1998a), and this might explain the apparent "intracellular" bacteria shown in, for example, Figure 1 of You and Zhou (1989). Conventional (as opposed to cryo) SEMs are also unreliable with respect to this because the preparation involved in SEMs disrupts the host cytoplasm, so that it is impossible to determine whether the cells are alive or dead (Sprent and James 1995). More reliable evidence of the endophytic occurrence of A15 has come from the study of Vermeiren et al (1998), which has shown, using resin-embedded material, that *gusA*-marked A15 will colonize the root surface, particularly at the root tips and lateral root junctions, and will also enter the epidermal cells. Within the latter cells, the bacteria have been shown to express *nzfH* (via *gusA;* Vermeiren et al 1998). Further studies using transmission electron microscopy (TEM) are now needed to determine whether the cytoplasm of the colonized host cells is intact.

Table 1. Diazotrophic bacteria confirmed by microscopy to be endophytic within rice.

Bacteria	Source[a]	Mode of infection and location	Effects of inoculation, and expression of nitrogenase	Reference
Alcaligenes faecalis (now renamed *Pseudomonas stutzeri*) A15	Roots	Mode of infection unknown, but it colonizes root cortical intercellular spaces and epidermal cells	*nifH* expression (via gusA) within root epidermal cells. ARA associated with roots.	You and Zhou (1989), You et al (1991), Vermeiren et al (1998)
Azoarcus sp. BH72	Kallar grass roots	Colonizes root aerenchyma, vascular system, and dead cells. Identity confirmed by *gusA* and IGL[b].	*nifH* expression (via *gfp*) within roots. Increased dry weight and protein content of seedlings, but not due to BNF.	Hurek et al (1994), Reinhold-Hurek and Hurek (1998a,b)
Azospirillum brasilense	Seeds	Localized within cavities. IGL.	Unknown.	Baldani et al (1993)
Azospirillum irakense	Roots and rhizosphere	Mode of infection unknown, but it colonizes root epidermal cells.	*nifH* expression (via *gusA*) within root epidermal cells.	Khammas et al(1989), Vermeiren et al (1998)
Burkholderia sp. M209	Roots, stems, leaves	Infectsvia "cracks" in the root epidermis and at lateral root junctions.	Unknown.	Baldani et al (1997)
Herbaspirillum seropedicae 267,294	Roots	Infects via lateral root junctions (Fig. 2A, B). Colonizes intercellular spaces and xylem vessels in roots, stems, and leaves (Fig. 2C, D). Also seeds. Identity confirmed by *gusA* and IGL.	Strain 294 increased N content of seedlings by 50%. Significant ARA associated with rice plants containing high numbers of *Herbaspirillum* spp.	Baldani et al (1986, 1995), Boddey et al (1995), Olivares et al (1996), Barraquio et al (1997). This study
Rhizobium leguminosarum bv. *trifolii* EII, E12	Surface-sterilized roots	Unknown, but possibly via cracks at lateral root junctions. Site of colonization yet to be confirmed.	Significantly increased dry weight, N content, and grain yield of plants in laboratory and field experiments. No nitrogenase activity (ARA).	Yanni et al (1997)
Serratia marcescens	Surface-sterilized roots	Unknown. Localized within root aerenchyma, stem aerenchyma, and xylem vessels (Fig. 1A–D).IGL.	*nifH* expression (via *gusA*) when associated with plants, and nitrogenase activity (ARA) when external carbon added.	Gyaneshwar et al (unpublished), Hurek et al (this volume). This study

Diazotrophic *Alcaligenes* strains have also been isolated from the rhizosphere of rice by Fujii et a1 (1987) and Oyaizu-Masuchi and Komagata (1988), as well as from surface-sterilized rice seeds (Mathan, unpublished). Further studies are needed to confirm their identities and their endophytic nature.

Azoarcus

Until recently, this bacterium has been thought to be an endophyte only of Kallar grass, as this is the only species from which it has so far been isolated (see reviews by Reinhold and Hurek 1998a,b). *Azoarcus* sp. strain BH72, however, can also infect gnotobiotically grown rice in laboratory experiments in which seedlings have been inoculated with the bacterium (Hurek et a1 1994, Reinhold-Hurek and Hurek 1998a,b, Egener et a1 1998). It appears to enter Kallar grass and rice via root tips and lateral root junctions and then enters the xylem, from where it is spread to the upper parts of the plants via the transpiration stream (Hurek et a1 1994, Reinhold-Hurek and Hurek 1998a,b). In both plants, *Azoarcus* colonizes mainly the lysigenous aerenchyma of the root cortex, within which it can express the Fe protein of nitrogenase (Hurek et a1 1994). Moreover, using a *nifH-gfp* fusion, Egener et a1 (1998) and Reinhold-Hurek and Hurek (1998b) have shown that *Azoarcus* can express nitrogenase-encoding genes while within, and in association with, rice roots.

More recently, Hurek et a1 (1997) have detected *nifH* DNA sequences of the genus *Azoarcus* within macerates of washed roots of wetland rice grown in Japan (Ueda et a1 1995b), and the presence of *Azoarcus* within wild rice in the Philippines and in Nepal has recently been confirmed (Hurek et al, this volume). Therefore, it now appears that *Azoarcus* is a genuine endophyte of rice as well as of Kallar grass.

Azospirillum

As stated earlier, this is one of the most common genera isolated from the rhizosphere of rice. Interestingly, although it is generally regarded as a rhizosphere bacterium, it has often been reported that the best results obtained with inoculation are with those strains that actually penetrate the roots (Sumner 1990), suggesting that some strains of *Azospirillum* are also endophytic within grasses (see reviews by Patriquin et a1 1983, James and Olivares 1998). In the case of rice, *A. lipoferum* and *A. brasilense* have both been isolated from the roots and the stems (Baldani and Dobereiner 1980, Ladha et a1 1982), and *A. amazonense* has been isolated from the roots (Pereira et a1 1988). More recently, Baldani et a1 (1993) and Vermeiren et a1 (1998) have presented microscopical evidence as to the endophytic nature of *Azospirillum* in rice. Baldani et a1 (1 993) used immunogold labeling to detect the bacteria in intercellular spaces and within dead cells in the leaves and roots. They were also observed within cavities in the seeds, alongside *Herbaspirillurn seropedicae.* Vermeiren et a1 (1998) used a *nifH-gusA* fusion of *A. irakense* (originally isolated from the rhizosphere and roots of rice; Khammas et a1 1989) to show that it could colonize and express *nif* genes within the root epidermal cells. Again, further studies with TEM sections are needed to confirm the results of Baldani et a1 (1993) and Verrneiren et a1 (1 998). Stoltzfus et a1 (1997) also isolated several putative endophytes of rice that are likely to be members of the

genus *Azospirillum,* although they do not appear to be closely related to any of the presently described species.

Finally, Christiansen-Weniger (1997) inoculated 2,4D-inducedpara-nodulated rice plants with an ammonium-excreting mutant of *A. brasilense* and suggested that the bacteria colonized the para-nodules in large numbers. The organisms may have initially entered the plants via cracks that had formed when the tumors emerged from the epidermis.

Burkholderia

A diazotrophic species of *Burkholderia* was isolated from rice fields in Vietnam and subsequently named *B. vietnamiensis* (Gillis et a1 1995). This bacterium appears to have potential as a rice inoculum as it has resulted in significant yield increases after field trials in Vietnam (Tran Van et al 1994, 1996, 1999). As *B. vietnamiensis* was isolated from rhizosphere macerates (roots + adhering soil), however, it cannot yet be described as an endophyte (Baldani et a1 1997). On the other hand, diazotrophic strains of a species of *Burkholderia* (e.g., strains M130 and M209) have been isolated from the interior of rice roots, stems, and leaves in Brazil. Preliminary results with optical microscopy suggest that they can enter rice roots via cracks in the root epidermis, particularly at the points of emergence of secondary roots, and subsequently "massively" colonize their interior (Baldani et al 1997). Strains similar to M130 have since been isolated from surface-sterilized plants by Stoltzfus and de Bruijn (this volume).

Enterobacteriaceae

Diazotrophic enterobacters are frequently isolated from the rhizosphere of rice, particularly *E. cloacae* and *E. agglomerans* (now renamed *Pantoea agglomerans*), and, along with *Pseudomonas* spp. (see later), may be the dominant N_2-fixing heterotrophs in the rhizosphere (Thomas-Bauzon et al 1982, Ladha et al 1983, Fujii et al 1987, Oyaizu-Masuchi and Komagata 1988, Omar et al 1989, Bilal et al 1993, Achouak et al 1994, Barraquio et al, this volume). The strains isolated by Thomas-Bauzon et al (1982), Ladha et a1 (1983), Oyaizu-Masuchi and Komagata (1988), and Omar et a1 (1989), however, came from non-surface-sterilized plant material, and Bilal et al(1993) and Achouak et al(1994) only observed them colonizing the root surfaces. Therefore, there is not much evidence as yet that *Enterobacter* spp. are endophytic in rice. On the other hand, Quadt-Hallmann and Kloepper (1996) have shown micrographs confirming the endophytic nature of *E. asburiae* strain JM22 within several nonlegumes (although not rice), suggesting that enterobacters can be endophytic per se (although it must be stated that diazotrophy by strain JM22 has yet to be demonstrated). Moreover, Gyaneshwar et al (unpublished) and Hurek et al (this volume) have recently shown that a diazotrophic strain of *Serratia marcescens* (IRBG500) can be isolated from surface-sterilized wetland rice. These are the first reports of N_2 fixation by a species of *Serratia.* A *nifH-gusA* fusion of this bacterium was constructed and was inoculated into gnotobiotically grown rice seedlings. The bacteria, expressing *nifH,* colonized the surfaces of the roots and were also observed associated with the stems and leaves. Further examination using light microscopy and TEM (coupled with

immunogold labeling using a polyclonal antibody raised against *S. marcescens* IRBGSOO) showed that the bacteria colonized the root aerenchyma and intercellular spaces (Fig. lA), and were particularly abundant within the stem aerenchyma and xylem vessels (Fig. 1B–D).Further work is being undertaken to determine whether *S. marcescens* IRBGSOO expresses nitrogenase *in planta.*

In the case of *Klebsiella,* although substantial N_2 fixation (up to 19% of the plant's N requirements) has been demonstrated in rice inoculated by wild-type or genetically engineered strains of *K. oxytoca* (and *E. cloacae*) by Yoo et a1 (1986) and Fujii et a1 (1987), there is little evidence that *K. oxytoca* or any other *Klebsiella* spp. are endophytic. For example, *K. planticola* has been isolated from submerged portions of rice stems by Ladha et a1 (1983), but these bacteria may have originated from the floodwater. The only published microscopical evidence for the colonization of rice by a *Klebsiella* sp. comes from Boonjawat et a1 (1991), who showed it adhering in microcolonies to the root surfaces via mucigel. Further microscopical studies are needed to determine the location of these potentially important bacteria (Yoo et al 1986, Fujii et a1 1987).

Herbaspirillum

A bacterium commonly isolated from rice is *Herbuspirillum seropedicae.* It is also found in maize, sorghum, sugarcane, and several other Gramineae (Baldani et a1 1986, 1993, 1995, 1996, Olivares et a1 1996). *Herbaspirillum-like* bacteria have also been isolated from wetland rice in the Philippines (Barraquio et a1 1997, Barraquio and Mathan, upublished). In most of the plants in which it has been found, *H. seropedicae* appears to colonize mainly the roots (and stems in the case of sugarcane), but does not survive in the rhizosphere in a culturable form (Baldani et a1 1993, Olivares et a1 1996). The only other diazotrophic species of *Herbaspirillum, H. rubrisubalbicans,* is also endophytic but has a narrower host range than *H. seropedicae,* and so far has been isolated only from sugarcane leaves, stems, and roots, as well as the leaves of sorghum (Olivares et a1 1996). On the other hand, there have been recent reports that *H. rubrisubalbicans* has been isolated from rice and palm trees in Brazil (Baldani et a1 1997), along with the isolation of "*Herbaspirillum*-like" bacteria from C_4 energy crops such as *Miscanthus* and *Pennisetum purpureum* (Kirchhof et a1 1997).

Herbaspirillum spp. are particularly interesting because, although they are diazotrophic, they may also show (depending on plant genotype) some phytopathogenic potential on sugarcane and sorghum (Pimentel et a1 1991, Olivares et a1 1997, James et a1 1997). *H. rubrisubalbicans,* originally named *Pseudomonas rubrisubalbicans,* is the agent responsible for mottled-stripe disease in sugarcane leaves and red-stripe disease in sorghum leaves (Pimentel et a1 1991). *H. seropedicae* can also induce mild disease symptoms on leaves of some varieties of sorghum, sugarcane, and *Pennisetum purpureum* (Pimentel et a1 1991), but, so far, neither of the *Herbaspirillum* spp. has been shown to induce disease symptoms on rice.

The endophytic nature of *Herbaspirillum* spp. in sugarcane and sorghum has been confirmed using light and electron microscopy, together with immunogold labeling. In the case of mottled-stripe disease-susceptible sugarcane leaves, *H.*

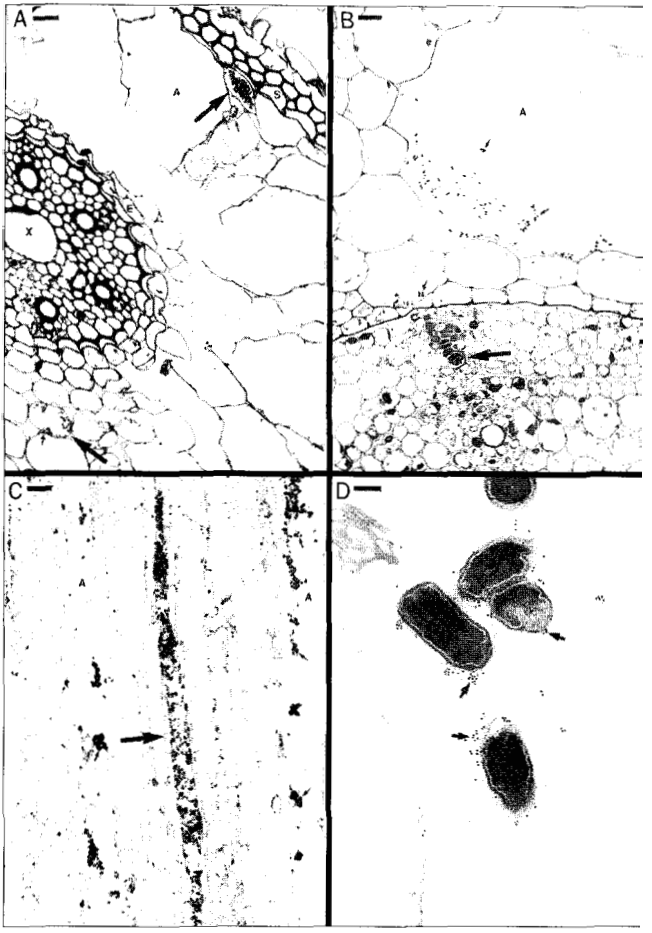

Fig. 1. Light and transmission electron microscope (TEM) study of the infection of gnotobiotically grown rice seedlings by a *nifH-GUS* fusion of a diazotrophic strain of *Serratia marcescens* originally isolated from rice. All micrographs are of sections taken at 5 d after inoculation of the rooting medium. (A) Transverse section (TS) of a primary root. The bacteria can be seen colonizing cells (large arrows) and intercellular spaces (small arrows) in the lysigenous aerenchyma layer within the cortex. E = endodermis, A = aerenchyma, X = xylem, S = sclerenchyma. Bar = 20 pm. (B) TS of the base of a stem. Bacteria (small arrows) can be seen in the large, lysed cells of the aerenchyma (A), and also within the xylem vessels (large arrow). Bar = 20 mm. (C) Longitudinal section of a stem. This section was immunogold-labeled (followed by silver-enhancement) using an antibody raised against *S. marcescens.* The bacteria (small arrows) can be seen as black-stained objects within the aerenchyma (A) and in a xylem vessel (large arrow). Bar = 20 pm. (D) TEM of bacteria within the stem aerenchyma. This section was immunogold-labeled with an antibody raised against *S. marcescens.* The surfaces of the bacteria are labeled with 15-nm gold particles (arrows). Bar = 20 nm.

rubrisubalbicans colonizes extensively the xylem and the intercellular spaces in the mesophyll, and also the substomatal cavities. Its growth, however, is restricted in leaves of mottled-stripe disease-resistant varieties, where it appears to only form microcolonies in the xylem, which are surrounded by host-derived material (Olivares et a1 1997, James and Olivares 1998). In contrast to *H. rubrisubalbicans, H. seropedicae* causes a hypersensitive response when it is inoculated into sugarcane leaves (Olivares et a1 1997), confirming that the leaves of this plant are not a compatible location for it (Olivares et a1 1996). On the other hand, both species will colonize the roots of sugarcane, where they enter via the lateral root junctions and subsequently colonize the root and stem xylem (James and Olivares 1998). In the case of sorghum, both *Herbaspirillum* spp. will colonize the leaves, where they appear to be restricted to the xylem vessels, with only *H. rubrisubalbicans* actually blocking them (James et a1 1997, James and Olivares 1998). *H. rubrisubalbicans,* but so far not *H. seropedicae,* has been shown to express the Fe protein of nitrogenase in leaves of sugarcane and sorghum (Olivares et a1 1997, James et a1 1997).

A preliminary study at the International Rice Research Institute (IRRI) on the infection of rice variety IR42 by a *gusA* fusion of *H. seropedicae* strain 267 (originally isolated from rice in Brazil by Baldani et a1 1986) has been presented by Barraquio et a1 (1997). In this study, at 28 d after inoculation, surface-disinfested roots of the seedlings were only sparsely colonized by the bacteria, although there was some staining of the subepidermal region, suggesting the presence of the bacteria. Similar results have been presented by Baldani et al (1997) with rice infected with *H. rubrisubalbicans.* In a more detailed study conducted at IRRI and the University of Dundee (UK) using the same *gusA*-labeled strain 267 of Barraquio et a1 (1997), we have shown that *H. seropedicae* will readily infect the lateral root junctions of variety IR72 at only 2 d after inoculation (Fig. 2A,B). At this stage, the bacteria can also be seen in the stem and leaf xylem vessels (not shown), as well as within the leaf intercellular spaces (Fig. 2C,D). At 7 d after inoculation, a host-defense reaction was observed in some of the stem xylem vessels (not shown). Throughout the study, the bacteria in the micrographs were confirmed to be *H. seropedicae* using immunogold labeling with an antibody raised against strain 267 (James et a1 1997) (Fig. 2C,D). The antibody was kindly donated by Dr. F.L. Olivares, EMBRAPA-Agrobiologia, Rio de Janeiro, Brazil. Uninoculated control plants showed no *gus* labeling, and no bacteria were visible on, or in, the plants when viewed under the microscope.

Pseudomonas

The diazotrophic nature of some members of this genus is still a matter of debate, and the genus as a whole contains many bacteria without clear taxonomic status and appears to be in a constant state of flux (see review by Chan et a1 1994). Nevertheless, several bacteria that have been placed within it are clearly diazotrophic, such as strains of *P. diazotrophicus, P. fluorescens, P. saccharophila,* and *P. stutzeri* (Chan et a1 1994). Moreover, other diazotrophs are now being moved into the genus, such as *Alcaligenes faecalis* strain A15, which has been recently renamed as a diazotrophic strain of *P. stutzeri* (Vermeiren et a1 1999) (see earlier). Although diazotrophic pseudomonads

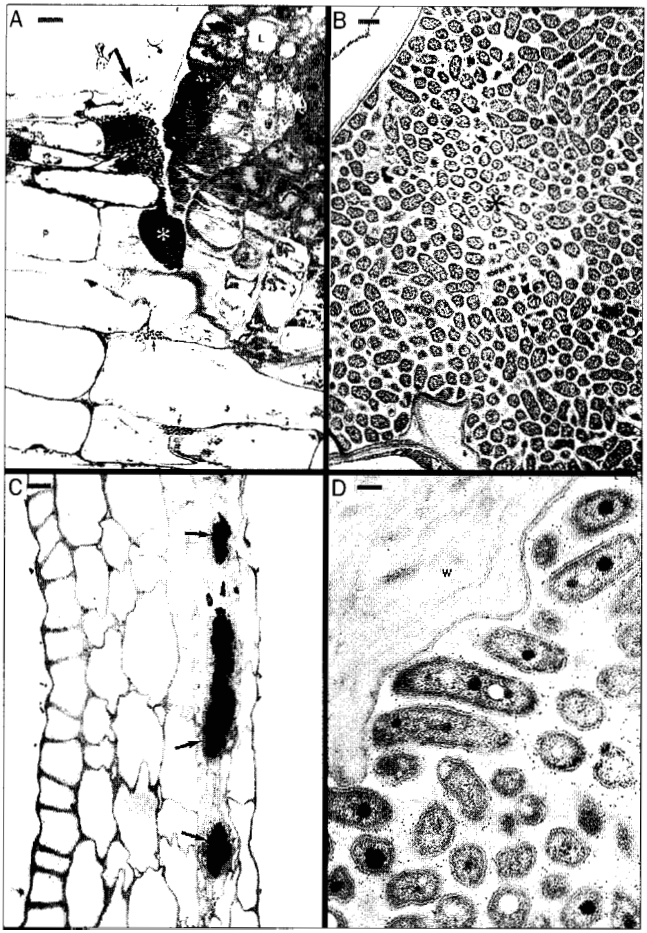

Fig. 2. Light and transmission electron microscope (TEM) study of the infection of gnotobiotically grown rice seedlings by a constitutively expressed *GUS* fusion of *Herbaspirillurn seropedicae* strain 267. All micrographs are of sections taken at 2 d after inoculation of the rooting medium. (A) Light micrograph showing bacteria (large arrow) entering the primary root (P) at a crack formed at the point of emergence of a lateral root (L). Bacteria can also be seen colonizing intercellular spaces within the inner layers of the primary root cortex (small arrows). Bar = 10 mm. (B) TEM of a large colony of bacteria (*) colonizing a cavity adjacent to the point of emergence of a lateral root (see area with * in Fig. 2A). Bar = 1 mm. (C) Light micrograph of a transverse section of a leaf. This section was immunogold-labeled (followed by silver-enhancement) using an antibody raised against *H. seropedicae.* The colonies of bacteria can be seen as densely black-stained areas within intercellular spaces (arrows). Bar = 20 pm. (D) TEM of bacteria within an intercellular space in a leaf (see Fig. 2C). This section was immunogold-labeled with an antibody raised against *H. seropedicae.* The surfaces of the bacteria are labeled with 15-nm gold particles. W = cell wall. Bar = 500 nm.

are commonly isolated from the rhizosphere of wetland rice (Bally et al 1983, 1990, Barraquio et al 1983, Watanabe et al 1982, 1987a), with the exception of *P. stutzeri* strain A15 (Vermeiren et al 1999), there is no direct evidence that any of them are endophytic. This is because surface sterilization appears not to have been used in most of the studies, and so far no microscopical evidence has been presented. On the other hand, among the putatively endophytic diazotrophs isolated by Stoltzfus et a1 (1997), one was closely related to *P. cepacia.* As with *Klebsiella* spp. (see above), further work on the potentially endophytic nature of diazotrophic Pseudomonas spp. is clearly needed, especially as they may be the predominant N_2-fixing heterotrophs in the rhizosphere of rice (Barraquio et a1 1983, Watanabe et al 1987a).

Rhizobia

Several researchers have attempted to construct artificial associations between rhizobia and rice (A1 Mallah et al 1989, Ridge et al 1993), particularly with *Azorhizobium caulinodans* (Christiansen-Weniger 1996, Webster et al 1997, Reddy et al 1997) and other rhizobia from *Sesbania* spp. (de Bruijn et al 1995). The "potentials" and "pitfalls" of this approach were reviewed extensively by de Bruijn et al (1995) and will not be discussed here. Recent evidence, however, suggests that there may also be "natural" associations between rhizobia and rice. For example, among the putatively endophytic bacteria isolated by Stoltzfus et al (1997) and Stoltzfus and de Bruijn (this volume), some showed similarity to *A. caulinodans,* and Ladha et al (1989) showed that this bacterium survives in the rhizosphere of rice when *Sesbania rostrata* is used as a green manure. Moreover, Yanni et al (1 997) have described in detail the isolation of *R. leguminosarum* bv. *trifolii* from wetland rice in Egypt, and shown conclusively that it is endophytic, although they have not yet described its exact location in the plant. In addition, Yanni et al (1997) showed that the same strains that were endophytes of rice were Fix+ and were also capable of nodulating the Berseem clover *(Trifolium alexandrinum)* that is commonly grown in rotation with Egyptian rice as a green manure.

Seed endophytes

Hallmann et al (1997) and James and Olivares (1998) have suggested that some endophytic bacteria may be transmitted from generation to generation via seeds. Certainly, seeds of many plants contain bacteria, including rice. Mukhopadhyay et al (1996) isolated strains of *Enterobacter, Serratia,* and *Bacillus* sp. and showed that *E. agglomerans* could be transmitted to the next generation of seeds. The bacteria were associated with the seed hull and husk, but were also observed by SEM within the embryonic tissue. Unfortunately, Mukhopadhyay et al (1996) did not test the strains for N_2-fixing ability and so they cannot as yet be described as endophytic diazotrophs. On the other hand, Baldani et al (1993) reported the presence of *H. seropedicae* and *A. brasilense* in cracks in the seed surface, as well as within cavities beneath the seed hull. Moreover, diazotrophic strains of *Alcaligenes, Pseudomonas,* and *Bacillus* have been isolated from rice seedlings germinated from surface-sterilized dehulled seeds, and hence could be seed-borne endophytes of rice. Interestingly, these strains form

separate clusters in bootstrap analysis from the known species and could represent novel strains (N. Mathan, unpublished). Further work is currently in progress at IRRI to confirm this.

Evidence for BNF by endophytes and effects of inoculation

App et al (1980, 1986) showed that heterotrophic BNF in the rhizosphere was enhanced by the presence of the rice plants, possibly by the latter contributing a carbon source. This suggests a close link between wetland rice and the diazotrophic bacteria found associated with it. The problem is to distinguish between N_2 fixation by rhizosphere diazotrophs on the one hand and endophytic diazotrophs on the other. There is probably a continuum between the two, with both making some contribution, but with the endophytes possibly making a more immediate one as they are so close to the plants themselves (i.e., within them). Unfortunately, field techniques, such as N-balances and ^{15}N isotope dilution studies (App et al 1980, Shrestha and Ladha 1996), cannot determine whether the measured BNF is "immediate," and ARAs say nothing about actual incorporation of fixed N. A possible means of showing whether there is a transfer of fixed N products to the plant over the short term, however, is the $^{15}N_2$ gas incorporation assay (van Berkum and Bohlool 1980, Boddey 1987, James 2000). For example, Yoshida and Yoneyama (1980) and Ito et al (1980) showed that significant amounts of ^{15}N could be incorporated into uninoculated rice plants, particularly the roots, basal stem, and outer leaf sheath, over a period of 7 to 13 d. In contrast, later studies by Eskew et al (1981) and Nayak et al (1986) showed relatively little incorporation into the plants, with most of the label (80–90%) being present in the rhizosphere soil. Eskew et al (1981) concluded that any ^{15}N uptake by the plants was most likely derived from the mineralization of rhizosphere diazotrophs rather than from any immediate fixation. Interestingly, Nayak et al (1986) also showed no significant increase in incorporation of ^{15}N with plants inoculated with *A. lipoferum.*

A problem with the $^{15}N_2$ incorporation assay is that few replicates can be used due to difficulties in setting up the experiments and to the high costs of the $^{15}N_2$ (Boddey 1987, Boddey et al 1995, 1998, James 2000). Therefore, it is difficult to determine whether the results of the studies performed so far are representative and so further experiments with more replicates are needed. Indeed, the $^{15}N_2$ assay could be used in combination with gnotobiotically grown plants inoculated with specific organisms, such as *Azoarcus, H. seropedicae,* and *P. stutzeri* A15, to determine whether particular endophytic diazotrophs really are capable of transferring fixed N products to the plants. *nif* mutants of these bacteria, if available, would make excellent controls. This is well illustrated by the study of Hurek et al (1994), which demonstrated that increases in dry weight and protein content shown by rice plants inoculated with *Azoarcus* sp. BH72 were not due to BNF because a nif mutant gave a response similar to that of the wild type. Several other recent studies have demonstrated growth increases after inoculation with various diazotrophs, but have not yet shown conclusively that they are due to BNF. For example, Yanni et al (1997) inoculated rice with *R. leguminosarum* bv. *trifolii* strains Ell and E12 and showed a significant increase

in plant dry weight and in grain yields, but could not attribute the response with any certainty to BNF. Hormonal effects are one of several possible alternative mechanisms by which endophytic bacteria could enhance plant growth (Hurek et al 1994, James and Olivares 1998). On the other hand, *Herbaspirillurn* has shown some promise as a rice inoculant and the response may be at least partly due to BNF. For example, significant acetylene reduction activity has been shown in rice plants (variety IR42) and fluctuations in nitrogenase activity were correlated with variations in the numbers of *Herbaspirillum* spp. within the plants (Boddey et al 1995). More specifically, in experiments testing various strains with axenically grown seedlings, Baldani et al (1995, 1997) showed that *H. seropedicae* strain 294 could contribute more than 50% of the plant's N requirement, and even showed that the potential phytopathogen, *H. rubrisubalbicans* strain M4, could provide the plant with about 30% of its N. These results have been supported by recent work from Pakistan by Mirza et al (this volume), who inoculated seedlings with a *Herbaspirillum* isolate from rice (RR8, species unknown) and obtained 39% of nitrogen derived from air (Ndfa) using the ^{15}N isotope dilution technique.

Further work and questions to be answered

A consistent theme with all work on heterotrophic BNF in rice is the importance of the plant genotype (App et al 1986, Watanabe et al 1987b, Wu et al 1995, Shrestha and Ladha 1996, Malik et al 1997, Malarvizhi and Ladha 1999). This suggests that BNF is a trait that could be selected for and improved upon (Wu et al 1995, Shrestha and Ladha 1996). The plants, however, are only one side of the story, and the diazotrophs, which actually do the fixing, must also be identified and maybe even enhanced. The challenge that now faces research on heterotrophic BNF by rice, and other Gramineae (James 2000), is to find the "best" combination(s) of plant genotypes and diazotrophs, and to establish beyond a doubt that they can fix significant quantities of N_2. This can only be achieved via an integrated approach, with more field and greenhouse experiments to determine the best varieties in terms of BNF, combined with further attempts to isolate, characterize, and localize the most abundant diazotrophs associated with the plants.

Finally, and with regard to endophytic diazotrophs, many questions still need to be addressed (Fig. 3). Although current thinking considers them to make the most important contribution to BNF in some grasses, in rice (and possibly other grasses), they may be only a relatively small subpopulation of a much larger rhizosphere diazotroph population (see first part of this chapter), and hence their actual contribution could be minor. For example, the total number of (culturable) diazotrophic endophytes recently isolated from wetland rice in the Philippines was estimated at only 10^6-10^7 (Barraquio et al 1997). When it is considered that a soybean *(Glycine max)* plant contains approximately 10^{11} rhizobia1 bacteroids (on the basis of 10^9 nodule^{-1}; Lin et al 1988), the number of diazotrophs living *within* rice appears to be relatively trivial.

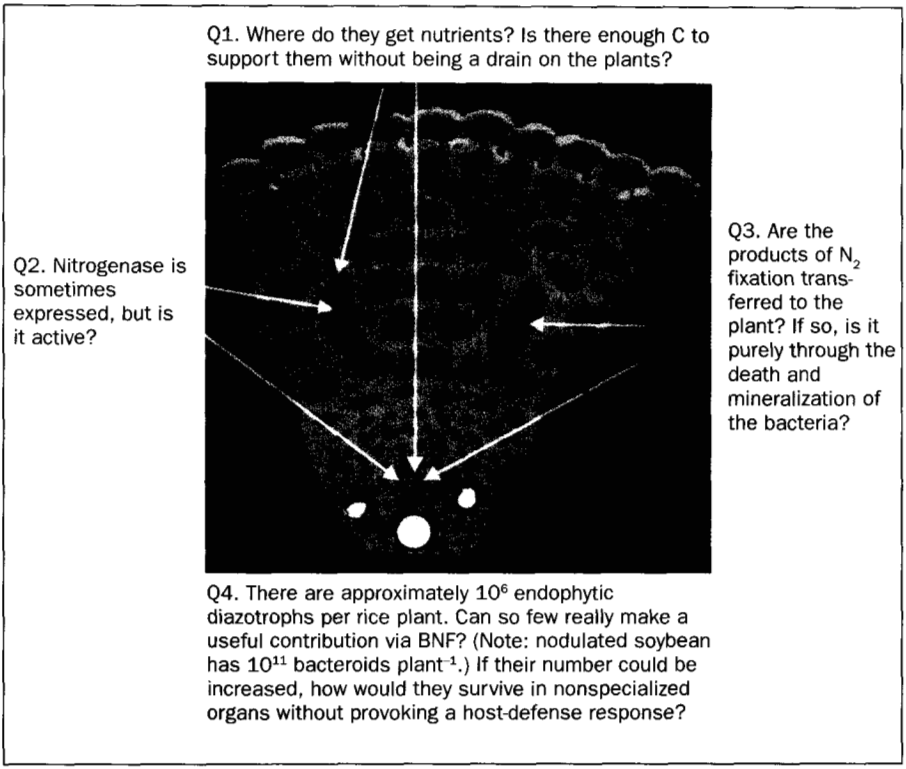

Fig. 3. Schematic diagram of a rice root colonized by endophytic diazotrophs. Questions (Q1-4) relating to the viability of "symbiotic" N_2-fixing associations between rice and these bacteria are indicated by arrows.

In addition to the "numbers problem," in a recent review of the evidence for endophytic "symbioses," James (2000) suggested that there are fundamental questions about how efficiently the endophytes can actually function within grasses when no obvious "symbiotic" structures appear to be present. In all N_2-fixing symbioses identified so far, specialized organs have evolved to house the diazotrophs, such as nodules on legumes and actinorhizal plants, and leaf cavities in *Azolla.* Such structures appear to be essential in allowing for the efficient exchange of metabolites between the partners (Mylona et a1 1995, James 2000). At present, it is difficult to see how the apparently random distribution of bacteria within intercellular spaces, aerenchyma, dead cells, and xylem vessels that typifies endophytic associations (James and Olivares 1998, and see Figs. 1–3) can perform functions analogous to those of such highly evolved organs. Clearly, the rapid transfer to the plants of the immediate products of N_2 fixation by endophytic diazotrophs must be demonstrated if we are to consider these associations as symbiotic (James 2000). In addition, if we want to improve endophytic associations between grasses and diazotrophs, it will presum-

ably be necessary to increase the numbers of bacteria within the plants. But how can this be done without provoking a host-defense response (Olivares et al 1997, James et al 1997)? Until these fundamental questions are answered (Fig. 3), it must be assumed that endophytic diazotrophs either do not make a significant contribution to grasses such as rice, or they do so via a means other than symbiotic N_2 fixation (James 2000).

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Xylem colonization of rice and Arabidopsis by Azorhizobium caulinodans ORS571

Edward C. Cocking

Our discovery that the xylem of the roots of *Sesbania rostrata* is colonized when inoculated with *Azorhizobium caulinodans* led us to investigate whether the xylem of rice and other nonlegumes could also be colonized. If so, this might provide a nonnodular niche for endophytic symbiotic nitrogen fixation in rice and other nonlegume crops, somewhat comparable to the naturally occurring nonnodular endophytic nitrogen fixation by diazotrophs in xylem vessels and intercellular spaces that other researchers have detected in sugarcane. Our earlier studies indicated the colonization of the xylem of lateral roots of wheat inoculated repeatedly with *Azorhizobium caulinodans.* We also observed the crack entry of *A. caulinodans* and intercellular colonization of the root systems of rice, wheat, and the model nonlegume *Arabidopsis thaliana,* and stimulation by flavonoids such as naringenin.

Recently, using the *lacZ* reporter gene, we have shown, for the first time, in rice inoculated with *Azorhizobium caulinodans,* that the xylem of roots can be colonized by azorhizobia. We have also shown, for the first time, that the xylem of the root system of the model nonlegume dicot *Arabidopsis thaliana* is extensively colonized, when inoculated with *Azorhizobium caulinodans.* Using this ability to induce xylem colonization by azorhizobia in rice and *Arabidopsis,* we are now investigating factors, including flavonoids, that might influence the extent of xylem colonization, and also the extent to which xylem colonization by azorhizobia will provide a niche for nonnodular symbiotic nitrogen fixation.

The inoculation of nonlegumes with various diazotrophic bacteria has been studied for many years with the expectation that these bacteria would fix dinitrogen gas and provide combined nitrogen to the plant for enhanced crop production. Measurements of nitrogen fixation in rice and wheat using ^{15}N, however, have confirmed that the majority of the fixed nitrogen remains in the bacteria within the root environment (Okon 1985). In most instances, bacteria colonize only the surface of the roots and remain vulnerable to competition from other rhizosphere microorganisms. In symbiotic systems, nitrogen-fixing bacteria colonize the plant internally and become endo-

phytic. They are thus protected from competition with rhizosphere microorganisms and have the possibility of more intimate metabolic exchange with host plants. Quispel (1991) suggested that only in endophytic systems are the prerequisites for effective nitrogen fixation likely to be fulfilled in interactions between nonlegumes and diazotrophic bacteria. In this respect, it is of interest that, more than a century ago, Schneider (1893) undertook experiments to force an interaction between the rhizobia and the roots of maize and other nonlegume crops. He obtained extensive infection by rhizobia in parenchymatous cells, at the crack entry point, near the vicinity of the emergence of maize lateral roots, but further invasion did not occur and the rhizobia did not colonize the plant internally and become endophytic.

Strategic approaches

One approach currently being explored with sugarcane and with rice is to seek naturally occurring endophytic diazotrophs and to attempt to optimize such endophytic nitrogen fixation. The alternative approach, which we are investigating, is to determine in a range of nonlegumes whether any rhizobial strains can be induced to colonize nonlegumes internally and fix nitrogen endophytically, either in a nodular or in a nonnodular niche. While rhizobia are well known for their ability to induce nodules and form nodular symbiotic endophytic associations with legumes, relatively little is known about whether they can form any type of nodular or nonnodular association with nonlegumes, other than *Parasponia* spp. (Webster et al 1998a). Moreover, little is known about the early infection events during root nodulation of *Parasponia* spp. by rhizobia, including *R. fredii* USDA 257 (Pueppke and Broughton 1999). Lancelle and Torrey (1984) reported invasion of the rhizobial endophyte through the intercellular spaces of the epidermis into the cortex and Bender et al (1987) detected a zone of elongating cells directly behind the root tip susceptible to infection.

Our earlier studies indicated that rhizobia entered rice, maize, wheat, and oilseed rape plants at low frequency at points of emergence of lateral roots (Cocking et al 1992, 1994). Among the rhizobia we have used, *Azorhizobium caulinodans* ORS571, which forms nodules on the stems and roots of the tropical legume *Sesbania rostrata,* is especially interesting. ORS571 forms nodules after crack-entry invasion, that is, bacteria enter intercellularly between adjacent cells (Tsien et al 1983, Ndoye et al 1994). It is also able to fix nitrogen in the free-living state without differentiation into bacteroids, in up to 3% oxygen (Kitts and Ludwig 1994). Our discovery that the xylem of the roots of *S. rostrata* is colonized when it is inoculated with *A. caulinodans* ORS571 (O'Callaghan et al 1997) led us to investigate whether the xylem of rice and other nonlegumes would be colonized if they were inoculated with ORS571 under suitable conditions. If so, this might provide a nonnodular niche for endophytic nitrogen fixation in rice and other nonlegumes somewhat comparable to the situation arising from the naturally occurring colonization of intercellular spaces and xylem of sugarcane by endophytic diazotrophs (James and Olivares 1997). We have observed the crack entry of *A. caulinodans* ORS571 and intercellular colonization of the cortex of root systems of rice (Webster et al 1997), wheat (Webster et al 1998b), and the

model nonlegume *Arabidopsis thaliana* (Gough et al 1997a,b) and stimulation of colonization by flavonoids such as naringenin (Gough et al l996, 1997a,b, Webster et al 1998b).

Xylem colonization of *Sesbania rostrata* by *Azorhizobium caulinodans* ORS571

Agrobacteria, diazotrophic plant pathogens closely related taxonomically to rhizobia, are known to invade the xylem of several species, including *Sesbania rostrata* (Vlachova et al 1987). In our study of the interaction of ORS571 with *S. rostrata,* we found that ORs571 colonizes xylem elements, in addition to inducing and invading nodules in the root cortex. Azorhizobia were detected microscopically and their presence was confirmed by the expression of a *lacZ* reporter gene (O'Callaghan et al 1997). We also examined whether rhizobial Nod factors were involved in xylem colonization in *S. rostrata,* and whether xylem colonization was dependent upon or followed nodulation. We found that the mutant ORS571::*nodC* (pXLGD4), which is deficient in Nod factors and which does not induce nodules in *S. rostrata,* still colonized lateral root xylem in this legume. Light and electron microscopy revealed bacterial colonization to be similar to colonization by ORS571 (pXLGD4). The *nodC* mutant invaded the plant through the annular crack at the bases of emergent lateral roots by the same route as the wild-type bacterium (Ndoye et al 1994). Our finding that crack-entry and xylem colonization of *S. rostrata* lateral roots by ORS571 is Nod factor independent is particularly interesting in view of our observations that the colonization of lateral root cracks of several nonlegumes by ORS571 is also Nod factor independent (Gough et a1 1996, 1997a,b, Webster et al 1997, 1998b).

From these studies on the colonization of root xylem of *S. rostrata* by ORS571, and our finding that rhizobia can colonize concomitantly both xylem and nodules, arises the novel perspective that some rhizobia can exist as symbionts in nodules and as benign vascular endophytes. Alternatively, as we have suggested (O'Callaghan et a1 1997), the possibility exists that the vascular rhizobial endophytes could also be symbiotic and contribute fixed nitrogen to their host. Currently, in this respect, we are evaluating whether xylem colonization in *S. rostrata* contributes to nitrogen fixation using ORS571::*nodC* (pXLGD4), which, as we have shown, colonizes the xylem but does not nodulate *S. rostrata.*

These studies on xylem colonization of *S. rostrata* by ORS571 suggested to us that it might be possible, with further experimentation, to find conditions that would enable the xylem of nonlegumes such as rice, wheat, sorghum, oilseed rape, and *Arabidopsis thaliana* to be extensively colonized by azorhizobia, with the possibility that this might provide a low pO_2 for nitrogenase activity and a site for metabolic exchange resulting in nitrogen fixation of benefit to the nonlegume. Several lines of evidence encouraged us to explore this possibility. As mentioned previously, xylem colonization by bacteria has already been demonstrated for diazotrophic agrobacteria in S. rostrata (Vlachova et al 1987) and for *Acetobacter diazotrophicus* in sugarcane (James et al 1994). Many plant species exhibit xylem colonization by bacteria without plant disease symptoms and xylem colonization is increasingly being seen as a

common aspect of plant-microbe interactions (Hallmann et a1 1997). Moreover, it is becoming increasingly realized that the xylem may be more robust structurally and physiologically than previously envisaged and that "the xylem is not a vulnerable pipeline on the edge of disaster" (Canny 1998). We were encouraged when we observed the xylem colonization of lateral roots of pot-grown wheat that had been inoculated repeatedly with ORS571 (Sabry et a1 1997). This suggested to us that under some growth conditions ORS571 might be able to colonize the xylem of plants other than *S. rostrata.* We were also encouraged to try to find conditions that would enable the xylem of rice and other nonlegumes to be colonized by ORS571 when we observed reproducible internal colonization of the root cortex by ORS571 of rice, wheat, and *Arabidopsis* at high frequency. This resulted from stimulation by flavonoids, such as naringenin, of lateral root crack colonization by ORS571 (Gough et a1 1996, Webster 1998b, Gough 1997a,b). We have shown that the xylem of *S. rostrata* is also colonized by *Sinorhizobium teranga* ORS604 and *S. saheli* ORS611 (O'Callaghan et a1 1999) and by NGR234 carrying a *gus* reporter gene (O'Callaghan et a1 1998). The studies with rice and *Arabidopsis* described here have only used inoculation with ORS571.

Intercellular colonization of nonlegume roots by *Azorhizobium caulinodans* ORS571

Our initial studies showed that *A. caulinodans* could colonize the cracks at the points of emergence of lateral roots of wheat and *Arabidopsis thaliana* and could subsequently intercellularly colonize adjacent cortical tissue. We also demonstrated that this colonization is nod gene-independent. Our demonstration of this in both the monocot wheat and the dicot *Arabidopsis* implied that this intercellular colonization by ORs571 was likely to be a general phenomenon in nonlegumes. Encouragingly in our initial studies with rice inoculated with ORS571, large intercellular pockets of bacteria were observed at the base of emerging lateral roots (Webster et a1 1998b). Our finding that lateral root crack (LRC) colonization (including crack colonization and intercellular colonization) of both wheat and *Arabidopsis* was significantly stimulated by flavonoids, such as naringenin, at low concentrations suggested that these flavonoids were probably acting as signals, and not nutritionally. These initial studies provided an experimental foundation of reproducible LRC colonization at high frequency, enabling us to begin to define the various genetic and physiological factors limiting for both rhizobia1 colonization and nonnodular endophytic symbiotic nitrogen fixation in nonlegumes.

More detailed studies of the intercellular colonization of wheat roots by ORS571 and other diazotrophic bacteria used strains marked with the *lacZ* reporter gene to facilitate their detection and identification. Naringenin (10^{-5} M) was shown to significantly stimulate wheat LRC colonization by ORS571 and it was confirmed that *nod* genes were not involved in LRC colonization (Webster et a1 1998b). It has been reported that the colonization of lateral root cracks of rice by ORS571 is also Nod factor independent (Reddy et a1 1997). Interestingly, naringenin significantly stimu-

lated LRC colonization of wheat more than any other flavonoid tested. The flavonoids quercetin, hesperetin, and chrysin did not increase the level of LRC colonization (Webster et al 1998b). The intercellular colonization of wheat roots by ORS571 was shown to resemble closely the first two stages, intercellular infection (crack entry) and the formation of large intercellular pockets, already well established for crack-entry infection of azorhizobia in the roots of the legume host, *S. rostrata* (Ndoye et al 1994). From these studies, we suggested that it would be interesting to study ORs571 intercellular colonization further and to determine, using the *lacZ* reporter gene, whether azorhizobia are able to spread deeper within the root system and, in particular, into the xylem when plants are grown longer and under better physiological conditions.

Studies on rice (cv. Lemont) indicated a similar stimulatory effect of naringenin on LRC colonization (Webster et al 1997) and this has been shown recently in other rice varieties (Gopalaswamy et al 1999). In rice inoculated with ORS571, in the presence of naringenin, toluidine blue staining of sections showed that bacteria had entered at LRCs, resulting in the presence of large intercellular pockets of bacteria in the region of emerging lateral roots. The further colonization of rice roots by azorhizobia resulted from their intercellular spread inward to a distance of three to four plant cell layers and the formation of larger pockets of azorhizobia within the main root cortex (Webster et al 1997).

Rice has been suggested as a model to assess the potential for extending endophytic biological nitrogen fixation to cereals (Ladha and Reddy 1995). For nonlegume dicots, *Arabidopsis thaliana* is the model of choice. *Arabidopsis* is readily amenable to genetic analysis and is currently by far the best nonlegume for use in identifying and studying the role of genes involved in a given plant response to interaction with diazotrophic bacteria (Gough et al 1997b).

We showed that ORS571, and some other diazotrophic bacteria, were able to internally colonize roots of *A. thaliana.* Strains tagged with *lacZ* or *gusA* reporter genes were used and the principal colonization sites were the points of emergence of lateral roots, lateral root cracks. We observed that after LRCs were colonized bacteria moved into intercellular spaces between the cortical and endodermal cell layers. As had been observed in wheat, specific flavonoids such as naringenin (10^{-5} M) significantly promoted colonization of *Arabidopsis* by ORS571. Moreover, it was also shown that Nod factors are not involved in flavonoid-stimulated colonization. It was noteworthy that, despite the high levels of LRC colonization, once the azorhizobia reached the intercellular cortical spaces of *A. thaliana,* multiplication appeared to be limited. There was no evidence that azorhizobia progressed further into the *Arabidopsis* roots than the area immediately adjacent to the LRCs (Gough et al 1997a).

James et al (1994) suggested that xylem vessels are potential sites of nitrogen fixation by diazotrophic bacteria because they could provide both the low pO_2 for nitrogenase activity and a site for metabolic exchange. It was encouraging that we were able to demonstrate that conditions within the intercellular spaces of colonized LRCs of wheat (Webster et al 1997) and of *Arabidopsis* (Gough et al 1996) were appropriate for nitrogen fixation by the expression of a *nifD-lacZ* fusion of ORS571, and that ORS571-inoculated wheat plants grown in pots exhibited high levels of acety-

lene reduction activity and increased dry weight and total nitrogen content when compared with uninoculated control plants (Sabry et al 1997). Overall, this suggested that an approach was needed that would increase the ability of azorhizobia to better exploit plant resources for colonization, thereby perhaps enabling ORS571 to colonize xylem elements.

Xylem colonization of rice by *Azorhizobium caulinodans* ORs571

The demonstrated ability of ORS571 to colonize the xylem of *S. rostrata,* and also to intercellularly colonize the cortex of rice roots, following crack-entry invasion, led us to undertake a detailed microscopic study of sections of rice roots inoculated with ORS571 in seeking conditions under which invasion of the xylem might occur. This was part of a collaborative project between Tamil Nadu Agricultural University and the University of Nottingham, initially supported by the British Council, with the subsequent award of a visiting FAO fellowship to Dr. Gopalaswamy. As mentioned previously, we confirmed, in a range of rice varieties, our earlier observation that the colonization of lateral root cracks by ORS571 (pXLGD4) was significantly stimulated by the flavonoid naringenin (10^{-5} M). We also observed that the number of lateral roots of a range of rice varieties, following inoculation with ORS571 (pXLGD4), was also significantly increased in the presence of naringenin (10^{-5} M). By detailed microscopic analysis of sections of rice roots inoculated, in the presence of naringenin (10^{-5} M), with ORS571 (pXLGD4), we detected colonization of the xylem of primary and lateral roots by azorhizobia (Gopalaswamy et al 1999). Surface-sterilized rice seeds (cv. ADT36 and cv. CR1009) were germinated and grown aseptically in tubes containing nitrogen-free Fåhæus medium (0.8% w/v agar) with 10^{-5} M naringenin, inoculated with ORS571 (pXLGD4) carrying a constitutive *lacZ* reporter gene, and maintained in a growth chamber. ORS571 expressing *lacZ* were localized easily in plant tissue, as they produced a dark blue precipitate in the presence of X-Gal. Plants were fixed in glutaraldehyde and processed for light and electron microscopy. Toluidine blue-stained sections of roots of plants inoculated with ORS571 (pXLGD4) showed numerous bacteria in the xylem of primary and emergent lateral roots. Electron microscopy verified the presence of bacteria in the xylem, in those regions where the blue precipitate had been observed by light microscopy (Gopalaswamy et al 1998).

With this system for xylem colonization of rice by ORS571, it will now be possible to address several important questions relating to the possible use of this system for assessments of endophytic symbiotic nitrogen fixation in rice:

1. Will the xylem of rice provide a suitable pO_2 level and nutritional niche for the expression of *nif* genes?
2. Will xylem colonization be restricted to the root system or will xylem colonization become systemic?
3. Will the number of azorhizobia, even if expressing *nif* genes, be sufficient to provide beneficial quantities of fixed nitrogen to the rice plant?

Investigations of the pathway of invasion of the xylem by ORS571 will now be required to assess, for instance, the role of cellulases and pectinases produced by

azorhizobia in facilitating intercellular invasion of the cortex and penetration through the endodermis and into the xylem. A detailed study of the role of flavonoids in these interactions will also be needed. It may be possible to enhance xylem colonization and systemic invasion of rice by azorhizobia using transgenic rice with modifications of the phenylpropanoid pathway; moreover, techniques are now available for using flavonoids as biochemical markers in the genus *Oryza* (Boyet et a1 1998).

Xylem colonization of *Arabidopsis thaliana* by *Azorhizobium caulinodans* ORS571

Following our finding that the xylem of rice could be colonized by azorhizobia, we decided to reassess the situation in the model dicot *Arabidopsis thaliana* in which we had already demonstrated extensive crack-entry invasion and intercellular colonization of the cortex by ORS571 (Gough et a1 1997a). We investigated whether improved growth conditions for *Arabidopsis,* together with the addition of naringenin, would result in xylem colonization. Our investigation was facilitated by the thinness of the roots of *Arabidopsis,* which enabled direct observation of xylem invasion following histochemical staining of ORS571 (pXLGD4) without the initial need for embedding and sectioning of the inoculated roots (Stone et a1 1999). Surface-sterilized seeds (ecotype Col-0) were germinated and grown aseptically in jars with improved aeration, containing Murashige and Skoog medium plus 3% w/v sucrose (0.8% w/v agar) with 10^{-5} M naringenin. Plants were inoculated with ORS571 (pXLGD4), which carries the *lacZ* gene and produces a dark blue precipitate in the presence of X-Gal. Sections examined by light microscopy showed very large numbers of dark blue bacteria in some xylem elements of both the primary and lateral roots and electron microscopy confirmed that these dark blue regions were composed of bacteria in the xylem (Stone et a1 1998, 1999).

Arabidopsis thaliana inoculated with ORs571 will be an attractive system for addressing the questions, already discussed in relation to rice, relating to the possible use of xylem colonization for nonnodular endophytic symbiotic nitrogen fixation (Stone et a1 1999). The architecture and developmental biology of *Arabidopsis* roots have been extensively investigated (Van der Berg et a1 1998). The genetics and molecular biology, including the production of *Arabidopsis* mutants, have also been extensively investigated. It may be possible to resolve these questions more readily in *Arabidopsis* than in cereals such as rice, maize, wheat, and sorghum, and in other dicot nonlegume crops.

Assessing nonnodular endophytic nitrogen fixation

It will now be of interest to use these systems, described here for rice and *Arabidopsis,* to investigate the extent of intercellular cortex colonization and xylem colonization by other rhizobia, particularly those that infect legumes and nonlegume *Parasponia* spp. by crack entry (Boogerd and Van Rossum 1997). The extent to which colonization will be stimulated by flavonoids, such as naringenin, will also be of interest

(Hungria and Stacey 1997). The finding that rhizobia, such as ORS571, can colonize the xylem of the dicot legume *S. rostrata,* a monocot nonlegume such as rice, and a dicot nonlegume such as *Arabidopsis* suggests that xylem colonization is likely to be a general characteristic of rhizobia such as ORS571. While this indicates the likelihood that xylem colonization by ORS571 of a range of cereals and other nonlegume crops will be possible, the extent to which this xylem colonization by ORS571 will contribute to endophytic nitrogen fixation of benefit to the nonlegume is yet to be determined. Now, however, there is an experimental system to evaluate the possibility that xylem colonization by specific rhizobia will provide a nonnodular niche for endophytic symbiotic nitrogen fixation in both monocot and dicot nonlegumes.

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Colonization of rice and other cereals by *Acetobacter diazotrophicus* an endophyte of sugarcane

Myrna Sevilla and Christina Kennedy

Our recent work established that *Acetobacter diazotrophicus* can colonize, fix N_2 within, and promote growth of its natural host plant, sugarcane, after inoculation of sterile plantlets. In the new work presented here, the potential of *A. diazotrophicus* to colonize and promote the growth of rice, maize, and wheat was investigated using Nif^+ and Nif^- strains tagged with the *uidA* gene. Three wide host range plasmids each expressing a different marker gene from a constitutive promoter were constructed for this work. While the *uidA* gene product GUS was detectable in *A. diazotrophicus,* the fluorescent products of the other two, *cobA* and *gfp,* were not. *A. diazotrophicus* was able to colonize maize, rice, and wheat, but the colonization was apparently restricted to root tissues. Differences in the colonization patterns and persistence of *A. diazotrophicus* inside these plants were also observed. Inoculation with *A. diazotrophicus* did not enhance the growth of wheat seedlings. Rice seedlings inoculated with the wild-type strain grew to be significantly taller 30 days after inoculation than plants inoculated with the Nif^- mutant or uninoculated plants under N-deficient conditions. When N was not limiting, there were no differences between inoculated and uninoculated plants, indicating that, unlike in sugarcane, *A. diazotropbicus* may not benefit plant growth in the presence of sufficient N.

The recent isolation and study of endophytic nitrogen-fixing bacteria from several grasses (Baldani et al 1997) represent an exciting period in the field of nitrogen fixation. These diazotrophs offer a promising tool by which synthetic nitrogen fertilizer use might be decreased in the cultivation of important cereal crops. The association between endophytic diazotrophs and their host grasses may parallel in some respects the *Rhizobium*-legume symbioses in which the bacteria are provided with photosynthate by the plant in exchange for fixed nitrogen. Unlike in the legume symbioses, however, specialized structures similar to nodules or other gross morphological changes are not found in grasses colonized by these diazotrophic bacteria. Although this characteristic represents a unique and interesting aspect of the association, it also poses difficulties in understanding the mechanism by which these bacteria colonize grasses.

This is particularly important for feasibility studies concerning whether these endophytes might be used for inoculation of other cereal crops.

Current techniques used to detect and isolate endophytes in plants mostly involve destructive methods such as maceration and centrifugation of surface-sterilized plant tissues (Hallmann et al 1997). While these methods can give estimates of bacterial populations and extent of colonization, they fail to describe the sites of infection or the colonization process. Another method is the use of electron microscopy, which has been successful in showing *in situ* colonization sites (Dong et al 1994, James et al 1994, Sevilla et al 1998). Preparation of samples for electron microscopy, however, are tedious and time-consuming and therefore cannot be used in routine examination of multiple plant samples.

In the past decade, the use of reporter genes such as the *Escherichia coli uidA (gusA)* gene has facilitated investigations of plant-microbe interactions, including those involving grass-associated diazotrophs (Christiansen-Weniger1997, Vande Broek et al 1993). uidA encodes b-glucuronidase, the activity of which can be detected easily using a wide variety of glucuronide substrates (Jefferson 1987). More recently, the jellyfish green fluorescent protein (GFP) has also become popular as a marker for protein or bacterial localization (Chalfie et al 1994, Galbraith et al 1995). GFP has the advantage of not requiring external substrates or co-factors for its activity. It was used to successfully visualize the endosymbiont *Sinorhizobium meliloti* within alfalfa infection threads and nodules and to detect *nif* gene expression of *Azoarcus* sp. in rice roots (Egener et al 1998, Gage et al 1996). *Acetobacter diazotrophicus* is one of the newly discovered endophytic diazotrophs isolated from sugarcane roots and stems (Cavalcante and Dobereiner 1988). This bacterium had been reported to be present in relatively high numbers inside sugarcane plants reaching up to $10^6 - 10^8$ cells g^{-1} fresh weight tissue (Baldani et al 1997, Sevilla et al 1998). Since its first isolation from Brazilian sugarcane, it has also been isolated from other sugarcane varieties grown in many parts of the world and from other plants such as coffee, tea, and banana (Bellone et al 1997, Dong et al 1994, Fuentes-Ramirez et al 1993, Jimenez-Salgado et al 1997, Li and MacRae 1991, Matiru and Thomson 1998).

We recently determined that *A. diazotrophicus* enhances plant growth, probably by providing both fixed N and another growth factor to very young sugarcane plants (Sevilla et al 1998). As part of the long-term goal of extending these benefits to other cereal crops, we began a study to investigate the colonization process of *A. diazotrophicus* in sterile sugarcane plants and to determine whether *A. diazotrophicus* can colonize other grasses such as rice, wheat, and maize using bacteria constitutively expressing three marker genes—*uidA,* gfp, and *cobA.* Of the three, only the uidA (GUS)-marked strains were useful in plant studies. Additionally, the effect of *A. diazotrophicus* inoculation on growth of rice, maize, and wheat was also evaluated.

Materials and methods

Construction of *A. diazotrophicus* strains constitutively expressing marker genes

Three different genes were used to tag *A. diazorrophicus* strain PA15—*uidA* (ß-glucuronidase), *gfp* (green fluorescent protein), and *cobA* (uroporphyrinogen III methyltransferase).

uidA. A promoterless 3.8-kb *uidA:cat* gene cassette was excised from pWM4 (Metcalfe and Wanner 1993) using *Sma*I and purified using the GeneClean kit (BIO 101, La Jolla, Calif.). The purified fragment was then inserted into the *Sma*I site of the wide host range plasmid pKT230 (Bagdasarian et a1 1981), disrupting the kanamycin resistance gene. The resulting construct, pMS600 (Fig. 1A), was then introduced into *A. diazotrophicus* PAL5 by electroporation (Sevilla and Kennedy 1999). *A. diazotrophicus* cells carrying pMS600 were selected on DYGS (Stephan et a1 1991) with chloramphenicol (500 µg mL^{-1}), streptomycin (50 µg mL^{-1}), and X-Gluc (50 µg mL^{-1}). The ability of *A. diazotrophicus* cells to fix nitrogen and grow in N-free media was not affected by the plasmid.

gfp. The plasmid carrying GFP-mut2, a more highly fluorescent derivative of GFP than the wild type (Cormack et a1 1996), was cut with *Hind*III and the entire plasmid was inserted into the *Hind*III site of pKT230 (Bagdasarian et a1 1981). The resulting co-integrate, pHSK230 (Fig. 1B), was introduced into *A. diazotrophicus* by conjugation, followed by selection on DYGS plates containing streptomycin (50 µg

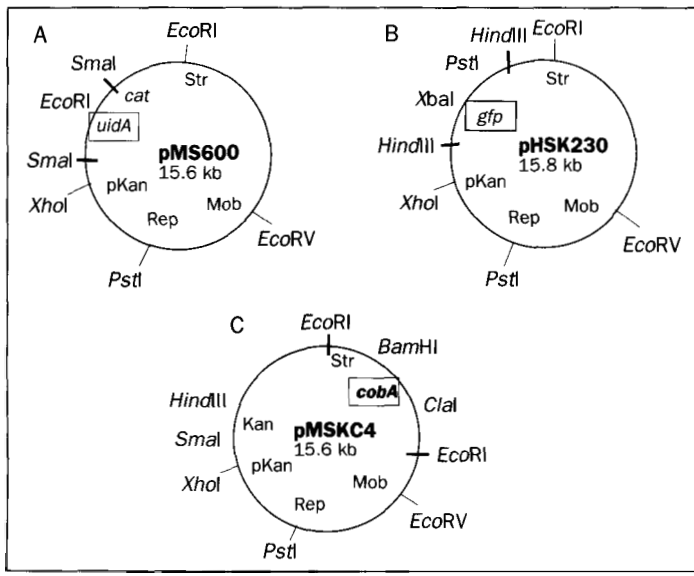

Fig. 1. Restriction maps of the three plasmids constructed for tagging *A. diazotrophicus* as markers *in planta.* Bold lines show sites of fragment or cointegrate insertion.

mL^{-1}) and ampicillin (200 mg mL^{-1}). The Amp^R and Sm^R cells were checked for fluorescence under ultraviolet (UV) illumination.

In a separate experiment, plasmid pTB93F, which was successfully used to visualize *S. meliloti* cells inside alfalfa nodules (Gage et al 1996), was introduced into *A. diazotrophicus* by electroporation and selection with 60 mg mL^{-1} of spectinomycin and 500 mg mL^{-1} of chloramphenicol. The transformed cells were examined under a fluorescence microscope (Leitz Diaplan, Leica) with the excitation filter set at 470–490 nm.

cobA. Uroporphyrinogen III methyltransferase catalyzes the S-adenosyl-L-methionine (SAM)-dependent addition of two methyl groups to uroporphyrinogen III, yielding porphyrinoid compounds (sirohydrochlorin and trimethylpyrrocorphin), which fluoresce bright red under UV (Roessner and Scott 1995). These compounds are intermediates in heme and vitamin B12 biosynthetic pathways. The plasmid pISA417 containing the *cobA* gene from *Propionibacterium freudenreichii* (Roessner and Scott 1995) was linearized with *EcoRI* and then ligated into the *EcoRI* site of pKT230. The resulting co-integrate, pMSKC4 (Fig. 1C), was introduced into *A. diazotrophicus* by conjugation, and selected on DYGS medium with ampicillin (200 mg d^{-1}) and kanamycin (200 mg mL^{-1}). Cells were examined for fluorescence under UV.

Plant material and inoculation

Sterile sugarcane plants were obtained from meristem tissue culture as described in Sevilla et a1 (1998). Seeds of rice (cv. Maratelli) and maize (line VA99+) were surface-sterilized by soaking in 95% ethanol for 10 min, followed by soaking in 10% bleach with 0.05% Tween 20 v/v for 10 min and rinsing with sterile distilled water three times with 10–15 min for each wash. Wheat seeds (cv. Penawawa) were surface-sterilized by soaking in 75% bleach for 2 min and rinsing with sterile distilled water five times with 1 min for each wash (E. Pierson, personal communication). All seeds were plated on water agar and Luria–Bertani(LB) medium and kept in the dark at 28 °C for germination. The plates were checked periodically for contamination and all contaminated seeds were discarded. Germinated seeds were immediately transferred into magenta boxes containing a modified Moth-strength sucrose-free MS medium with or without N and solidified with 0.7% agar (Murashige and Skoog 1962, J. Irvine, personal communication). These plants were kept on growth shelves at 28 °C with a 12 h light and dark diurnal cycle.

Bacterial inoculum of *A. diazotrophicus* wild-type strain (PA15) and tagged strains *PA15::uidA, PA15::gfp,* and *PA15::cobA* were prepared as described in Sevilla et al (1998). Sugarcane plants were inoculated as previously described (Reis et al 1995, Sevilla et al 1998). Rice, wheat, and maize were inoculated at an early seedling stage (just emergent) with 100 mL of the bacterial inoculum prepared and concentration adjusted as previously described (Sevilla et al 1998) and incubated on the growth shelves. Control plants were inoculated with 100 mL of LGI salt solution. Plants were checked for colonization at 2-d intervals for up to 14 d. The seeds co-cultured with *A. diazotrophicus* were transferred after 14 d to sand and incubated for 1 mo in the growth chamber.

Evaluation of colonization

Histochemical analysis of plants for *uidA* expression was performed by staining whole plants or excised parts for 12–16h in 0.1 M phosphate buffer, pH 7.0, with 1 mM X-Gluc and 0.1 % Triton X-100. Plants were stained in the dark at 37 °C with gentle agitation and then examined for blue color under the microscope. When necessary, plants were destained in 10% bleach for 5–10min before examination. Stained plants were also sectioned and observed under a light microscope to look for the presence of *A. diazotrophicus* in GUS-positive tissues. Additionally, small pieces of sugarcane tissues (<1 mm^3) that stained positive for GUS were fixed in 50 mM cacodylate buffer with 1 % glutaraldehyde. The fixed tissues were embedded in LR White acrylic resin following the procedures of Hurek and Villiger (1992). Semithin sections (0.5–2 µM) were cut and collected on a glass microscope slide. The sections were counter-stained with 0.5% basic fuchsin in 25 mM phosphate buffer, pH 7.0, for 5 s to provide contrast between plant tissues and bacterial cells and were then rinsed in distilled water and heat-dried before microscopic examination. Plants inoculated with PA15::*gfp* and PA15::*cobA* were sectioned and examined by fluorescence microscopy. Numbers of bacteria inside the plants were determined by the most probable number (MPN) and plate counts using homogenized surface-sterilized plant tissues as described previously (Sevilla et al 1998, 1999).

Evaluation of inoculation effect

Rice, wheat, and maize plants were inoculated with *A. diazotrophicus* wild type as described above. For comparison, plants were also inoculated with a Nif^- mutant, MAd3A (Sevilla and Kennedy 1999). Ten days after inoculation, rice and wheat seedlings were transferred in bleached and washed conical pots with sterile sand and grown for 30 d in the growth chamber with the same conditions as the growth shelves. Maize seedlings were directly transferred into pots with sterile sand in the greenhouse. Plants were irrigated with half-strength MS medium once every week with or without N and with distilled water when necessary. To determine the persistence of *A. diazotrophicus* in the different plants, MPN and plate counts were again taken at 30 d after transfer into sand. The identity of the reisolated bacteria was evaluated by microscopic examination, cultural characteristics, acetylene reduction assay, and amplification of the *A. diazotrophicus* 23S rRNA using species-specific primers as described in Sevilla et al (1998).

Results and discussion

Expression of marker genes in *A. diazotrophicus*

All three of the reporter genes, *uidA, cobA,* and *gfp,* expressed from the Kan^r constitutive promoter in pKT230 were initially transformed into *E. coli. E. coli* cells carrying the co-integrates pHSK230 (*gfp*) and pMSKC4 *(cobA)* fluoresced green and red, respectively, when illuminated by UV light. In addition, when cells were centrifuged, the cell pellet showed the distinctive colors of the marker genes they carried, suggesting high levels of expression of these genes (data not shown). Cells with pMSKC4

were red, whereas those carrying pHSK230 were yellow-green. In *A. diazotrophicus,* however, the GFP and CobA gene products were much less detectable than in *E. coli* despite the constitutive promoters and the relatively high copy number of pKT230.

When illuminated under UV, the fluorescence of *A. diazotrophicus* cells carrying pHSK230 or pMSKC4 was only about 10% of that of *E. coli* transformants carrying these plasmids. Similar results were obtained in *A. diazotrophicus* carrying pTB93F, a *gfp* plasmid used successfully to observe *S. meliloti* in alfalfa infection threads (Gage et a1 1996). In addition, *A. diazotrophicus* cells carrying pMS600 *(uidA)* were also about 50% less blue than *E. coli* cells in X-Gluc plates (data not shown). The reason for the quenching of the fluorescence or blue color is unknown, but similar observations have been made concerning *lacZ* expression in *A. diazotrophicus* (unpublished results). *A. diazotrophicus* produces significant amounts of exopolysaccharide (EPS), especially when grown in high sucrose concentrations. It is possible that the EPS produced by *A. diazotrophicus* prevents the chromogenic X-Gluc from entering the bacterial cell. Analysis of GUS activity requires the penetration of the substrate X-Gluc to the site of localization, and the precipitation of the product of hydrolysis at this site (Jefferson 1987). When the sucrose and glucose concentrations of the culture media were decreased to minimize the production of EPS, a slight increase in the intensity of the blue color in pMS600 transformants was achieved, but no change in fluorescence of pHSK230 or pMSKC4 transformants was observed (unpublished results).

The optimum starting pH for *A. diazotrophicus* growth is 5.5 and this organism also acidifies its medium to a much lower pH (Stephan et a1 1991). This low pH may be inhibitory to gene expression and/or might also affect the breakdown of the substrates used (X-Gluc) or gene products examined (CobA, GFP) in this study. CobA was reported to have optimum activity at pH 8.0 in *Salmonella typhimurium* (Suh and Escalente-Semerena 1995). With GFP, this low pH may result in inefficient folding and chromophore formation of the protein (D. Galbraith, personal communication). When *A. diazotrophicus* was grown in media with pH close to 7.0 and/or in mannitol-containing medium, which is not easily acidified by *A. diazotrophicus,* however, only a slight increase in fluorescence was observed, suggesting that other factors may be involved as well.

Logarithmic-stage *Azoarcus* cells carrying *gfp* constructs fluoresced brighter than did cells in lag-phase, whereas stationary-stage cells did not fluoresce at all (Egener et a1 1998). No difference in fluorescence was observed when actively growing cells of *A. diazotrophicus* were compared with cells in the stationary stage, indicating that fluorescence is not affected by the age of the cells. Analysis of the *A. diazotrophicus nifHDK* and *lsdA* genes, coding for nitrogenase and levan sucrase, respectively, revealed a higher GC content than the GC content of *gfp* (Sevilla and Kennedy 1999). That this different codon usage is responsible for the low levels of expression in *A. diazotrophicus* is possible. In addition, poor GFP fluorescence has been observed in other Gram-negative bacteria, indicating the need for careful optimization of conditions for the best *gfp* expression in each bacterial species (Egener et a1 1998, Matthysse et al 1996).

When *A. diazotrophicus* cells carrying any of the three plasmids were grown in the absence of antibiotics and then plated on media with and without antibiotics, the proportion of antibiotic resistant cells declined in each generation (data not shown). This suggested that the constructed plasmids cannot be maintained stably in *A. diazotrophicus* in the absence of selection. Although other strains of *A. diazotrophicus* have indigenous plasmids, PA15 has none. All tagged PA15 strains were used in preliminary inoculations with sugarcane to determine whether there would be a difference when the genes are expressed *in planta.* In the case of *R. meliloti,* cells carrying pTB93F fluoresced brighter inside the nodules, which was suggested to be related to the low oxygen tension in these nodules (Gage et a1 1996).

Expression of marker genes *in planta*

A preliminary experiment in sugarcane revealed that only *uidA (gusA)* was expressed inside the plant. Sugarcane plants incubated for up to 14 d after inoculation still showed strong blue color after staining with X-Gluc, indicating that the plasmid might be more stable in bacteria inside the plant. In contrast, GFP and CobA were not detected in planta and *A. diazotrophicus* cells progressively lost what little fluorescence they developed initially after several subcultures, even when the media used contained antibiotics. Interestingly, plasmids with the expected size and restriction sites were isolated from bacteria in these plants (data not shown). Thus, the loss of fluorescence cannot be explained by the loss of plasmids in these bacteria. Even if plasmids of correct size were obtained, however, it is still possible that some other modifications in the plasmids might have occurred and such modifications may account for the loss of fluorescence. It was also not determined, for example, by using Northern blot analysis, whether *gfp* and *cobA* were transcribed inside the plants. Although these genes are expressed from constitutive promoters, no studies report gene expression in *A. diazotrophicus* inside the plant. Because only the *uidA* gene product was detected in plants, *A. diazotrophicus* cells carrying the *uidA* gene were used in all other plant inoculation experiments.

Plant colonization

In all experiments, uninoculated plants and those inoculated with wild-type PA15 with no marker genes did not show any blue color.

Sugarcane. Sugarcane plants inoculated with PA15::*uidA* showed the characteristic blue color when stained with X-Gluc, whereas uninoculated plants and plants inoculated with the wild-type PA15 did not show any blue staining. Examination of the roots 2 d after inoculation revealed intense blue staining on the tear wounds created when the sugarcane plantlets were separated before inoculation and wounds caused by emerging lateral roots, which confirmed our earlier observations as well as those of others that *A. diazotrophicus* gains entry into micropropagated sugarcane plantlets through these wounds (James et a1 1994, Sevilla et a1 1999). After 8 d, blue color was also observed in other parts of the plants, including areas immediately above the root stem junction. At 14 d, *A. diazotrophicus* was also observed in the leaves of the sugarcane plantlets. Examination of fixed sections of GUS-positive sugarcane stems and

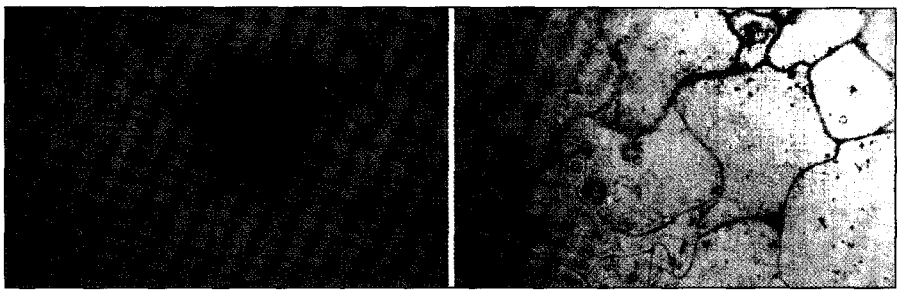

Fig. 2. *A. diazotrophicus* cells (PA15::*uidA*) expressing the *uidA* gene primarily in the leaf intercellular spaces of sugarcane (arrow). Plants were examined 3 d after inoculation by light microscopy after staining with X-Gluc and basic fuchsin as described in materials and methods section: (A) magnification 40X, (B) magnification 100X.

leaves showed that *A. diazotrophicus* cells were concentrated in the intercellular spaces (Fig. 2). Sugarcane plants grown in N-deficient condition appeared to be colonized earlier and better than plants grown with N. Later examination of plants, however, showed no significant difference in the extent of staining of inoculated plants, suggesting that *A. diazotrophicus* colonized micropropagated plants equally well regardless of the N condition. Similar results were obtained with *Azospirillum brasilense* in wheat colonization studies (Vande Broek et al 1993). This observation also confirmed our earlier results in which we had shown that there was little difference in the number of bacteria reisolated from inoculated micropropagated plants grown with or without added N (Sevilla et al 1998, 1999). Root tips were colonized, but not root hairs, agreeing with the observations of James et al (1994).

Maize. Two days after inoculation, the maize kernel was heavily colonized by *A. diazotrophicus*. The lower portion of the primary root of the maize seedling, as well as numerous root hairs, was also heavily colonized. Caballero-Mellado et al (1998) also found similar colonization patterns in their maize inoculation experiment. The heavy colonization of root hairs may indicate that *A. diazotrophicus* can enter plants through them, as has been reported for other bacterial endophytes (Hallmann et al 1997). Microscopic examination of all GUS-positive maize tissues revealed the presence of *A. diazotrophicus* cells expressing the *uidA* gene, that is, blue-colored bacteria (data not shown). In contrast to the sugarcane results, the leaves and stems of maize were not found to be colonized after 14 d, suggesting that *A. diazotrophicus* did not spread in maize as it did in sugarcane.

Rice. Unlike the maize kernels, rice seeds were not as heavily colonized; however, the portion of the roots just emerging from the seeds was almost always heavily colonized just after 2 d of incubation. Some, but not all, root hairs were also colonized. As in sugarcane, *A. diazotrophicus* was also found concentrated in the junction where lateral roots were emerging (Fig. 3). This again suggests that crack entry is one way by which *A. diazotrophicus* can get into the plant. It was also common to find *A. diazotrophicus* lined up along the grooves of the epidermal cells of the root. Similar observations were reported by Rolfe et al (1997), who examined DAPI-stained roots of rice grown with 2,4-D and inoculated with *A. diazotrophicus*. This may represent

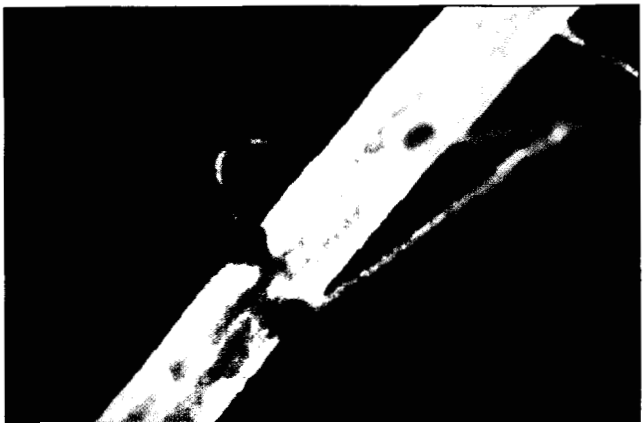

Fig. 3. *A. diazotrophicus* (PA15::uidA) colonization of rice. Bacterial cells concentrated on lateral root emergence points in rice after staining with X-Gluc.

another way for bacterial entry as epidermal conjunctions have also been reported as potential routes of entry in addition to crack entry (Sprent and de Faria 1989).

Blue *A. diazotrophicus* cells were observed when sections of GUS-positive root tissues were examined under the microscope. Rolfe et al (1997) also showed that the colonization of one rice cultivar was enhanced by the addition of auxin. The results of the present study showed that added auxins were not needed for successful colonization of the rice cultivar used in this study (data not shown). There is a clear need to define the basis of any specificity and the potential role of auxins in enhancing colonization. As with maize, *A. diazotrophicus* was not observed in the leaves and upper stems, indicating its limited spread in rice.

Wheat. The patterns of colonization in wheat were similar to those of rice; however, more root tips were colonized in wheat than in rice or maize (data not shown). The significance of this difference is not known and is probably host-specific because sugarcane root tips were also heavily colonized. Examination of GUS-positive root tip sections revealed the presence of blue *A. diazotrophicus* cells, indicating *in planta* expression of the *uidA* gene. Root tips are also believed to be potential routes of entry for bacteria (James et al 1994). It is possible that *A. diazotrophicus* uses different ways to gain entrance into different plants. The leaves and stems were not colonized in any of the 15 plants examined.

The choice of colonization site is believed to be species- and strain-specific for many bacterial endophytes (Hallman et al 1997). Whether this is true for *A. diazotrophicus* will have to be verified in future experiments involving other bacterial strains. What is apparent in the present study is that systemic colonization by *A. diazotrophicus* only occurred in its natural host, sugarcane. While there were not enough plant sections examined in this study to positively say that, in maize, rice, and wheat, *A. diazotrophicus* is only limited in the root cortex, this observation, if confirmed in future experiments, could explain the reason for the limited spread of *A.*

diazotrophicus in these plants. In sugarcane, *A. diazotrophicus* is present in the xylem as well as intercellular spaces and other workers proposed that bacteria are translocated through the transpiration stream to other parts of sugarcane (James et al 1994, Reis et al 1995). However, *A. diazotrophicus* cannot enter the xylem vessels of the other grasses in this study, as there may be a barrier in the root endodermis in these plants such that *A. diazotrophicus* cannot traverse it and this could prevent their spread to other parts of the plants.

Effect of inoculation on plant growth

It should be pointed out that there were no macroscopic symptoms of a disease reaction in any plants inoculated with *A. diazotrophicus* wild type or mutant strain. Details of the effect of *A. diazotrophicus* inoculation on sugarcane growth were or will be reported elsewhere (Sevilla et al 1998, 1999).

Maize. Most of the maize plants transferred to the greenhouse died before growth data could be taken (16 out of 24) because of extreme temperatures during July. *A. diazotrophicus* failed to be isolated from maize plants grown in the greenhouse 40 d after inoculation (30 d after planting in sand) although, at 14 d after inoculation, plate counts showed that an average of 2.3×10^3 colony-forming units per gram fresh tissue could be isolated from inoculated maize plants. This observation suggests that *A. diazotrophicus* did not persist in this plant. It is not known how long the bacteria persisted in the plant after the last day of examination (14 d after inoculation) for *uidA* gene expression. In another study, *A. diazotrophicus* was isolated from maize plants 12 d after inoculation, but no growth enhancement was observed with inoculation (Caballero-Mellado et al 1998). It is not known, however, whether *A. diazotrophicus* was still present in plants when plant growth was measured in this study. If the data were taken when *A. diazotrophicus* was no longer significantly present in the maize plants, then it is possible that any positive effect of inoculation would not have been observed. In addition, differential response to bacterial inoculation by different maize cultivars cannot be ruled out as has been reported in other bacterial-plant interactions (Baldani et al 1995, Rolfe et al 1997). Although *A. diazotrophicus* was recently isolated from other plants that are not known to be sucrose-rich (Jimenez-Salgado et al 1997, Matiru and Thomson 1998), it has been isolated in only one other grass, *Pennisetum purpureum,* which is also sucrose-rich (Dobereiner et al 1995). The ability of *A. diazotrophicus* to hydrolyze sucrose and grow in high-sucrose concentrations (Stephan et al 1991) may mean that, at least with grasses, those with high-sucrose contents are the preferred hosts. It will be interesting to see whether *A. diazotrophicus* will be able to persist in maize with higher sucrose content (e.g., sweet corn varieties). That it can colonize maize for at least a short time is a promising result that justifies more studies on the effect of *A. diazotrophicus* inoculation on maize.

Rice. The height of rice plants as well as the length of their roots were measured 30 d after planting in sterile sand. As Table 1 shows, both *A. diazotrophicus* wild-type and mutant strains were reisolated from inoculated plants and no bacteria were isolated from the uninoculated control plants. The isolated bacteria were confirmed to be

Table 1. Effect of *A. diazotrophicus* inoculation on plant height and root length of rice seedlings 30 d after planting[a].

Treatment	Number of bacteria g^{-1} fresh tissue[b]	Height (cm)[c]	Root length (cm)
0 – N	0	12.80 d (6)	17.00 c
PA15 – N	14.5×10^3	19.00 c (9)	19.00 bc
MAd3A – N	5.9×10^3	12.07 d (7)	18.67 bc
O+N	0	29.08 b (6)	26.83 a
PA15 + N	12.5×10^3	32.94 ab (8)	21.86 ab
MAd3A + N	5.3×10^3	35.58 ab (7)	23.00 ab

[a]Means in the same column followed by the same letter are not significantly different; multiple comparisons performed with Student-Newman-Keuls method, P = <0.0001. [b]Determined by most probable number (MPN); means of two plants. [c]Number in parentheses represents number of plants in each treatment.

A. diazotrophicus based on microscopic examination, growth on selective media with or without N, acetylene reduction, and kanamycin resistance. The number of bacteria isolated was considerably less than what has been reported and what we have measured for sugarcane (Sevilla et al 1998). This could indicate the presence of *A. diazotrophicus* in significantly fewer numbers or that individual colony-forming units are much more difficult to separate and isolate from macerated surface-sterilized rice plants. That *A. diazotrophicus* is able to proliferate better in sugarcane than in rice might be related to differences in the sucrose content of these two plants. Other host-specific factors, however, could also be involved. Nonetheless, our results indicate that the association between *A. diazotrophicus* and rice can be established for at least up to 40 d after inoculation.

In one test of effect on growth, inoculation with the *A. diazotrophicus* wild-type strain resulted in a significant increase in the height of N-limited plants compared with uninoculated plants or plants inoculated with the Nif$^-$ mutant (Table 1). This suggests that *A. diazotrophicus* can enhance rice growth and that growth promotion might be related to the transfer of biologically fixed N although other factors such as auxin production could be involved. More experiments are obviously required to determine the basis for the apparent enhancement of rice growth by *A. diazotrophicus,* including N measurements and isotope dilution analysis. Currently under investigation also is the ability of mutants of *A. diazotrophicus* deficient in indole acetic acid production *(iaa$^-$)* and double mutants *(nifiaa$^-$)* to promote growth of sugarcane and rice. When rice plants were grown in the presence of N, there was no significant increase in the height of inoculated plants compared with uninoculated plants, suggesting that *A. diazotrophicus* did not influence plant growth when fixed N was sufficient. In sugarcane, when N supply was sufficient, significant increases in plant height were usually observed in inoculated plants regardless of whether the inoculum was the wild type or Nif$^-$ mutant (Sevilla et al 1998).

When root lengths were measured, there were no significant differences observed in uninoculated and inoculated plants (Table 1). Visual inspection of the root

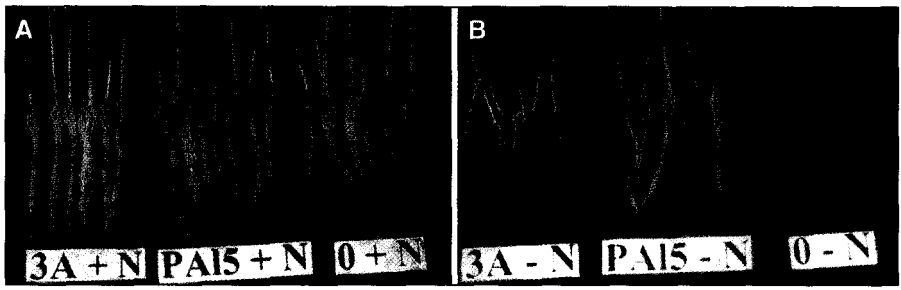

Fig. 4. Effect of *A. diazotrophicus* inoculation on root mass of rice seedlings measured 30 d after inoculation: (A) plants supplied with fixed nitrogen, (B) plants under N-limiting conditions.

mass, however, suggested a positive effect in inoculated plants compared with uninoculated plants under N-deficient conditions (Fig. 4). In addition, differences were observed between plants inoculated with wild-type *A. diazotrophicus* and plants inoculated with the Nif$^-$ mutant. There were no differences observed in the root mass of plants grown in N–sufficient conditions.

Wheat. The number of *A. diazotrophicus* cells isolated from wheat 30 d after planting in sand was, even lower than that in rice (average MPN count of 6.8×10^2 bacteria per gram fresh tissue). This again suggested that some host factors are lacking in wheat and rice that can support a high population of *A. diazotrophicus.* That an association between wheat and *A. diazotrophicus* can be established even for a short period, however, confirmed the potential of this bacterium to colonize other grasses not rich in sucrose. In contrast with rice plants, inoculation with *A. diazotrophicus* did not result in an increased height of wheat plants (data not shown).

The experiments presented here must be repeated to measure all important growth parameters and confirm the positive effects of *A. diazotrophicus* inoculation observed in preliminary results. It will be important to establish whether growth promotion in rice can also be demonstrated in long-term experiments. These further studies are justified because of the demonstrated potential of *A. diazatrophicus* to colonize other grass crops in addition to sugarcane and its possible ability to promote plant growth through nitrogen fixation.

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Notes

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Progress in multinational collaborative studies on the beneficial association between Rhizobium leguminosarum bv. trifolii and rice

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This chapter summarizes our collaborative project to search for natural, intimate associations between rhizobia and rice *(Oryza sativa),* assess their impact on plant growth, and ultimately exploit those that can enhance grain yield with less dependence on nitrogen fertilizer inputs. Two cycles of field and laboratory studies have indicated that diverse indigenous populations of the clover root-nodule symbiont, *Rhizobium leguminosarum* bv. *trifolii,* intimately colonize rice roots in cultivated fields of the Egyptian Nile Delta, where rice has been rotated successfully with berseem clover *(Trifolium alexandrinum)* since antiquity. Certain strain/variety interactions significantly expand rice root architecture, enhance the uptake of several plant nutrients, and increase plant biomass under laboratory and greenhouse conditions. Preliminary results indicating statistically significant increases in grain yield and agronomic fertilizer N-use efficiency following inoculation have been obtained, We are now examining various basic and applied aspects of this beneficial *Rhizobium* -rice association, such as its ecology, physiology, biochemistry, and molecular biology and the identification of the underlying mechanisms of plant growth promotion operative in this beneficial association. We are also further assessing selected rhizobial strains to perform as beneficial biofertilizer inoculants for rice under field conditions. Certain strains of these riceadapted rhizobia colonize the surface and, within limited regions of the interior of lateral roots of rice seedlings, secrete indoleacetic acid and gibberellin phytohormones in vitro, and extracellularly solubilize precipitated phosphates. Various acetylene reduction assays and ^{15}N-based studies do not support a role of biological nitrogen fixation in the positive plant growth-promotion response of this *Rhizobium* -rice association. This natural, intimate *Rhizobium* - rice association represents a unique experimental system suitable for both basic and applied studies on beneficial rice-bacteria interactions. This association of dissimilar organisms living together may also turn out to offer potential benefits to enhance the sustainable agriculture of rice, the most important cereal crop of the developing world,

Cereals are the world's major source of food for human nutrition. Among these, rice (*Oryza sativa* L.) is the most prominent and represents the staple diet for more than two-fifths (2.4 billion) of the world's population, making it the most important food crop of the developing world (IRRI 1996). Rice production, and hence global food security, depends on reaching even higher levels of sustainable grain production, which is currently not possible without major additional nutrient input. Indirectly, rice is able to use a basal level of fixed N as a source of its N nutrition from the N_2-fixing activities of diazotrophs in its agronomic ecosystem (Ladha 1986, Roger and Ladha 1992). If rice were able to establish a more direct and efficient symbiotic association with N_2-fixing organisms, serious economic and ecological problems associated with the use of inorganic and organic fertilizers to enhance rice production could be mitigated.

A remarkable diversity of N_2-fixing bacteria naturally associate with roots of field-grown rice (Bally et al 1983, Ladha 1986, Ladha et al 1993, Roger and Watanabe 1986, Stoltzfus et al 1997, Ueda et al 1995, Yanni et al 1997). During the past few years, interest has increased in exploring the possibility of extending the beneficial interactions between rice and some of these N_2-fixing bacteria. This line of investigation came into full focus in 1992, when the International Rice Research Institute hosted an international workshop to assess knowledge on the potential for nodulation and nitrogen fixation in rice associated with symbiotic bacteria (Khush and Bennett 1992). An umbrella Frontier Project was formed as a spin-off of that workshop to pool the expertise of various researchers to support international collaborative efforts on establishing more intimate, beneficial associations of nitrogen-fixing bacteria and rice.

One of the mandates for future research identified at that workshop was to explore and define the extent of natural colonization of rice roots by rhizobia. Rather than just address whether rhizobia are stimulated as a competent rhizosphere colonizer, the "frontier" question posed was whether rhizobia can intimately colonize and even invade rice roots in nature. Predictably, rhizobial isolates naturally adapted to rice would be good germplasm for further exploitation by genetic manipulation. This bold "frontier" subproject challenges the normal view that rhizobia persist only as soil saprophytes in a physiological state of starvation survival during periods in which the host legume is absent. The idea that certain rhizobia may already be able to intimately colonize rice roots in nature is derived from the general concept that roots of healthy plants grown in natural soil eventually develop a continuum of root-associated microorganisms extending from the rhizosphere to the rhizoplane, and even deeper into the epidermis, cortex, endodermis, and vascular system (Balandreau and Knowles 1978, de Bruijn et al 1995, Klein et al 1990, Old and Nicolson 1975,1978). Although little is known about the physiological effects of the natural microflora that develop within the interior of healthy roots grown in soil, these endophytes typically do not induce obvious symptoms of disease.

Originally described as *endorhizosphere* microorganisms, the microflora that colonize this specialized habitat inside healthy roots are now more commonly referred to as endophytes or internal *root colonists* (Kloepper et al 1992). This habitat has already been identified as an important reservoir for the isolation of N_2-fixing

plant growth-promoting rhizobacteria (PGPR) in other cereal roots. Examples that illustrate this point are the isolation of *Azospirillum* strains from "inside" host roots (after surface-sterilization), which efficiently promote yield when inoculated on that homologous host (Boddey and Dobereiner 1988), and the diazotrophic endophytes of *Azoarcus* inside Kallar grass (Bilal and Malik 1987, Hurek et al 1994) and *Acetobacter diazotrophicus* inside sugarcane (Dobereiner et al 1993). Presumably, nature selects endophytes that are competitively fit to occupy compatible niches within this nutritionally enriched and protected habitat of the root interior without causing pathological stress on the host plant.

Our long-range goals for this frontier subproject have been to search for natural, intimate associations between rhizobia and rice, assess their impact on plant growth, and ultimately exploit those that can enhance grain yield with less dependence on nitrogen fertilizer inputs. Our working hypotheses going into this project were twofold: (1) that natural associations of rhizobia and rice likely occur where this cereal crop is successfully rotated with legumes that sustain the rhizobial populations at a high inoculum potential for the next rice-growing season; and, if so, (2) this type of association should provide inoculant strains that are already able to competitively occupy colonization niches on rice roots under field conditions.

Site selection

A region of the world that has been ideally suited to address these questions is in the irrigated Nile Delta of Egypt, the major producer of rice in North Africa. For between 7 and 14 centuries (exact date within this range is unknown), most of the rice cultivated in this region has been rotated with the legume Egyptian berseem clover *(Trifolium alexandrinum* L,). Currently, about 60–70% of the 500,000+ ha of land area used in Egypt for rice production is in a rice-clover rotation. This clover species is well adapted to the Middle East, where it is believed to originate, and its high yields, protein content, and N_2-fixing capacity enhance its use as a forage and green manure crop (Graves et al 1987). In the Nile Delta region, both indica and japonica rice varieties are cultivated in late spring by transplantation from small nurseries into irrigated lowland fields with levees immediately after harvest of the clover forage. Typically, irrigation of the rice fields from the Nile River is stopped to partially dry the soil 15–20 d before harvest of the mature transplanted rice. After harvest of the rice grain and straw in early fall, berseem clover seed is broadcast in the same fields (most often without tillage) and becomes naturally nodulated by indigenous rhizobia (*Rhizobium leguminosarum* bv. *trifolii).* Sporadic vegetative regrowth of the rice roots produces so-called "ratoon rice" intermingled among the clover plants. During the next spring season, the aboveground forage of berseem clover is harvested by hand at flowering to repeat the rotation cycle. The clover root residues are left belowground to decompose and release their fixed nitrogen for the next rice crop.

Extensive agronomic studies indicate that this practice of clover rotation in the Nile Delta replaces 25–33% of the recommended rate of fertilizer-N application for optimal rice production. Therefore, the rotation is of immense benefit to sustainable

agriculture of that rice-cropping system, not only for the increased yields with less fertilizer-N input but also for the lower level of groundwater nitrate pollution derived from leached fertilizer N applied to the crop.

There is an interesting enigma, however, for this ancient scenario of successfully farming rice. Agronomic N-balance studies have shown that this benefit of clover rotation for rice production cannot be explained solely by the increased availability of fixed N through mineralization of the N-rich clover crop residues. For these reasons, and the many years in which native clover rhizobia have had the opportunity to evolve adaptations to interact with rice, we chose this agronomic system of the Egyptian Nile Delta to test our working hypotheses. Now that we have accumulated sufficient data providing affirmative answers for both working hypotheses, we predict that this rice-legume cropping system has the potential to derive even greater benefit from real-world applications of the knowledge gained through this research project to optimize the *Rhizobium*-rice associations.

Experimental approaches and findings

Discovery and confirmation of the natural, intimate *Rhizobium*-rice association

We took an ecological approach involving two cycles of field and laboratory studies to test our primary working hypotheses (Dazzo et al 1998, 1999, Yanni et al 1997). Rice plants of approximately the same height were sampled at different field sites during two rice-growing seasons; the first from one site of vegetatively regenerated ratoon rice well into the clover growing season, and the second from four different sites in flooded fields of transplanted rice. Field-sampled roots were promptly taken to the laboratory, washed free of soil, cut at the stem base, blotted, weighed, and surface-sterilized sufficiently with penetrating sodium hypochlorite solution until viable rhizoplane organisms could no longer be cultivated. These surface-sterilized roots were macerated in sterile diluent and five replicates of each decimal dilution were inoculated directly on axenic roots of berseem clover seedlings grown on N-free Fahraeus agar slopes in enclosed tube cultures. Nodulated plants were scored after 1 mo of cultivation. This experimental design took advantage of the clover "trap" host to perform the labor of selecting infective clover rhizobia present among the other naturally occurring rice endophytes that survived surface sterilization of the roots, providing most probable numbers (MPNs) of the clover-nodulating rhizobial populations per unit of root biomass, plus an easy route for isolating the numerically dominant, rice-adapted rhizobia from within the harvested clover root nodules. We knew from past experience that the MPN plant infection technique was quite sensitive with white clover as the trap host, with a threshold inoculum of only 14 viable cells of *R. leguminosarum* bv. *trifolii* being sufficient to generate a positive score for a nodulated plant (Dazzo 1982).

The results of both experiments indicate that the clover root-nodule symbiont *R. leguminosarum* bv. *trifolii* does intimately colonize rice roots in the Egyptian Nile Delta, where this cereal has been rotated successfully with berseem clover since an-

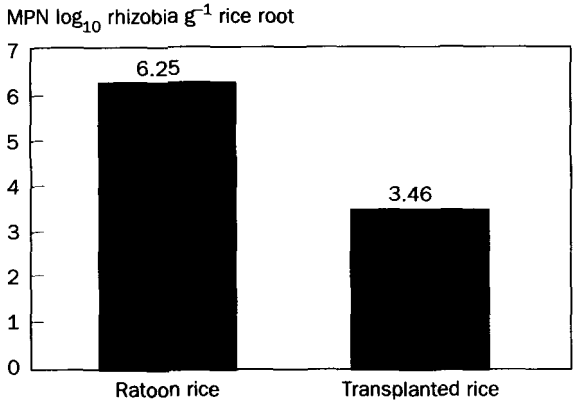

Fig. 1. Populations (log_{10}) of clover-nodulating rhizobia colonizing the root interior of field-grown ratoon rice intermingled with berseem clover in drained soil and transplanted rice in flooded soil (Yanni et al 1997, Y. Yanni and F. Dazzo, unpublished data). Values were obtained by the Mube MPN plant infection technique on diluted macerates of surface-sterilized rice roots inoculated on berseem clover as the trap host. Nodule reisolates were verified as *Rhizobium leguminosarum* bv. *trifolii.*

tiquity. The native population density of *R. leguminosarum* bv. *trifolii* surviving surface sterilization of roots of field-grown rice was on average 2- to 3-logs higher in the ratoon rice growing among the clovers in a field of drained soil than in the transplanted rice of the same height in the flooded fields (Fig. 1).

Strain diversity of rice-adapted *R. leguminosarum* bv. *trifolii* populations in the Nile Delta

A total of 39 "E" (for Egyptian endophyte) isolates of rice-adapted *R. leguminosarum* bv. *trifolii* (El-El2 from the first cycle, E13-E39 from the second) were obtained in pure culture from clover nodules sampled from different plants receiving the highest diluted inocula of rice root macerates. Each isolate represents a different clone of numerically abundant rhizobia from intimate, natural associations with rice roots. As expected, each of the 39 isolates nodulated berseem clover on N-free Fahraeus agar slopes under microbiologically controlled conditions. Of the isolates, 35 (90%) were effective in nitrogen fixation and four (from the second cycle of isolation) were ineffective on this host, based on a statistical comparison of total plant dry weight and Kjeldahl N content with those of uninoculated control plants. Berseem clovers inoculated with all the effective isolates were green and healthy, whereas those inoculated with the four ineffective isolates produced white root nodules and stunted leaves that eventually turned purple. Two of the clover-nodulating effective isolates (E11 and E12) were examined further and proven taxonomically to be authentic strains of R. *legurninosarum* bv. *trifolii* (Yanni et al 1997) based on their complete 16S rRNA sequences that were almost identical to the official type strain of *R. legurninosarum* bv. *trifolii* (differing at only one base position in the entire sequence; Willems and

Collins 1993, Young and Haukka 1996), their growth characteristics in BIII-defined medium (Dazzo 1982), their encapsulation and characteristic acidic EPS molecular structure (Dazzo 1982, Philip-Hollingsworth et al 1989), and their production of cell-bound cellulases and pectinase (Jiminez-Zurdo et al 1996, Mateos et al 1992).

The strain diversity and relatedness of these 39 E isolates of *R. leguminosarum* bv. *trifolii* are being examined by several molecular techniques: pattern recognition of plasmids in Eckhardt agarose gels (Eckhardt 1978) modified by Espuny et al (1987), restriction fragment length polymorphism (Corich et al 1991), genomic fingerprinting using the BOX-PCR technique (Versalovic et al 1994), and analysis of low molecular weight stable RNA (5S and tRNA) by staircase electrophoresis (Cruz-Sanchez et al 1997). The results obtained so far cluster them into 10 different groups of strain-specific genotypes: three groups from the first cycle of isolations from ratoon rice roots (Yanni et al 1997) and seven other groups from the second cycle of isolations from transplanted rice roots. Although this study of strain diversity is not yet complete, sufficient data are already available to conclude that diverse populations of *R. leguminosarum* bv. *trifolii* can be isolated from surface-sterilized rice roots in both flooded and drained soils in the rice-berseem clover rotation in the Nile Delta of Egypt.

Confirmation of the ability of these rhizobia to associate intimately with rice

To fulfill Koch's postulates, two different strains (E11 and E12) were cultured on roots of rice plants under microbiologically controlled conditions, and then reisolated from surface-sterilized roots after 32 d of incubation. Strain identification tests using plasmid profiling and BOX-PCR genomic fingerprinting showed that these reisolates were the same as the original inoculant strains, fulfilling Koch's postulates and confirming that they can form intimate associations with rice roots without requiring the assistance of other soil microorganisms (Yanni et a1 1997).

Some of these rhizobia can promote rice growth

During the course of these initial experiments, we found that rice plants were taller and had longer roots when inoculated with strains E11 and E12 (Yanni et al 1997). More studies using other combinations of rice and E strains of rhizobia clearly indicated that some of these rhizobia behave as plant growth-promoting rhizobacteria (PGPR) on rice. The degree of plant growth promotion (PGP)-exhibited strain/variety specificity on rice was more pronounced when tested in Hoagland's No. 2 plant growth medium (containing ammonium nitrate) than in N-free Fahraeus medium, and did not necessarily correlate with the size of the "endophytic" population of rhizobia that could be cultured from surface-sterilized roots of plants grown in the enclosed tubes (Yanni et al 1997).

Each of these results has important implications in the study and exploitation of this *Rhizobium*-rice association. For instance, they indicate that the identification of superior combinations of rhizobia and rice genotypes for optimal growth responses will likely require PGP bioassays rather than just an assessment of the degree to which

the bacterial endophyte can colonize the root interior. In addition, manipulations to increase the endophyte population per se above the level that rice can normally support will not guarantee that the resultant growth promotion response is improved. In essence, this result emphasizes that the rhizobia1 endophyte's genotype is more important in determining the outcome of the PGP responses than just its population size *in planta.* We must also leave open the possibility that intimate rhizoplane populations of these rhizobia may also benefit the plant without having to colonize the root interior, although their competition for nutrient resources would be more intense because of the diverse microbial community in this same external root environment. Nevertheless, even small numbers of superior PGP inoculant strains on the rhizoplane may still do wonders for the plant if their mechanism of PGP is functional and potent.

More extensive lab and greenhouse studies have since confirmed the ability of selected strains of rice-adapted rhizobia to promote the growth of certain japonica, indica, and hybrid rice varieties from Egypt, IRRI, the United States, and Australia under a variety of experimental conditions. These variations include growth chamber experiments in which the plant roots were grown under microbiologically controlled conditions in a layer of flooded sand above Hoagland's No. 2 plant nutrient agar within large enclosed tubes, in larger semienclosed cylindrical jars almost completely filled with agar to provide more "growing space," as well as in greenhouse experiments using potted soil (Biswas et al 1999,2000a,b, Dazzo et al 1998, 1999, Prayitno et al 1999, Yanni et al 1997). Each of these methods to assess the PGP activity of rhizobia on rice has its merits and disadvantages, but the bottom line is that each method reveals positive growth promotion of rice by certain strains of rhizobia and provides useful new information that enhances our understanding of this *Rhizobium*-rice association. From an ecological and evolutionary perspective, the finding of specific combinations of these dissimilar organisms that benefit by living together suggests that they may represent the evolution of a loose mutual plant-bacteria symbiosis in these fields of the Nile Delta.

Inoculation of selected rhizobia on rice under field conditions

The ultimate assessment of the potential importance of this newly described plant-microbe association for rice productivity requires extensive, repeated experimentation under field conditions. To date, we have conducted two separate field inoculation trials on this project at experimental farms of the Sakha Agricultural Research Station in the Egyptian Nile Delta. These first two field trials included a comparison of inoculation responses of rice to strain E11 and other strains of *R. leguminosarum* bv. *trifolii,* the first in 1997 using Giza-175 rice variety (a hybrid of japonica cultivar Giza-I4 and indica cultivar IR-28 from IRRI) (Yanni et al 1997) and the second in 1998 using Giza-177 (an improved hybrid of japonica and indica rice) in a different field. In both cases, the increases in agronomic responses of grain yield and agronomic fertilizer-N use efficiency to inoculation were very encouraging, but a larger number of repeated field trials over several years need to be done before making hard conclusions about their efficacy as biofertilizer inoculants for rice.

Strategic planning for continuation of this project

Strategic planning calls for a good balance of basic and applied research because this combination increases the probability of ultimate success in exploitation of the *Rhizobium*-rice association to improve rice production with less N-fertilizer input. Therefore, because of the profound importance of rice to world human nutrition and the potential of this newly discovered plant-microbe interaction to benefit rice production, we have created an international network of research collaboration that adds unique expertise focused on basic and applied studies to increase our understanding of this natural *Rhizobium*-rice association. This network currently involves 16 different research groups in 10 countries representing all continents where rice is grown (none in Antarctica). Through the sharing of rhizobial strains isolated from surface-sterilized rice roots in Egypt, as well as other native and engineered strains from IRRI and Australia, four major ongoing lines of collaborative investigation have come to the forefront under this frontier subproject. These thrust group areas are (1) the ecology of the association, (2) plant growth promotion, (3) the biochemistry and molecular biology of the association, and (4) the agronomic testing of *Rhizobium* as a potential biofertilizer for rice-cropping systems.

The ecology of the *Rhizobium*-rice association

Ecological studies under way or planned for this area include (1) assessment of the population density, diversity, and biogeography of rhizobia that intimately colonize rice roots under field conditions; (2) microscopical analysis of the spatial patterns and mode of rice root colonization by rhizobia, including a critical assessment of the "endophytic" state within the root interior; (3) assessment of the range of intimate *Rhizobium*-cereal associations that occur naturally; and (4) evaluation of the compatibility of rhizobia introduced into living rice tissue. Results obtained for item 1 were described earlier. Currently, staircase electrophoresis analysis of the low molecular weight stable RNAs (Cruz-Sanchez et al 1997) is being performed to determine whether the diversity of the larger groups of isolates can be split further.

Two efforts have been made so far to address item 2. First, collaboration involving the training of IRRI staff in plant microscopy at Michigan State University served as the catalyst to study the mode of colonization and entry of the *Sesbania* nodule symbiont *Azorhizobium caulinodans* ORS571 into rice roots grown gnotobiotically. We performed these studies by bright-field microscopy using reporter strain derivatives expressing *lacZ* and *gusA,* laser-scanning confocal microscopy in the epifluorescence mode using acridine orange staining, scanning electron microscopy, and transmission electron microscopy of plants grown gnotobiotically with ORs571 (Reddy et al 1997). Key accomplishments were the documentation of extensive colonization of the rice rhizoplane by the azorhizobia (especially just above the root meristem and at lateral root axils), their "crack entry" mode of primary host infection in natural wounds of the epidermis at the fissure site where lateral roots have emerged, their limited colonization within intercellular spaces and host cells of the outer root cortex, and the high magnification resolution of their "endophytic" state within dead

host cells adjacent to well-preserved intact cells of the root cortex (Figs. 2A-2D; Reddy et a1 1997). Similar results were reported for the invasion of wheat roots by *A. caulinodans* when the cultures were supplemented with the flavonoid naringenin (Webster et a1 1998).

Item 2 is now being addressed more thoroughly to examine rice root colonization by selected strains of rice-adapted rhizobia. B.G. Rolfe and colleagues at the

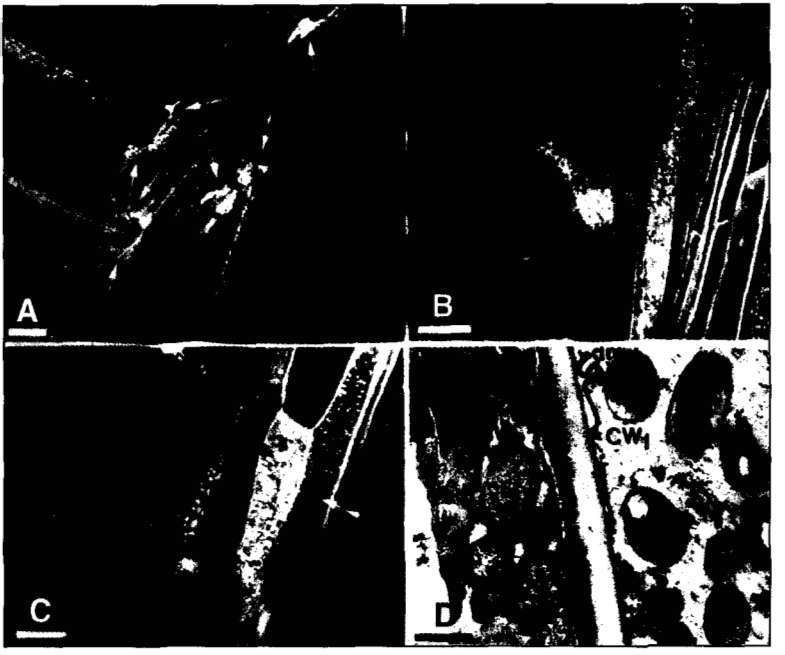

Fig. 2. Colonization of rice root tissue by *Azorhizobium caulinodans* ORS571 in gnotobiotic culture (Reddy et al 1997). (A-C) Roots were stained with acridine orange and examined by laser-scanning confocal microscopy to detect fluorescent bacteria. (A) Low magnification view of a branched root segment showing a high density of brightly fluorescent bacteria colonizing around sites of lateral root emergence (arrows). (B,C) Higher magnification views showing the extensive colonization of bacteria at the fissure site junction of an emerged lateral root (single white arrow). This is a major route for crack entry of *A. caulinodans* into rice roots. (B) is a Z series of 10 confocal optisection overlays covering a depth of approximately 20 mm. (C) is a single optisection that samples into the outer cortex of the main root. Note that one cortical cell is filled with a high density of fluorescent bacteria (black arrow), a second deeper cortical cell contains a moderate level of fluorescent bacteria (double white arrow), and an adjacent cortical cell appears to be uninvaded (white asterisk). (D) Transmission electron micrograph of a section within the rice root cortex showing the interface of a dead, invaded host cell (ic, on the right) and an adjacent intact, uninvaded host cell (uc on the left). The invaded host cell contains a degraded cell membrane (dm), several bacteria (b), and a cell wall (CW_1) whose electron density is much less than the wall (CW_2) of the adjacent uninvaded cell. The cristae of the intact mitochondria (m) in the uninvaded host cell are re solved, indicating good preservation of the tissue prior to sectioning. Bar scales are (A) 250 mm, (B and C) 10 mm, and (D) 0.5 mm.

Australian National University have constructed reporter strain derivatives that express the gene encoding the green fluorescent protein (GFP) of a marine jellyfish (Prayitno et al 1999). Examination of GFP-tagged derivatives of strains E11 and E12 on rice roots by conventional epifluorescence microscopy has shown that they efficiently colonize seedling root surfaces, forming microcolonies on epidermal cells and grooved junctions between them, and form larger masses of expanding biofilms at the emerging lateral root zone and root tips. Certain rhizobial strains also display some intercellular colonization in lateral roots formed on the main roots. The frequency of this type of interior root colonization varied among strains. In the most frequent case, the GFP-tagged rhizobia multiplied within the lateral rootlets and migrated to form long fluorescent lines of bacterial cells inside of those growing roots (Prayitno et al 1999). Related to this finding is earlier work with laser-scanning confocal microscopy using epifluorescence with acridine orange staining of inoculated rice seedlings grown gnotobiotically, which revealed numerous E11 cells between leaf whirls at the neighboring stem base (Yanni et al 1997). Using scanning electron microscopy, we have also confirmed that E11 efficiently colonizes the root epidermis (often with polarly attached cells), including growth and multiplication of bacteria in fissures opened at junctions between epidermal cells. The fact that these preferred sites of rice root colonization by E11 and E12 differ from that of *A. caulinodans* ORS571 (at axils of smaller roots and not necessarily epidermal cell junctions, see Figs. 2A-2C; Reddy et al 1997) implies that different rhizobia may have different preferred sites of rice root colonization; therefore, multiple, spatially discrete, nonoverlapping ecological niches may be available for rhizobial colonization of rice roots.

These GFP-tagged derivatives of the Egyptian rice-adapted rhizobia have significantly facilitated their detection within the interior of rice roots (Prayitno et al 1999). We plan to follow up on these findings using laser-scanning confocal microscopy on live tissue, excise and process the tissues of interest, and perform transmission electron microscopy to further resolve the "endophytic" state of selected E strains in internal root colonization. A similar protocol was successfully used to locate sites of crack entry of *A. caulinodans* (Reddy et al 1997) and other bacteria (de Bruijn et al 1995) within the rice root interior, and also to locate sites of crack entry suitable for examination of the root-nodule symbiont *Allorhizobium undicola* within the root interior of the aquatic legume *Neptunia natans* in the aquatic environment (Subba-Rao et al 1995).

Immunofluorescence microscopy will also be used to examine the colonization of rice roots by wild-type E11 by making use of specific fluorescent antibodies that detect surface antigens on these genetically unmodified cells (Dazzo and Wright 1996). We plan to quantify the kinetics and spatial distributions of early colonization of rice roots by wild-type Ell using *in situ* immunofluorescence with laser-scanning microscopy in the epifluorescence confocal mode and independent confirmation with scanning electron microscopy (Dazzo and Wright 1996, Dazzo and Wopereis 1999). Time-resolved microscopy of root colonization by the bacteria will be followed by digital image analysis to obtain statistically defendable data on their spatial depen-

dence during root colonization (Dazzo and Petersen 1989, Dazzo and Wopereis 1999), using custom CMEIAS© (Center for Microbial Ecology Image Analysis System) software that we are developing. Although CMEIAS© was originally designed to compute the morphological diversity of bacteria in digital images of microbial communities (Liu et al 2000), its newly added measurement features of microbial abundance (numbers and biomass) and their spatial relationships to the underlying substratum make this custom image analysis software ideally suited to perform geostatistical analyses of the spatial distributions of microbial colonization of roots (Dazzo and Wopereis 1999; J. Liu, F.B. Dazzo, A. Jain, manuscript in preparation).

Immunofluorescence microscopy will also be used to examine the colonization of rice roots by E11 in selected fields of the Nile Delta region. This will provide useful information on the autecology of this inoculant strain on field-grown rice roots in the presence of indigenous rhizoplane microflora (Dazzo and Wright 1996). We have already obtained rice roots sampled from the Egyptian fields and from potted soil at IRRI for these experiments.

Related work on this project is being done to explore whether other types of rhizobia develop natural, intimate root associations with rice and other cereals. The extent of intimate associations between cowpea rhizobia and rice will be examined in Venezuela, where rice and cowpea have been rotated extensively. We are also examining whether rhizobia intimately associate with other cereals in rotation with legumes, especially wheat rotated with various leguminous crops in Egypt.

Other studies independent of this project have also contributed information on natural endophytic associations of rhizobia and rice. Dreyfus (1998) has found a natural beneficial association of endophytes of aeschynomene bradyrhizobia that colonize inside wild rice roots in fields of Senegal where these two plants intermingle. Populations of *Azorhizobium caulinodans* have recently been found within the diazotrophic endorhizosphere community of rice cultivars growing in Nepal (B. Hurek-Rinehold, personal communication). Stoltzfus and de Bruijn (this volume) have isolated an organism like *A. caulinoduns* but without a *nodC* from surface-sterilized rice roots at IRRI. Y. Jing (personal communication) is exploring whether astragalus rhizobia invade rice roots in fields of China, where rice and astragalus have been rotated since antiquity. Considered collectively, these surveys should provide a larger picture of whether the natural intimate association of *Rhizobium* and rice (plus other cereals) is widespread.

Related to the question of the range of nonlegumes that exhibit positive growth responses to inoculation with rhizobia, other significant results have been reported for wheat, maize, radish, and mustard inoculated with *R. leguminosarum* bv. *trifolii* (Holflich et al 1995), canola and lettuce inoculated with *R. leguminosarum* (Noel et al 1996), and maize and lettuce inoculated with *R. leguminosarum* bv. *phaseoli* (Chabot et al 1996).

It is obviously of interest to know whether our "E" strains of *R. leguminosarum* bv. *trifolii* that are PGP^+ on rice can also promote the growth of other nonlegume crops. A first set of inoculation trials conducted at the University of Wisconsin-Madison has provided preliminary evidence that this may be the case, at least for certain

specific genotypes of maize (E. Triplett, unpublished data). In this first study, inoculation with strain Ell resulted in statistically significant increases in dry weight of three of six tested maize genotypes in the greenhouse, and one of seven maize genotypes in experimental field plots receiving no N fertilizer. Interestingly, a cross between the high-responding genotype and a different nonresponding genotype resulted in a hybrid maize genotype with an intermediate level of growth responsiveness to inoculation with Ell. These results are very preliminary and must be repeated with additional field trials before final conclusions are drawn. They do reinforce the earlier finding that induction of positive growth responses in cereals by rhizobia is very genotype-specific even in maize, and further work with these crosses may help to identify the genes in cereals necessary for expression of these growth responses to rhizobia.

For item 4, experiments involving viable plate counting of strains El1 and El2 introduced into rice leaves have shown that these rhizobia are compatible with rice, and can grow and survive more than R. leguminosarum bv. trifolii wild-type strain ANU843 within rice tissue (Prayitno et a1 1999). This result suggests that these E strains of rhizobia may possess "rice adaptation" genes that are worthy of further molecular analysis and application. This information should help to explain how these specialized rhizobia are able to foil or otherwise suppress host defense responses during invasion of the rice root interior, a concept derived from current thinking about host-symbiont compatibility in the *Rhizobium*-legume symbiosis.

Plant growth promotion

Studies on this topic have sought to identify optimal strain/variety interactions, the range of growth responses to selected rhizobia1 inoculant strains, the developmental stages in which these growth responses occur, and the underlying mechanisms of plant growth promotion in this *Rhizobium*-rice association (Biswas et a1 2000a,b, Prayitno et a1 1999, Reddy et a1 1997, Yanni et a1 1997). This information is vital to future basic and applied research activities designed to exploit the *Rhizobium*-rice association for sustainable agriculture.

Identification of optimal strain/variety combinations. We are assessing the PGP activity of the various genotypes of rice-adapted rhizobia on selected rice varieties from Egypt, the United States, and IRRI. The Egyptian varieties chosen for the current tests are Giza- 177 and Sakha-102, representing two different improved japonica/indica rice hybrid varieties that are currently preferred for consumption and well adapted to cultivation in the Nile Delta. The U.S. rice lines being evaluated are L204 (indica) and M202 ('japonica). Final decisions on the IRRI rice varieties for these studies have not been made as of this writing. These PGP bioassays are performed using the cylinder jar technique (Prayitno et a1 1999) under controlled conditions in growth chambers, and the plants are harvested at approximately mid-vegetative stage. Measurements of PGP responses of rice plants involve their cumulative shoot height and leaf area, root architecture including number of roots, cumulative root length, root surface area, and root volume, and whole-plant biomass.

This effort to further evaluate PGP responses in different strain/variety combinations is important for several reasons. From the applied perspective, this information assists in reducing the number of potential combinations that require costly field inoculation trials to identify superior combinations that may prove useful in promoting rice yields under agronomic conditions. It also eliminates from field testing any strain/variety combinations that may be deleterious (Prayitno et a1 1999). From the basic perspective, this effort helps to identify the most suitable combinations of rhizobia and rice useful for follow-up mechanistic investigations on plant growth promotion. An example of a value-added result of this assay was the finding that strain E15 induces strong PGP^+ responses on a few rice varieties despite being totally ineffective in BNF on berseem clover (Y.G. Yanni and F.B. Dazzo, unpublished data). Thus, the ability of this particular clover-nodulating *Rhizobium* strain to express strong PGP^+ responses in rice can function independently of its Fix^- phenotype on berseem clover. Because this *R. leguminosarum* bv. *trifolii* strain is actually deleterious to berseem clover, we will not use it in field inoculation trials and thereby avoid increasing its population burden on this legume crop.

Promotion of plant growth ut various stages of rice development. Figure 3 summarizes the various growth responses of rice to rhizobia found at different stages of plant development (Biswas et a1 l999,2000a,b, Prayitno et a1 1999, Yanni et a1 1997). Time-course studies that follow the entire life cycle of inoculated rice plants in potted soil indicate that many of the early stimulatory responses to inoculation that enhance rice vigor at the seedling stage result in a carryover of significantly increased straw and grain yields at maturity, even when the culturable population of the inoculant diminishes below detectable levels before this late stage of rice development (Biswas et a1 2000a).

Underlying mechanisms of plant growth promotion. We have found evidence of various possible mechanisms of PGP operative in the *Rhizobium*-rice association. These include the induction in development of an expansive root architecture and enhanced efficiency in plant mineral nutrient uptake, production of extracellular growth-regulating phytohormones by rhizobia, and solubilization of precipitated phosphate complexes by rhizobia that are likely to increase the bioavailability of this important, relatively insoluble plant nutrient in soil.

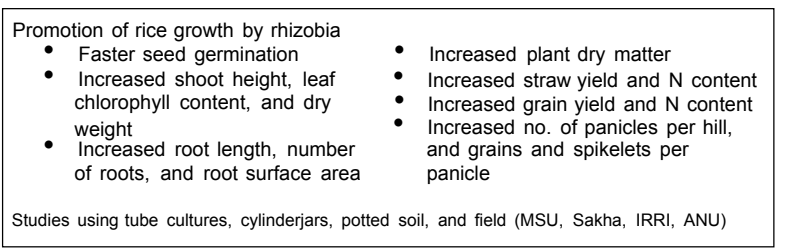

Fig. 3. List of various types of rice growth responses to inoculation with rhizobia. Results summarized from Yanni et al (1997), Biswas et al (2000a,b), and Prayitno et al (1999).

Even the very first studies on this natural association showed that certain strains of rhizobia induce a more expansive root architecture on rice plants in gnotobiotic culture and increase grain N when grown in field soil (Yanni et a1 1997). These inoculation responses have subsequently been repeated in semienclosed cylinder jars and potted soil (Biswas et a1 2000a, Prayitno et a1 1999). We predicted that the expansive root architecture of rice inoculated with *Rhizobium* is likely to increase the plant's ability to take up plant mineral nutrients, somewhat analogous to the *Azospirillum*-grass association (Okon and Kapulnik 1986, Umali-Garcia et a1 1980). This hypothesis is supported by experiments showing that Rhizobium-inoculated rice plants in potted soil were enhanced in accumulation of not only N but also P, K, and Fe (Biswas et a1 2000b). Recently, more comprehensive elemental analyses of inoculated rice plants grown in gnotobiotic tube cultures with defined and controlled levels of plant nutrient sources have confirmed the positive effects of E11 inoculation on the accumulation of N, P, and K, and have shown that Ca, Mg, Na, Zn, and Mo were also significantly increased in inoculated rice plants (Y.G. Yanni and F.B. Dazzo, unpublished data). This ability of El1 to enhance the uptake of these plant nutrients in rice will likely influence many physiological activities that affect plant growth and development. Interestingly, the accumulated levels of several other minerals present within these same growth-stimulated rice plants were not significantly influenced by inoculation with E11. These results suggest that the mechanism(s) of enhanced plant mineral uptake functional in El1 -inoculated rice plants exhibits some selectivity for certain nutrients rather than just a nonspecific across-the-board consequence of enhanced growth.

Other bioassays have shown that culture supernatants of E11 grown in certain media will promote the growth of the rice root system, resulting in a more expansive architecture with a significantly greater absorptive surface area. Figure 4 illustrates the typical root architecture of untreated plants grown in Hoagland's No. 2 medium, and of plants grown identically, except that the plant growth medium contained a diluted sterile sample of the E11 culture supernatant. Image analysis of these silhouette root images using CMEIASO software indicated that their cumulative lengths, cylindrical biosurface areas, and cylindrical biovolumes were 425 mm, 815 mm^2, and 135 mm^3 for the control root, respectively, and 625 mm, 1,563 mm^2, and 482 mm^3 for the root grown with E11 excreted metabolites. These results illustrate the ability of E11 to produce extracellular bioactive metabolites that can promote rice root development resulting in an expansive root architecture.

These results have logically led us to determine whether the E strains of *R. leguminosarum* bv. *trifolii* produce growth-regulating phytohormones. Several studies indicate that they do so in vitro and in the rice root environment, and it is likely that these bioactive molecules affect the *Rhizobium*-rice association. Tentative identification of indoleacetic acid (IAA) in cultures of E11 and other rhizobia1 strains was initially obtained by colorimetric assays, high-performance liquid chromatography, and thin-layer chromatography. Physiological growth studies have identified new media formulations that optimize IAA production by strain E11 in vitro. A comprehensive chemical analysis of the culture supernatants of E11 grown under these conditions is

Fig. 4. Induction of an expansive root architecture in rice by extracellular metabolites of *R. leguminosarum* bv. *trifolii* strain Ell. Plants were grown axenically in Hoagland's No. 2 plant growth medium with or without a diluted sterile sample of the bacterial culture supernatant. Image analysis of these root silhouette im ages using CMEIAS© software indicated that the Ell metabolites cause 47%, 92%. and 257% increases in cumulative root length, cumulative cylindrical root surface area, and cylindrical root biovolume, respectively. Unpublished data of M. Vega-Hernandez and F. Dauo.

under way. So far, we have obtained definitive identification of IAA production by E11 using combined gas chromatography/mass spectrometry (GC/MS) of the corresponding trimethylsilyl derivatives. In addition, these same extracts of E11 culture supernatants have been shown by combined GC/MS to contain gibberellin(s). These findings open new areas of investigation on the molecular basis of plant growth promotion in the beneficial *Rhizobium*-rice association.

Solubilization of precipitated phosphate complexes. Most of the soil in rice cultivation in the Nile Delta contains about 1,000 ppm phosphorus, primarily in the unavailable form of precipitated tricalcium phosphate ($Ca_3[PO_4]_2$), and only 3-4 ppm P (= Olsen P) is available to rice. Therefore, solubilization of precipitated phosphates by rhizobacteria *in situ* would enhance phosphate availability to rice in these soils, thus representing a possible mechanism of PGP for rice under these field conditions. We therefore examined whether the E strains exhibited phosphate-solubilizing activity by culturing them in agar media impregnated with various insoluble phosphate precipitates. Improvements in plating methods to detect the extracellular solubilization of precipitated tricalcium phosphate in vitro indicated that several of the E strains of rice-adapted rhizobia were positive in this metabolic activity (Fig. 5; E. Martínez-Molina and F.B. Dazzo, unpublished data). These E strains also exhibit extracellular phytase activity that can solubilize precipitated phytate (inositol hexaphosphate), a

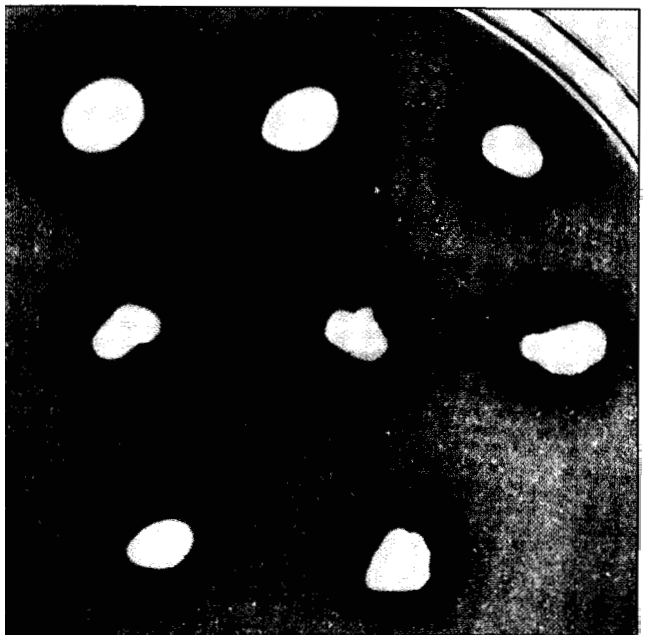

Fig. 5. Demonstration of extracellular solubilization of precipitated tricalcium phosphate by isolated colonies of various E strains of rice-adapted *R. leguminosarum* bv. *trifolii.* The relative intensities of this activity vary among the strains. Unpublished data of E. Martinez-Molina and F. Dauo.

major form of phosphorus stored in plants (E. Martinez-Molina and F.B. Dazzo, data not shown). If extended to rhizosphere soil, this PGP activity may potentially increase the availability of phosphorus for rice, and thereby promote rice growth when soil phosphorus is limiting. Because rice can develop endophytic mycorrhizal symbioses and these are known to facilitate the uptake of phosphorus into mycorrhizal roots, we intend to measure the effect of Ell inoculation on mycorrhizal development in rice in the next field inoculation trials in Egypt.

Other topics of the *Rhlzobium* - rice association being addressed by biochemistry and molecular biology

P. Mateos and colleagues are studying the cell-bound cellulases and pectinase of strain E11. Reddy et al(1997) have obtained evidence that rhizobia-produced IAA modifies rice root growth and development. Further studies indicate that IAA production by rhizobia is stimulated in the external root environment of rice (Biswas et al 2000b), and that axenically collected rice root exudate stimulates the production of IAA by rhizobia (M. Vega-Hernindez and F.B. Dazzo, unpublished data). To study this further, B.G. Rolfe and colleagues are applying proteomic studies to map most of the proteins in cells of selected *R. leguminosarum* bv. *trifolii* strains (including E11, E12, and ANU843) and identify the conditions regulating their synthesis. These studies are

likely to uncover fundamental information on rhizobial genes and gene products important to the *Rhizobium*-rice association, including those induced by chemical signals in rice root exudate.

Various experiments have been done to test whether biological nitrogen fixation can contribute to the positive PGP responses in this natural *Rhizobium*-rice association. No consistent evidence indicating their involvement has yet been found. Experiments to detect BNF in this association have included acetylene reduction assays of nitrogenase activity at various times after plant inoculation, measurements of N content accumulated in inoculated and uninoculated plants when grown in N-free media (mature plants look yellow and sick under these conditions), and ^{15}N-based measurements of the amount of combined nitrogen derived from BNF in inoculated versus uninoculated plants. For these latter experiments, the delta-^{15}N natural abundance method was performed on soil, roots, straw, and grain of plants grown under field conditions at Sakha, and the ^{15}N isotope-dilution method was performed on straw and grain from plants grown in potted soil at IRRI. Quantitative $^{14}N/^{15}N$ measurements of these plant samples were obtained by isotope ratio mass spectrometry using modem analytical instruments at Michigan State University and IRRI, respectively.

The results of these tests for BNF have been consistently negative, with one exception (Biswas et al 2000a,b, P. Ostrom, Y.G. Yanni, and F.B. Dazzo, unpublished data). In one of the ^{15}N isotope-dilution experiments of uninoculated and inoculated rice grown in ^{15}N-enriched potted soil at IRRI, the ^{15}N excess was 12% lower in the rice grain of E11-inoculated plants, indicating that a statistically significant amount of the grain N is derived from BNF, however, this positive result was not repeated in a second duplicate experiment (Biswas et al 2000b). Considered collectively, the results of these various tests argue against a major role of biological nitrogen fixation in accounting for the significantly increased gains in straw N and grain N of rice when inoculated with E11.

An alternate working hypothesis is that the physiological responses of rice to some of these rhizobia increase its aboveground photosynthetic capacity and its belowground ability to absorb combined N as well as certain other essential plant nutrients by an expanded root architecture capable of exploiting a larger reservoir in the soil. In other words, some of our rhizobial endophytes may make rice roots better "miners" of the plant nutrients in the soil resource. At this workshop, it was reported that the E12 strain did indeed stimulate photosynthetic capacity of inoculated rice plants, as well as increase their grain yield (Biswas et al 1999, 2000a). Studies conducted under gnotobiotic conditions indicate that the PGP^+ activity of E11 does not require the assistance of other soil microorganisms, although in soil they will likely influence these processes. This explanation relates to what has been proposed to account for the BNF-independent growth response in *Azospirillum brasilense*-grass associations (Tien et al 1979, Umali-Garcia et al 1980, Okon and Kapulnik 1986), but the bioactive molecules responsible for these PGP responses may differ in these two types of diazotroph-graminae associations. For instance, bioassays completed so far have not found a concentration of IAA standard (over a 10^3-fold range) that can du-

plicate the growth responses of rice to culture supernatants of Ell adjusted to an equivalent IAA content, suggesting that IAA alone cannot account for the growth responses of rice roots to secreted E11 growth-regulating substances (M. Vega-Hernandez, E. Marshall, T. Jain, and EB. Dazzo, unpublished data). More reconstitution experiments of this type are needed to sort out the molecular mechanisms of BNF-independent growth promotion of rice by rhizobia.

At this workshop, a novel PGP mechanism for sinorhizobia was described in which the organism converts riboflavin into lumichrome in the external root environment, which then stimulates root respiration and hence CO_2 enrichment, photosynthesis, and larger shoots of the associated plant (Phillips et al, this volume).

There now seems to be a consensus of agreement among workers in this field that diazotrophs (rhizobia included) possess the ability to promote rice growth through more mechanisms than just biological nitrogen fixation. Multiple mechanisms of growth promotion are likely operative, and this has a major impact on the directions of research designed to exploit biofertilizers to enhance rice production with less N-fertilizer input.

Agronomic testing of *Rhizobium* as a biofertilizer for rice-cropping systems

A third field inoculation trial is currently being performed on Giza-177 and Sakha-102 rice varieties at the Sakha Agricultural Research Station in Egypt, using some new strains of *R. leguminosarum* bv. *trifolii* obtained from our second cycle of isolations from surface-sterilized field-grown rice roots. These new strains are Fix+ on berseem clover and induce positive PGP^+ responses on these Egyptian rice varieties in lab bioassays without obvious adverse reactions. In these field experiments, subplots are 20 m^2 with four replications each in a random design, given several different levels of supplementary N fertilizer, and the entire yields of the subplots are evaluated to obtain the yield data. In the experiments now in progress, we will also be keen to evaluate whether inoculation influences the undesirable tendency of higher N-fertilizer inputs to actually lower grain yield because of excessive vegetative growth resulting in poor light distribution, lodging, increased spikelet sterility, and the production of late tillers.

Our future plans are to continue to conduct field inoculation trials using selected E strains of rhizobia on rice varieties Giza- 177 and Sakha-102 at the Sakha Agricultural Research Station in Egypt to achieve adequate replication for multiple years so that recommendations of superior inoculant strains can be made for implementation in rice-cropping systems in the Nile Delta. We also plan to conduct field inoculation trials at IRRI and in California at the University of California-Davis (facilitated by rice agronomist J. Hill), but decisions on which rice varieties to include have not been made as of this writing. Lab PGP bioassay tests would be performed beforehand to assist in strain/variety selection in advance of these field trials. Field inoculation trials conducted at these three different locations will enable us to assess the efficacy of the rhizobia1 inoculant strains in improving grain yield of various preferred rice varieties with less N-fertilizer inputs under different edaphic, climatic,

and irrigation conditions, as well as different agronomic practices in these rice-cropping systems.

Small farmers who can only afford low to moderate levels of N-fertilizer inputs in marginally fertile soil (very common in the Philippines and other developing countries) are a valuable resource of information that has direct bearing on this project (Umali-Garcia et a1 1999). We plan to tap this resource in both the design of certain experiments and possible future implementation of this technology for rice-cropping systems. For instance, it would be informative to include at some point their preferred varieties in efficacy testing of the rice inoculants. Another important issue is the logistics of how to successfully deliver the inoculum to rice seedlings in a real-world farm setting, because the methods of raising the seedlings are different in the rice-cropping systems in Egypt, IRRI, and the United States. At IRRI, it would be valuable to compare the inoculation of seedlings raised by the wetbed and dapog methods to determine which would perform better. The dapog method may offer the advantage of more successful preemptive colonization by the inoculant strain before exposure to the entire soil community at transplanting. Past successes in using agricultural biofertilizers to improve rice yields with less N-fertilizer inputs can serve as a useful guide in this important future endeavor (Umali-Garcia et a1 1999).

Conclusions

During the past few years, we have made considerable progress in describing the nature of this newly discovered, beneficial *Rhizobium*-rice association, and have begun to evaluate the potential value of exploiting this association to sustain high-yielding rice-cropping systems with reduced N-fertilizer input. We have also delineated our plans of remaining studies and how they will enhance our understanding of this intimate and beneficial association of *Rhizobium* and rice at both the basic and applied levels. Success at the applied level on a larger scale will likely require further improvement of these strains. Participants in this subproject represent a network of international linkages on all continents where rice is grown, and are committed to making this goal a reality.

Our proposal to explore the use of rice-adapted *Rhizobium* biofertilizers for rice production is fully consistent with an important goal of sustainable agriculture, namely, to employ biological processes to enhance the crop's output without irreparably damaging the natural resource base where the crop can be grown. Leonardo da Vinci summarized it best: "Look first to Nature for the best design before invention." In this case, we have employed Nature to help select and identify rhizobia1 germplasm that already possesses the many important traits desired of an ideal biofertilizer inoculant for rice needed to fulfill the long-term real-world goal of this Frontier Project.

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Notes

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Beneficial effects of inoculated nitrogen-fixing bacteria on rice

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Nitrogen fixation in two rice varieties—Super Basmati and Basmati 385—was studied by the acetylene reduction (AR) assay and the ^{15}N dilution technique. In the roots and submerged shoots of field-grown plants of both varieties, a higher acetylene reduction activity and population of diazotrophs were detected at the grain-filling stage than at the panicle initiation stage. Nitrogen-fixing bacterial isolates from rice and other plants were differentiated by using ERIC- and BOX-PCR. A unique banding pattern of the PCR products was obtained from each isolate, confirming that distinct bacterial strains have been isolated. The beneficial effects of the bacterial inoculations were studied on rice plants grown in pots filled with nonsterile soil. Super Basmati showed a better response (plant biomass and grain yield) to bacterial inoculations than did Basmati 385. Maximum fixation (59% Ndfa) in plants of Super Basmati was obtained when *Azospirillum lipoferum* N-4 was used as inoculum, whereas *Herbaspirillum* RR8 showed maximum fixation (39% Ndfa) in Basmati 385.

High interest in nitrogen-fixing bacteria associated with rice and other graminaceous plants has been shown during the past few years to minimize the use of expensive chemical fertilizers in agriculture. One of the reasons for considering rice as a more promising candidate for research on nitrogen fixation is the observation that nitrogen status of wetlands under rice cultivation is sustained due to the activity of nitrogen-fixing bacteria that find the environment favorable for their growth in the submerged rice system (App et al 1980, Ladha et al 1993, Roger and Ladha 1992). Using the acetylene reduction (AR) method, nitrogen fixation in rice roots was for the first time detected by Yoshida and Ancajas (1971). Nitrogen balance studies (App et al 1980, Ventura and Watanabe 1983) and long-term fertility studies (Watanabe et al 1981) confirmed nitrogen gains in wetland rice soils, mostly from N_2 fixation. Nitrogen fixation associated with wetland rice includes N_2 fixation that occurs on or in the leaf sheaths, the basal portion of the shoot, and the root zone (Watanabe et al 1981, Yoshida and Rinaudo 1982). A large percentage of the aerobic heterotrophic bacteria in wetland rice roots are N_2-fixing (Watanabe et al 1979).

From rice and other members of the family Gramineae, several diazotrophic bacteria belonging to the genera *Azospirillum, Herbaspirillum, Enterobacter, Acetobacter, Azoarcus, Alcaligenes, Pseudomonas, Zoogloea,* and others have been isolated (Baldani et al 1986, 1996, Barraquio et al 1983, Bilal and Malik 1987, Cavalcante and Dobereiner 1988, Dobereiner 1992, Hassan et al 1998, Khammas et al 1989, Ladha et al 1983, 1987, Malik et al 1994, Reinhold-Hurek et al 1993, Reinhold et al 1987, Tarrand et al 1978, Watanabe et al 1979, You et al 1991). Some newly identified N_2-fixing genera such as *Acetobacter, Azoarcus,* and *Herbaspirillum* have been called endophytes because of their occurrence mainly within plant tissues (James and Olivares 1998). Endophytic diazotrophs have been isolated from several grasses in which significant biological nitrogen fixation has been demonstrated, particularly Brazilian sugarcane varieties, but also in rice, maize, and sorghum. Endophytic diazotrophs have been linked with the high N_2 fixation (up to 80% of the N incorporated) reported particularly in sugarcane where the bacteria are found in high numbers (Boddey et al 1991, 199Sa,b, Dobereiner et al 199Sa,b). Endophytic diazotrophs colonize the interior rather than the surface of the plants and hence are better placed to exploit carbon substrates supplied by the plants and avail a low pO_2 environment necessary for the expression and operation of nitrogenase (Baldani et al 1997, Gallon 1992, James and Olivares 1998, Patriquin et al 1983).

Incorporation of biologically fixed N and enhancement of crop yields of cereals by inoculation with nitrogen-fixing bacteria have been observed in many field experiments (Baldani et al 1983, 1987, Boddey et al 1986, 1991, Dobereiner et al 1993, Kapulnik et al 1981a,b, 1987, Lima et al 1987, Sarig et al 1984, Urquiaga et al 1992). Yield increases obtained in inoculated plants, however, have been attributed to biological N fixation and also to the production of plant growth substances by the root-colonizing bacteria. Increases in yields of wheat by inoculation with *Azospirillum* strain Cd have been consistent in field experiments, mainly in Israel and other semiarid regions (Kapulnik et al 1981a,b, 1987, Okon 1985, Smith et al 1984). In two cultivars of grain sorghum, inoculation with three *Azospirillum* strains, but not with a strain of *Herbaspirillum,* led to an increase in plant dry weight and total N in grain (Pereira et al 1989). Pereira et a1 (1 989) have also reported that, in rice, *A. lipoferum* Al 121 and *A. brasilense* sp 245, isolated from rice and wheat roots, respectively, when used as inoculum, did not affect plant growth or grain yield. A field study carried out by Ali et al (1998), in which N_2-fixing bacteria were used as inoculum for rice, indicated that a low input of chemical N fertilizer was useful for increasing rice yield, fertilizer-N-use efficiency, and BNF in rice grown under flooded soil conditions.

To reduce dependency on the use of chemical fertilizers, research efforts have also attempted to identify rice genotypes that naturally support high nitrogen-fixing activity or show the best response to inoculations with diazotrophs. Several studies indicate that significant genotypic differences exist in ability to support nitrogen fixation (App et al 1986, Hirota et al 1978, Ladha et al 1987. 1988, Lee et al 1977, Shrestha and Ladha 1996, Yoshida and Ancajas 1971). Ladha et al (1986) have suggested that these genotypic differences may be due to specificity of plant-bacterial associations,

differences in root exudation, and gaseous diffusion efficiency. Therefore, rice genotypes showing high N_2-fixing activity in the presence of indigenous populations of diazotrophs or showing the best response to bacterial inoculations may be selected on the basis of their lower requirement for chemical N input.

In the present study, AR activity was measured in two field-grown Basmati rice varieties to detect the presence of diazotrophic bacteria. The morphologically highly related bacterial isolates were differentiated by using BOX- and enterobacterial repetitive intergenic consensus (ERIC)-PCR (polymerase chain reaction). The main objective of the study was to evaluate the performance of diazotrophic strains isolated from rice and other grasses when used as inoculum for two rice varieties, Super Basmati and Basmati 385.

Materials and methods

Detecting N_2-fixing activity in roots and submerged shoots of rice

The acetylene reduction assay was used to detect the presence of nitrogen-fixing activity associated with rice varieties Super Basmati and Basmati 385 growing in farmers' fields at Ghakar, Gujranwala District, Pakistan. Roots and shoots of rice submerged underwater (5-cm pieces of shoots near the base) were collected from the field and washed with sterile water. Approximately 5 g of fresh weight root or shoot samples were transferred to 16-mL-capacity glass tubes and incubated in 10% acetylene at 30 °C for 24 h. Triplicate samples of roots and shoots were used for the AR assay. The tubes with plant material (roots and shoots) but without C_2H_2 were used as a control. Another set of tubes containing only 10% C_2H_2 and no plant material was also used as a control. Ethylene production was measured on a gas chromatograph (Gasukuro Kogyo model 370) using a Porapak N column. Root and shoot samples were dried in an oven at 70 °C to a constant weight.

Detecting bacterial populations in roots and submerged shoots of rice

The roots and lower parts of shoots submerged underwater were collected from the field and thoroughly washed with sterile water to remove adhering soil. Roots or shoots (1 g) were homogenized in sterile water and serial dilutions were prepared. These dilutions were used to inoculate semisolid combined carbon medium (CCM; Rennie 1981) and incubated at 30 °C for 48 h. Acetylene (10% V/V) was injected to the vials showing bacterial growth and acetylene reduction activity was measured to determine most probable number (MPN) counts on a gas chromatograph (Gasukuro Kogyo model 370) using a Porapak N column. For plate counts, the serial dilutions prepared from roots and shoots of rice were also used to inoculate Luria-Bertani (LB) plates.

Characterizing bacteria by ERIC- and BOX-PCR

Bacterial cells were grown in LB for 24 h at 30 °C and centrifuged at 13,000 rpm for 5 min. The cell pellets from 1.5-mL cultures were washed with TE buffer (10 mM TRIS Cl, 1 mM EDTA, pH 8) and then dissolved in 200 µL of TE. Cell lysis was

obtained at 37 °C for 30 min with lysozyme (2 mg mL^{-1} , final concentration) and by using sodium dodecyl sulfate (SDS) (1%). The lysate was extracted twice with phenol/chloroform followed by two extractions with chloroform/isoamyl alcohol (24:1). After adding 1/10 volume of sodium acetate (3 M, pH 5.2) and 0.5 volume of isopropanol, the supernatant was incubated at −20 °C for 30 min. The nucleic acids were then precipitated by centrifugation at 13,000 rpm for 20 min and the pellets were washed with 70% ethanol before drying under a vacuum. The nucleic acid pellets were then dissolved in TE and the DNA concentration of the samples was adjusted between 50 and 100 ng μL^{-1} . One µL of this DNA solution was used as a template for PCR. The PCR reaction was carried out in a 25-µL volume containing 50 pmole (I µL) for each primer, 1.25 mM deoxynucleoside triphosphate, and 2 units of *AmpliTaq* polymerase. For ERIC primers (ERIC1R 5'-ATGTAAGCTCCTGGGGATTCAC, ERIC2-S AAGTAAGTGACTGGGGTGAGCG; Versalovic et a1 1991), the first cycle at 95 °C for 7 min was followed by 30 cycles at 94 °C for 1 min, at 52 °C for 1 min and at 65 °C for 8 min, 1 cycle at 65 °C for 16 min, and a final soak at 4 °C. For BOX-PCR (BOX primer 5'-CTACGGCAAGGCGACGCTGACG-3'; Louws et a1 1994), the following cycles were used: 1 cycle at 95 °C for 7 min; 30 cycles at 90 °C for 30 s, at 53 °C for 1 min, and at 65 °C for 8 min; one cycle at 65 °C for 16 min; and a final soak at 4 °C.

After the reaction, 10 µL of the ERIC- and BOX-PCR products were used for electrophoresis on 1.5% agarose gels. The gels were stained with ethidium bromide to visualize the banding patterns of PCR products and then the gels were photographed.

For the analysis of the bacterial fingerprints, product moment-correlations and Dice coefficients software programs were used.

Inoculation of rice with bacterial isolates

For this experiment, rice plants (varieties Super Basmati and Basmati 385) were grown in nonsterilized soil filled into 25-cm diameter fiberglass pots. To fill each pot, 10 kg of air-dried soil was used to make about a 25-cm depth in the pot. The paddy soil used in this experiment was a sandy loam collected from the top 30 cm of a rice-growing area of the Institute and it had an electrical conductivity of saturation extract (ECe) 2.5 dS m^{-1}, pH 8.2, organic matter 0.60%, and total N 0.059%. The pots were kept flooded with canal water until 2 wk before the rice harvest.

In the 2nd week of July, 5-wk-old rice seedlings were transplanted. Ten seedlings were grown in each pot. At the time of transplanting, the seedlings were inoculated by keeping the root system submerged in liquid bacterial cultures (approximately 10^9 cells mL^{-1}) for 30 min. Two weeks after transplanting, ^{15}N-labeled ammonium sulfate of 5 atom % excess (0.72 g pot^{-1}) was added as a tracer to quantify nitrogen fixation. The plants were harvested at maturity and dried in an oven at 70 °C until no change in weight was noted. The dried plant samples were analyzed for ^{15}N excess on a double-inlet Mass Spectrometer (MAT GD 150). Uninoculated plants were used as a nonfixing reference to estimate ^{15}N dilution.

Results and discussion

With the AR assay technique, nitrogen-fixing activity was detected in roots as well as in submerged shoots of field-grown rice varieties Super Basmati and Basmati 385 (Table 1). This indicates colonization of rice roots as well as shoots by diazotrophic bacteria. The presence of nitrogen-fixing activity in lower parts of the shoot, in addition to the roots, has also been reported by other researchers (Watanabe et al 1981, Yoshida and Rinaudo 1982). In the aerial parts of rice plants above the level of floodwater, no AR activity was found. In the roots and submerged shoots of both varieties, more AR activity was detected at the grain-filling stage than at the panicle initiation stage. Relatively higher AR activity was detected in roots than in submerged shoots of both varieties at the panicle initiation stage. At the grain-filling stage, more activity was found in submerged shoots than in roots. The population of diazotrophs as estimated by AR assay-based MPN at the grain-filling stage was also higher in submerged shoots than in roots (Table 2). The high AR activity detected in shoots and the presence of diazotrophs in high numbers may be of practical significance as the isolation and use of these bacteria as biofertilizers along with root-colonizing bacteria may enhance the efficiency of such inocula.

We used the PCR technique with primers corresponding to repetitive DNA sequences, namely, the 124–127-bp ERIC and 154-bp BOX elements to generate specific DNA fingerprints of the nitrogen-fixing isolates from rice and other hosts with

Table 1. Detection of acetylene reduction activity[a] in roots and submerged shoots of field-grown rice.

Stage	Basmati 385		Super Basmati	
	Root	Shoot	Root	Shoot
	(Mean ± SD)[b]		(Mean ± SD)	
Panicle initiation	20 ± 6	15 ± 7	35 ± 9	25 ± 6
Grain-filling stage	75 ± 13	110 ± 21	95 ± 11	113 ± 17

[a]n.mole C_2H_4 g^{-1} dry weight 24 h^{-1}. [b]SD = standard deviation.

Table 2. Detection of the bacterial population (in the grain-filling stage) associated with roots and submerged shoots of field-grown rice.

Technique	Basmati 385		Super Basmati	
	Root	Shoot	Root	Shoot
	(Mean ± SD) × 10^7		(Mean ± SD) × 10^7	
AR assay-based MPN[a]	0.43 ± 0.12	0.06 ± 0.01	3.3 ± 1.1	0.05 ± 0.01
Plate counts on LB	820 ± 80	29 ± 7	770 ± 140	67 ± 10

[a]AR = acetylene reduction, MPN = most probable number, LB = Luria-Bertani.

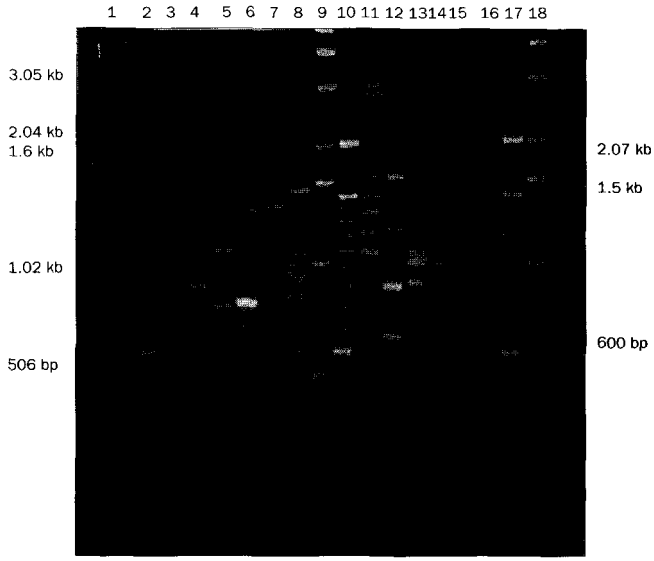

Fig. 1. Genomic fingerprinting of different bacterial strains using BOX primers in PCR. Lane 1 = 1-kb ladder; lane 2 = 100-bp ladder; lane 3 = *A. lipoferum* N-4; lane 4 = *Azospirillum* ER-20; lane 5 = *Pseudome nas* 96-51; lane 6 = *Zoogloea* Ky-1; lane 7 = Azoarcus K-1; lane 8 = *Azospirillum* sp. 4ATR; lane 9 = 1-kb ladder; lane 10 = 100-bp ladder; lane 11 = *Azospirillum* sp. AZLS12; lane 12 =*A. lipoferum* USA5; lane 13 = A. brasilense Sp7; lane 14 = A. brasilense Sp13; lane 15 = A. lipoferum 4T; lane 16 = *A. halopraeferens* AU4; lane 17 = 100-bp ladder; lane 18 = 1-kb ladder.

two objectives. The first was to differentiate morphologically highly related *Azospirillum* isolates and to compare these isolates with standard strains of this genus. The second was to make available for comparisons specific DNA fingerprints of bacterial strains for use in future ecological studies requiring reisolations of the inoculated bacteria. Five locally isolated nitrogen-fixing and plant growth hormone-producing strains—*Azospirillum* N-4, *Azospirillum lipoferum* ER-20, *Azoarcus* K-1, *Pseudomonas* 96-51, and *Zoogloea* Ky-1—were used for ERIC- and BOX- PCR fingerprinting. Banding patterns generated after amplification of genomic DNA with BOX and ERIC primers are given in Figures 1 and 2, respectively. PCR products with both types of primers were obtained from all the strains tested. Several strong and weak bands (some of the weak bands were visible on the gel but are not clearly visible in the photographs) were observed with both types of primers. Each strain showed its own specific banding pattern of PCR products. These unique banding patterns could be useful in future field experiments for comparisons with those obtained from reisolates for confirmation as the respective inoculated strain. The BOX- and ERIC-PCR patterns of morphologically highly related *Azospirillum lipoferum* strains N-4 and ER-20, isolated from rice and wheat, respectively, were different from each other. The amplified products of these two strains ranged from less than 450 bp to more than

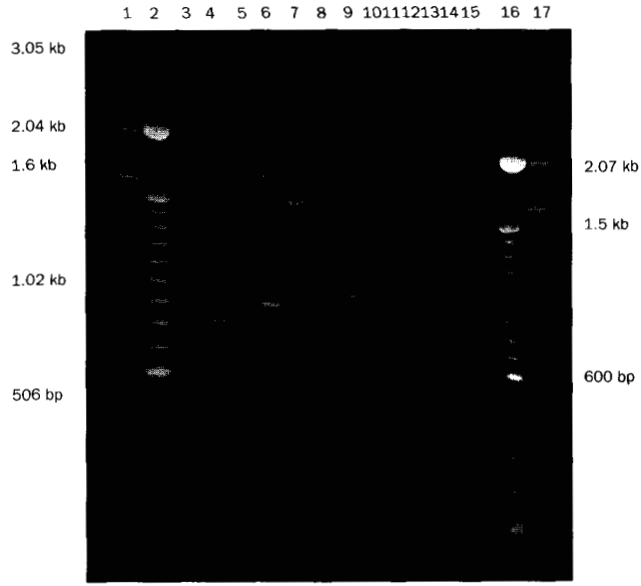

Fig. 2. Genomic fingerprinting of different bacterial strains using ERIC primers in PCR. Lane 1 = 1-kb ladder; lane 2 = 100-bp ladder; lane 3 = *A. lipoferum* N-4; lane 4 = *Azospirillum* ER-20; lane 5 = *Pseudomonas* 96-51; lane 6 = *Zoogloea* Ky-1; lane 7 = *Azoarcus* K-1; lane 8 = *Azospirillum* sp. 4ATR; lane 9 = *Azospirillum* sp. AZLS12; lane 10 = *A.* lipoferum USA5; lane 11 = *A. brasilense* Sp7; lane 12 = *A. brasilense* Sp13; lane 13 = *A. lipoferum* 4T; lane 14 = *A. lipoferum* 34 H; lane 15 =*A. halopraeferens* AU4; lane 16 = 100-bp ladder; lane 17 = 1-kb ladder.

4 kb with BOX primers and from less than 400 bp to more than 3.5 kb with ERIC primers. With BOX primers, 18 PCR bands were obtained from the template DNA of *A. lipoferum* N-4 and 19 PCR bands were obtained when the template of *A. lipoferum* ER-20 was used for amplifications. In *A. lipoferum* N-4, the size of the products ranged from about 600 bp to 2 kb, whereas in *A. lipoferum* ER-20, the size ranged from about 410 bp to 4 kb. When ERIC primers were used for amplification of DNA from *A. lipoferum* N-4, four strong and six weak bands of PCR products ranging from about 370 bp to 1.4 kb were visible on the gel. With these primers, four strong and seven weak bands were formed from the template of *A. lipoferum* ER-20 and the size of the bands ranged from about 550 bp to 3.5 kb. These different banding patterns of ERIC- and BOX-PCR obtained in this study indicate that *A. lipoferum* N-4 and ER-20 are different isolates and not the reisolates of the same strain.

To find out the degree of relatedness, the banding patterns of the PCR products of the local isolates were compared with the DNA fingerprints of standard *Azospirillum* strains by using Gel Compar and calculated using Pearson's software correlations and Dice coefficients. When the analysis was carried out using Pearson's correlations, BOX-PCR fingerprints produced by *Azospirillum* N-4 and ER-20 showed about

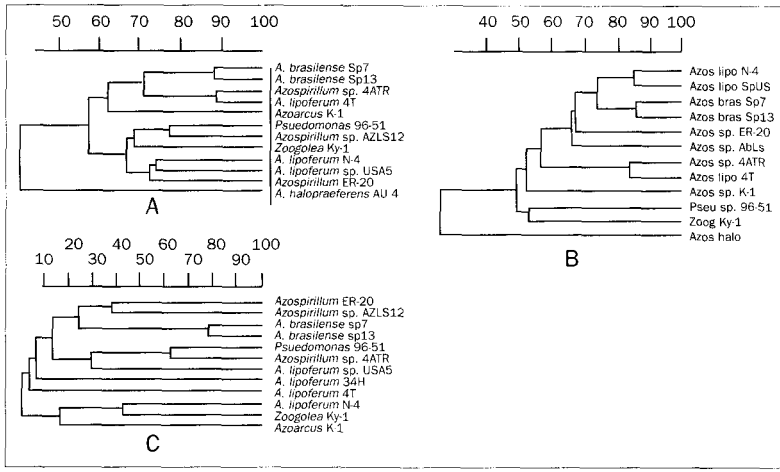

Fig. 3. (A) Dendrogram showing relationships between diazotrophic bacteria based on BOX-PCR fingerprints using product moment-correlations. (B) Dendrogram showing relationships between diazotrophic bacteria based on BOX-PCR fingerprints using Dice coefficients. (C) Dendrogram showing relationships between diazotrophic bacteria based on ERIC-PCR fingerprints using product moment-correlations.

74% similarity (Fig. 3A). When compared with the standard *Azospirillum* strains, the local isolates *Azospirillum* N-4 and ER-20 showed more similarity to *A. lipoferum* sp USAS, which was found to be 76% and 74%, respectively. Similar observations of 77% similarity have been reported between different *Rhizobium meliloti* strains when repetitive extragenic palindromic sequence (REP)-PCR-generated genomic fingerprints were compared (Rossbach et a1 1995). The remaining standard *Azospirillum* strains showed less than 60% similarity. Standard strain *A. halopraeferens* was not closely related with either our local isolates or with other standard *Azospirillum* strains. The same BOX-PCR patterns of the local isolates were also analyzed by using Dice coefficients (Fig. 3B). This analysis showed 67% similarity between *Azospirillum* N-4 and ER-20 strains. All the local and standard Azospirillum strains except one *(A. halopraeferens)* formed a cluster, which showed similarity values ranging from less than 60% to more than 85% among various strains. The analysis of ERIC-PCR banding patterns by using Pearson's correlations showed very low similarity values between local *Azospirillum* strains and between most standard *Azospirillum* strains (Fig. 3C). Similarity values of more than 70% were observed only between two *A. brasilense* strains—sp7 and sp13. Our results, especially when ERIC-PCR patterns were compared, support suggestions by Versalovic et a1 (1994) that these PCR-based methods are more important for differentiating among bacterial strains belonging to the same species than for studying relatedness at the species or genus level.

Beneficial effects of inoculations with nitrogen-fixing and plant growth hormone-producing bacteria on various plant growth parameters have been reported. In this study, four *Herbaspirillum* strains and one *Azospirillum* strain (N-4) isolated from rice, *Azourcus* K-1, *Zoogloea* Ky-1, and *Pseudomoms* 96-51 isolated from Kallar

Table 3. Effect of bacterial inoculations on rice variety Super Basmati.

Treatment	Root wt	Shoot wt (g plant^{-1})[a]	Grain wt
Control	3.2 b	17.8 b	8.3 c
Herbaspirillurn RS1	3.7 ab	24.3 ab	13.0 bc
Herbaspirillurn RS4	5.2 ab	25.7 ab	15.7 ab
Herbaspirillurn RR3	5.0 ab	25.7 ab	15.8 ab
Herbaspirillurn RR8	3.3 b	19.2 b	12.0 bc
Azoarcus K1	3.3 b	17.7 b	11.8 bc
Azospirillum lipoferum N-4	5.7 a	28.3 ab	15.0 ab
Azospirillum brasilense Wb3	4.7 ab	24.5 ab	14.5 b
Zoogloea Ky-1	5.2 ab	30.3 a	20.0 a
Pseudomonas 96-51	3.7 ab	20.0 ab	13.5 bc

[a] Average of 8 plants collected from 4 different pots. Means followed by the same letter are not statistically different at the 5% level.

grass, and *Azospirillum* wb3 isolated from wheat were used to inoculate plants of two rice varieties — Super Basmati and Basmati 385 — grown in unsterilized soil. All bacterial strains showed beneficial effects on root weight, shoot (straw) weight, and grain weight of Super Basmati (Table 3). Inoculation with *Azospirillum* N-4 isolated from rice resulted in the highest root weight, whereas Zoogloea Ky-1, an isolate from Kallar grass, proved to be the best strain as a higher increase in straw weight and grain weight was recorded in plants inoculated with this strain. These results clearly show that some nitrogen-fixing isolates from other hosts may show a better performance with rice than the isolates from the homologous host. The *Pseudomonas* strain used in this study as a nonfixer inoculant also resulted in increased plant biomass (root, shoot, and grain weight) over the control. As this strain is a phytohormone producer like all N_2-fixing strains included in this study, beneficial effects observed in this study may be due to combined effects of nitrogen fixation and growth hormone production by the inoculated strains. It has been reported in the literature that improved growth of plants inoculated with bacteria may be due to the effect of growth substances produced by inoculated strains (Tien et a1 1979, Umali-Garcia et a1 1980).

The response of Basmati 385 to bacterial inoculations was poorer than that observed in Super Basmati because the difference between the shoot weight and grain weight of inoculated and uninoculated plants was not statistically different. In Super Basmati, *Azospirillum* Wb3, an isolate from wheat, had the most beneficial effect on grain weight. The *Zoogloea* Ky-1 strain in this variety as well was the most promising strain as it showed a 20% increase in straw weight and 13% increase in grain weight over the control.

Quantification of nitrogen fixation was carried out by the ^{15}N isotopic dilution method in the two rice varieties. The effect of bacterial inoculation was more prominent in Super Basmati than in Basmati 385 (Table 4). In Super Basmati, Azospirillum N-4 and *Herbaspirillum* RR8 showed maximum fixation, where 58.9% and 58.2% Ndfa were recorded, respectively. In Basmati 385, *Herbaspirillum* strain RR8 was

Table 4. Quantification of nitrogen fixation (% Ndfa) in rice varieties Super Basmati and Basmati 385 inoculated with bacterial strains.

Treatment	Basmati 385[a]	Super Basmati
Herbaspirillum RS1	19.5 bc	38.1 ab
Herbaspirillum RS4	28.0 abc	51.6 a
Herbaspirillum RR3	28.5 ab	50.7 a
Herbaspirillum RR8	38.7 a	58.2 a
Azoarcus K-1	10.5 c	21.6 b
Azospirillum lipoferum N-4	20.0 bc	58.9 a
Azospirillum brasilense Wb-3	19.9 bc	47.1 a
Zoogloea Ky-1	22.3 abc	46.8 a
Pseudomonas 96-51	24.3 abc	44.0 ab

[a] Means followed by the same letter are not statistically different at the 5% level. The uninoculated control, used as a nonfixing reference for the estimation of ^{15}N dilution, has 0% Ndfa for both varieties.

the most efficient, where 38.7% Ndfa was observed. Thus, the performance of *Herbaspirillum* strain RR8 was excellent with both varieties. *Herbaspirillum* strains are expected to live endophytically in a protected, energy-rich environment and fix more nitrogen than diazotrophs (e.g., *Azospirillum*), which occur predominantly in the rhizosphere (James and Olivares 1998). For plants inoculated with a phytohormone producer, nonfixer *Pseudomonas* strain 96-51, Ndfa values of 44% and 24.3% were observed for Super Basmati and Basmati 385, respectively. This indicates stimulation of growth of diazotrophs or their nitrogen-fixing activity or enhanced root colonization when this growth hormone producer is used as inoculum. No prominent increase in root weight of plants inoculated with *Pseudomonas* 96-51 was observed, which suggested that a modification in root morphology, such as the formation of thin roots or more root hair resulting in more colonization by an indigenous diazotrophic bacterial population, might have occurred. Effects of plant growth hormones produced by inoculated strains on root growth and root morphology have been reported (Okon 1985, Tien et al 1979, Umali-Garcia et al 1980).

The results of this study suggest that genotypic differences among rice varieties Super Basmati and Basmati 385 exist as Super Basmati shows the best response to inoculated strains, which is reflected in an increase in plant biomass and high Ndfa values compared with those of the control obtained with this variety. From the performance of *Zoogloea* Ky-l, an isolate from Kallar grass, it can be concluded that in some cases the isolates from nonhomologous hosts may be even better than the inoculants obtained from the same host.

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Release of nitrogen: a key trait in selecting bacterial endophytes for agronomically useful nitrogen fixation

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A new method of screening for biological N_2 fixation (BNF) was tested with bacterial isolates from alfalfa and rice. Because agronomically useful BNF requires the transfer of bacterial N to the plant, the technique assessed N released from bacteria in N-free liquid medium using thin-layer chromatography (TLC). Many natural products with high N content (22–50% N) are lipophilic, and 7% of 21 mg N L^{-1} released by the alfalfa endophyte *Pantoea agglomerans* growing in N-free liquid medium was removed from the aqueous phase by C18 resin. Riboflavin was a minor lipophilic component (<0.1%), but it served as an easily detected indicator molecule because small amounts (10 pmol) fluoresced visibly on TLC plates. Tests with 66 putative endophytes from Chinese rice culms found that 38 formed pellicles in N-free medium, and 34 of those released riboflavin. Acetylene reduction was detected in 31 of the 34 isolates that released riboflavin. The C_2H_2-reducing activity and release of riboflavin were correlated significantly in the 38 pellicle-forming isolates (r^2 = 0.72, $P \leq 0.001$). Detailed studies of eight isolates showed that all released riboflavin during logarithmic growth in N-free medium and also later after increases in viable cell number ceased. Several endophytic isolates that reinfected rice had little effect on plant growth in the absence of combined N, but others impaired rice growth. Thus, riboflavin is an easily detected indicator of BNF in bacterial cultures, but beneficial interactions with plants must be confirmed.

Attempts to extend the benefits of biological N_2 fixation (BNF) to nonleguminous agronomic plants, such as rice, generally focus first on identifying plant-associated bacteria that contain nitrogen fixation *(nif)* genes and then on whether the bacteria reduce N_2 in the plant. The first step is often accomplished in part by testing for growth on an N-free medium (Dobereiner 1995, Kirchhof et al 1997). Acetylene-reducing activity, an indirect assay of BNF, is a useful, complementary technique for this purpose (Malik et al 1997), and polymerase chain reaction tests can detect the presence of *nif* genes (Stoltzfus et al 1997). Subsequently, BNF and agronomic effects of the selected bacterial isolate must be measured in plants growing in con-

trolled environments and field conditions (Malik et al 1997, Yanni et a1 1997). Unfortunately, major problems are often encountered between the initial demonstration of BNF in a bacterial isolate and final proof that the organism supplies reduced N to a host plant.

One of the most significant problems may lie in the fact that free-living N_2-fixing bacteria survive by retaining newly reduced N for their own growth (Silvester and Musgrave 1991). That situation contrasts sharply with the functioning of highly evolved microsymbionts in the Rhizobiaceae that excrete N_2-derived N after they differentiate into N_2-fixing bacteroids in legume root nodules (Bergersen and Turner 1967, Waters et a1 1998). Growing populations of N_2-fixing bacteria can produce an increase in total reduced N, but plants will benefit from the N only when it is released. Release may occur by death of the bacterial cells, but other products associated with cell death could cause a pathogenic response by the host. If bacterial growth is unrestricted, then they may become parasites on the plant. Despite these possible problems, N_2-fixing bacteria are found widely as plant endophytes (Barraquio et a1 1997, Triplett 1996). Thus, we are forced to consider how N_2-fixing endophytes function inside plants without causing these problems.

Successful N_2-fixing endophytic bacteria may use different strategies to avoid these problems. One strategy would be to repress their BNF potential inside the plant. Existing data, however, show clearly that some endophytes do reduce N, in the plant (Boddey et at 1991). Another possibility is that N_2-fixing endophytes may have mechanisms for transferring N_2-derived N from living cells to the plant. To explore the latter possibility, this project screened putative bacterial endophytes from alfalfa and rice for release of N_2-derived N. Because different species of endophytic bacteria may release various N-rich transfer molecules, we tried to identify an easily detected compound that could serve as an indicator of total N status and BNF.

In addition to BNF, endophytic bacteria may supply key regulatory factors or essential vitamins to plants (Fuentes-Ramirez et al 1993, Rodelas et a1 1993). It may be possible to identify beneficial bacteria by direct screening for release of such compounds. A newly identified mutualistic molecule released by *Sinorhizobium meliloti* cells in the rhizosphere and in culture is one such factor (Phillips et a1 1999a). This compound was recognized first as being capable of increasing root respiration (Volpin and Phillips 1998); then, because many Rhizobiaceae require exogenous CO_2 to grow (Lowe and Evans 1962), it was studied further in an effort to increase root colonization and promote development of the BNF symbiosis in legumes. The mutualistic nature of this factor, which was termed "compound D" for its chromatographic retention time, became evident when it was found that the increased root respiration was associated with an enhancement of net photosynthesis (Phillips et a1 1999b). When compound D was identified as lumichrome, a direct degradation product of riboflavin, its close relationship to the results described here was recognized. The metabolic relationship of lumichrome to riboflavin as well as the relatively high N content of the molecule justify its inclusion in this study.

Here we describe the development of a checkpoint for selecting agronomically useful BNF bacteria, which may serve as an alternative to current indirect tests for

nitrogenase activity. This step involves assessing the release of N-rich compounds in an N-free medium. The technique was developed using an endophytic bacterium isolated from alfalfa, and then the methods were applied to bacteria isolated from rice growing in California (USA) and China.

Materials and methods

Isolation, culture, and identification of bacteria

One bacterial isolate, alfalfa endophyte AEl2, was obtained from internal tissues of Moapa 69 alfalfa (*Medicago sativa* L.) roots on seedlings growing under controlled conditions. Other bacteria were isolated from vigorously growing wetland rice (*Oryza sativa* L.) in marginal agricultural fields located in northern California and China.

Putative alfalfa endophyte AE12 was recovered from inside a root segment after sonicating the root for 3 min in water, treating it for 3 min in 1% chloramine T, and washing it three times in sterile distilled water. The alfalfa seedling from which the root was collected had developed from a seed that had been surface-sterilized for 60 min with 5% $NaHClO_3$. Endophytic bacteria were collected from field-grown rice culms excised at various stages from young seedlings to postanthesis. Stems were cut into 8-cm segments, immersed for 1 h in 1% chloramine T, and rinsed once in 0.1% Tween 20 and three times in sterile distilled water. Discolored ends of the culms were removed aseptically, and then the segments were centrifuged sterilely at 8,000 g for 20 min to produce droplets of apoplastic solution. Aliquots (100 µL) of the apoplastic solution were plated on half-strength TSA medium (1.5% tryptic soy broth and 1.5% agar) supplemented with nystatin (100 mg mL^{-1}) to inhibit fungi. The efficacy of sterilization and isolation was verified by coating culm segments with cells of S. *meliloti* 1021 before sterilization and checking for their absence by known antibiotic resistance markers after sterilization. Rhizoplane isolates were collected from rice seedling roots by vortexing and sonicating root segments in 0.1 % Tween 20 before plating serial dilutions on TSA with nystatin (100 mg mL^{-1}).

Bacterial isolate AE12 was determined to be a true endophyte of alfalfa by its capacity to reinfect sterile seedlings. Test plants were grown from surface-sterilized seeds. The AE12 isolate used for the test was selected for intrinsic resistance to ampicillin (50 mg L^{-1}) and was identified by that marker and morphological traits after reisolation.

Bacterial isolates were identified by amplifying the 16S-rRNA gene with primers fD1 and rD1 (Wang et al 1999). Nucleotide sequences were obtained automatically (ABI377, Applied Biosystems, Foster City, CA) and compared with standard databases by BLAST software (NCBI, USNIH).

Analytical procedures

To select bacteria for potential BNF, isolates were cultured in a semisolid BNF test medium containing the following major components (g L^{-1})–K_2HPO_4 (1.0), KH_2PO_4 (3.0), $MgSO_4$ (0.065), $FeCl_3$•6 H_2O (0.01), $CaCl_2$•2 H_2O (0.07), and dextrose (5.0)– and minor components (µg L^{-1})–Na_2MoO_4•2 H_2O (240), H_3BO_4 (3,000), $MnSO_4$•H_2O

(1,830), $ZnSO_4$•7 H_2O (290), $CuSO_4$•5 H_2O (130), and $CoCl_2$•6 H_2O (120). Initial experiments to determine pellicle-forming capacity used a semisolid medium containing 1.8 g L^{-1} agarose type II (Sigma, St. Louis, MO) to slow O_2 diffusion and facilitate N_2 fixation. Subsequent experiments used BNF liquid medium without agarose.

Aqueous supernatant remaining after bacteria had been removed by centrifugation was passed through C18 resin (Sep-Pak cartridges, Waters, Milford, MA) after activation according to the manufacturer's instructions. Solutes that were not retained on the cartridge were referred to as hydrophilic eluent, while those that eluted subsequently in methanol were referred to as the lipophilic fraction. TLC on silica-gel-coated glass plates (0.2-mm thick, 100-mm wide, and 50- or 100-mm long) (HPTLC Silica Gel 60, Alltech, Deerfield, IL) separated compounds in the lipophilic fraction using either of two solvent systems: I, chloroform:methanol:water (v:v), 17.5:12.5:1.5, or II, l-butanol:2-propanol:water (v:v), 10:20:2.5. After drying, the TLC plates were viewed in UV light using either a hand-held, 4-W, 366-nm lamp (model UVL-21, UVP Inc., San Gabriel. CA) or a UV-light box (model T1202, Sigma). Photographs were made using either black-and-white (Polaroid 667 3000iso) or color (Fujicolor Superia ISO 400) film. N analyses were performed by microkjeldahl (Colombo and Giazzi 1979). Visual estimates of riboflavin on TLC plates were calibrated by eluting samples and measuring absorbance relative to standards in a spectrophotometer (Lambda 6, Perkin-Elmer, Norwalk, CT).

Acetylene-dependent ethylene production. that is, C_2H_2 reduction, was measured as an indirect assay for nitrogenase activity (Bethlanfalvay and Phillips 1977) in bacteria growing in 3 mL of semisolid BNF test medium in a 5-mL vial. Replicate cultures were used for measurements made at various growth stages.

Plant tests

Seeds of California rice cultivar M202 or Chinese rice cultivar Hua Gui, generously supplied by Dr. D.J. Mackill, were dehulled, rinsed for 1 min in 70% ethanol, and sterilized in 5% $NaHClO_3$ (15 min for Hua Gui, 30 min for M202) before being germinated for 24 h on TY agar medium (Beringer 1974). Seeds judged to be externally sterile after 24 h on agar were transferred to sterile, 50-mL Erlenmeyer flasks containing sterile vermiculite and covered with clear plastic wrap after delivering 25 mL of sterile plant nutrient solution (De Jong and Phillips 1981) with or without 0.5 mM NH_4NO_3 into the vermiculite. Bacteria for inoculation tests were grown in TY liquid medium, removed by centrifugation, and rinsed with water before being suspended in the plant nutrient solution and inoculated into the flask (10^6 cfu $flask^{-1}$). All plants were grown in a controlled environment chamber with 250 mmol photosynthetically active radiation m^{-2} s^{-1} in a 12/12 h, 25/20 °C day/night cycle.

Database searches

Potential N-transfer molecules were obtained from the Beilstein chemical database using Beilstein CrossFire Minerva version 3.1 at http://www.library.wisc.edu:4001.

Results

Development of the released-N assay

The putative endophyte AE12 isolated from alfalfa was identified as Pantoea *agglomerans* by nucleotide sequence analysis of the 16s-rRNA gene. The DNA identity of AE 12 with the reference strain *P. agglomerans* A29 (GenBank AFl30907) was more than 99.8% across the entire length of the amplified fragment. This strain reinfected alfalfa roots under microbiologically controlled conditions and thus qualified as an actual endophyte. Its good growth in the BNF liquid medium (Fig. 1) suggested that it had N_2-fixing activity. Analyses of the culture supernatant after logarithmic growth ceased indicated that approximately 7% of the N released was retained on C 18 resin (Table 1). Riboflavin in that fraction accounted for less than 0.1 % of the total extracellular N (Table 1). Such low levels of riboflavin were clearly vis-

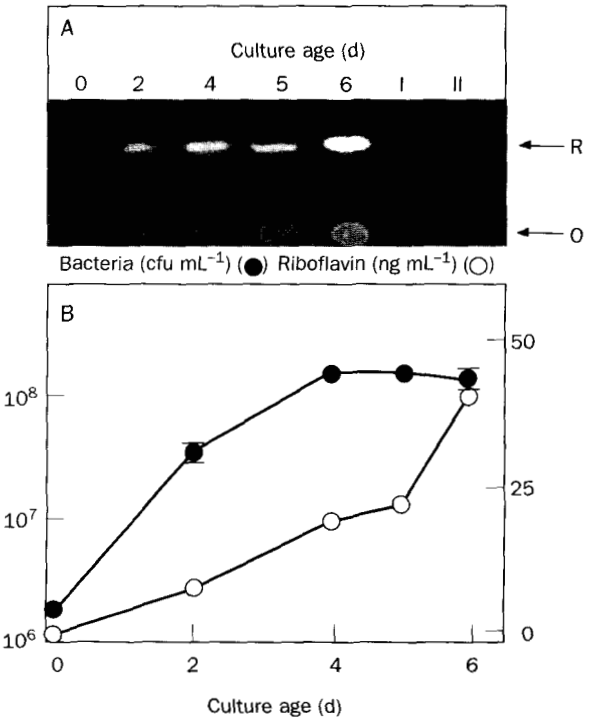

Fig. 1. Growth and riboflavin release by the alfalfa endophyte *Pantoea agglomerans* AE12 in liquid N-free medium. (A) Thin-layer chromatography (TLC) analysis in solvent system II of lipophilic components from the supernatant of a 2-mL culture. 0 = origin, R = riboflavin. Riboflavin standards: I, 3.8 ng; II, 5.0 ng. The TLC plate was photographed in ultraviolet light. (B) Viable cell counts and riboflavin released.

Table 1. Nitrogen fractions released by *Pantoea aggromerans* AE12, an alfalfa endophyte. Culture supernatant was analyzed after logarithmic growth in N-free medium ceased. N content of the lipophilic fraction that was retained on C18 resin and the hydrophilic eluent that passed through the C18 were determined by microkjeldahl analysis. Riboflavin, one component of the lipophilic fraction, was quantified by thin-layer chromatography.

Fraction	N content	
	(mg L^{-1})	(% of total)
Hydrophilic eluent	19.6	93
Lipophilic components	1.5	7
Total	21.1	100
Riboflavin	0.02	< 0.1

ible on TLC plates (Fig. 1A). Indeed, minimum levels for reproducible visual detection of riboflavin in this system were the 10 and 13 pmol standards in lanes I and II, respectively.

Numerous N-rich molecules that might be present in the lipophilic fraction were located in the Beilstein chemical database. Approximately 2,400 compounds that satisfied three criteria (isolation as a natural product, molecular weight < 400, and % N $\geq$ 13) were examined. Eleven common, N-rich lipophilic molecules, in addition to six compounds previously reported as N-transfer or N-transport molecules in plant-microbe systems, are listed in Table 2. Two other lipophilic amino acids, phenylalanine (8.5% N) and tyrosine (7.7% N), are not listed in Table 2 because they contain considerably less N than tryptophan. Many compounds in the Beilstein database were ignored because they have not been reported in bacteria. One characteristic of many compounds in Table 2 is that they absorb UV light. Thus, they can be monitored easily when they are present in high concentrations. Riboflavin has the additional advantage that its fluorescence allows it to be detected in low amounts (Fig. I A). This latter fact was used to screen bacterial isolates from rice.

Application of the released-N assay

Of 200 original isolates from rice roots and culms, 98 grew in the semisolid BNF test medium. Detailed studies with 66 isolates collected from rice culms in China showed that 38 formed pellicles in the BNF liquid medium, and 31 of these reduced acetylene. All 3 l pellicle-forming, acetylene-reducing isolates also released riboflavin. Three other pellicle-forming isolates also released riboflavin, but no riboflavin was detected in culture supernatants taken from the 32 non-pellicle-forming isolates tested. Thus, the very low levels of riboflavin detected in some cultures were not associated with death of the initial inoculum. The C_2H_2-reducing activity and release of riboflavin were correlated significantly in the 38 pellicle-forming isolates ($r^2 = 0.72$, P £ 0.001).

Table 2. Common N-rich molecules in plant-microbe systems.

Compound	Composition	Percent N	Category[a]
Ammonia	NH_3	82.3	A
Adenine	$C_5H_5N_5$	51.8	B
Guanine	$C_5H_5N_5O$	46.3	B
Cytosine	$C_4H_5N_3O$	37.8	B
Allantoin	$C_4H_6N_4O_3$	35.4	A
Isopentenyladenine	$C_{10}H_{13}N_5$	34.4	B
Zeatin	$C_{10}H_{13}N_5O$	31.9	B
Allantoic acid	$C_4H_8N_4O_4$	31.8	A
Histidine	$C_6H_9N_3O_2$	27.1	B
Uracil	$C_4H_4N_2O_2$	25.0	B
Lumichrome	$C_{12}H_{10}N_4O_2$	23.1	B, C
Thymine	$C_5H_6N_2O_2$	22.2	B
Asparagine	$C_4H_8N_2O_3$	21.2	A
Glutamine	$C_5H_{10}N_2O_3$	19.2	A
Alanine	$C_3H_7NO_2$	15.7	A
Riboflavin	$C_{17}H_{20}N_4O_6$	14.9	B, C
Tryptophan	$C_{11}H_{12}N_2O_2$	13.7	B

[a]A = previously reported N-transfer or N-transport molecules; B = lipophilic compounds; C = fluorescent compounds.

Twenty-four isolates with C_2H_2-reducing activity were separated into five groups according to colony color and morphology (Table 3). All 24 isolates released riboflavin during logarithmic growth, and larger amounts were released after logarithmic growth ceased. All but two of the 24 isolates exhibited C_2H_2-reducing activity in cells after logarithmic growth ceased, and most cultures showed no major decline in the number of colony-forming units during the period of maximum riboflavin release and C_2H_2 reduction. Based on its fluorescence and chromatographic mobility, lumichrome was also judged to be present in culture supernatants from all of these 24 isolates. Because its fluorescence was less intense than that of riboflavin, however, no attempt was made to estimate the amount of lumichrome in these cultures.

Eight isolates, including representatives from each of the five major morphological groups of bacteria isolated in this study (Table 3), were selected for more detailed tests. The eight isolates released a variety of UV-absorbing and fluorescing compounds into the culture medium, and, in every case, some riboflavin was detected on the TLC plate. Visual estimates of riboflavin quantities were quite reproducible and could be verified by eluting riboflavin from the TLC plate and measuring absorbance with a spectrophotometer. Four of the isolates, including the highest and lowest riboflavin producers, offered examples of the variety of compounds that can be detected with this simple method (Fig. 2). When these eight isolates were tested for growth effects on rice seedlings in the absence of combined N, there was little effect on plant growth. In the presence of combined N, however, there were strong interactions between individual bacterial strains and the form of combined N, which are being studied further. Several isolates clearly reduced rice growth relative to

Table 3. Cultural traits of putative endophytic bacteria isolated from field-grown rice culms in China. All isolates showed C_2H_2-reducing activity during logarithmic growth in N-free medium, and that activity continued after logarithmic growth ceased in all but two isolates. Riboflavin was detected in all cultures during logarithmic growth, and it was released in larger amounts after logarithmic growth ceased, even though most cultures showed no major decline in viable cell number.

Group	Isolates (no.)	Colony color	Acetylene reduction[a] (nmol culture^{-1} h^{-1})	Riboflavin released
A	8	Creamy	2.5–5.0	All
B	7	Yellow	0–2.5	All
C	6	White	0–0.5	All
D	2	Orange	0.5–2.5	All
E	1	Black	0.5	All

[a]Determined in 3-mL cultures after logarithmic growth ceased.

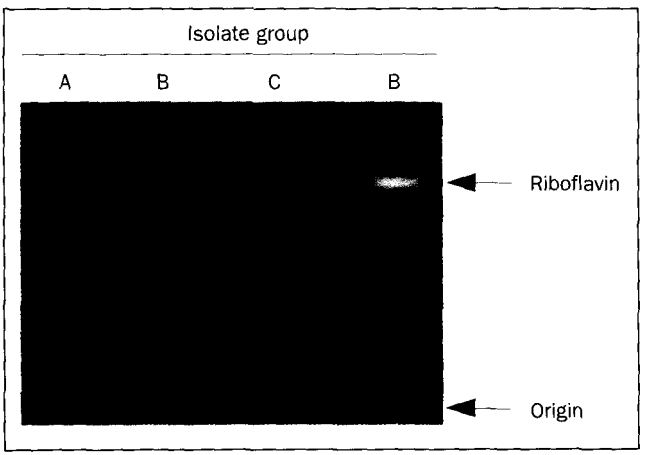

Fig. 2. Representative thin-layer chromatography analyses of four rice endophytes from three groups in Table 3. Samples were separated in solvent system I. Riboflavin was visible under ultraviolet light in original chromatograms from all samples, including the isolate from group C shown here. Products of isolates within any one group, such as the two different bacteria from group B shown here, often differed greatly.

uninoculated controls. Other experiments established that several isolates tested for reinfection of rice seedlings were true endophytes (E. Martinez-Romero, in preparation).

Discussion

Results from this study show that endophytic bacteria release lipophilic N-rich compounds from populations of N_2-fixing cells that maintain a relatively constant number of total cells (Fig. 1). Nearly all isolates in this study continued to reduce C_2H_2 and to release riboflavin for several days after increases in cell number ceased. The factor limiting growth in these cultures may have been oxygen or some quorum-sensing signal, such as a homoserine lactone (Huisman and Kolter 1994). How the plant might stimulate BNF either by limiting oxygen availability or by inducing *nif* genes is unknown. It is evident, however, that at least one crop plant, sugarcane, has evolved a mechanism that results in BNF (Boddey et al 1991).

One sensitive measure of a positive N status and continued BNF in bacterial cells examined here was the release of riboflavin (Fig. 1A, Table 3). While riboflavin has a moderately high N content (Table 2), it must be viewed as only an indicator of N status, not as a major N-transfer molecule (Table 1). In this role, riboflavin offers several advantages in screening for BNF capacity of unknown bacterial isolates. First, its fluorescence on TLC plates allows easy detection in small cultures (Figs. 1A, 2). Second, unlike many compounds in Table 2, riboflavin is not released as a major breakdown product of most proteins or nucleic acids during cell death. Assays for riboflavin production will not replace the primary initial test for BNF, which is growth on an N-free medium (Table 4). Riboflavin assays, however, may help identify cultures with particularly high amounts of reduced N. For this reason, riboflavin measurements may be preferred over microkjeldahl determinations and may be useful for some investigators as an alternative to other indirect BNF assays, such as C_2H_2 reduction or PCR tests for *nif* genes, when large numbers of isolates are being screened. The only significant disadvantage in working with riboflavin is that it degrades in light (Yagi 1956). This problem can be surmounted by working in a reproducibly subdued light environment and by covering samples with aluminum foil at all times.

As more facts about plant-microbe interactions become known, it seems likely that microbiologists will screen endophytic isolates for production of other molecules that enhance plant productivity. For this reason, we view TLC analyses as potentially helpful techniques for detecting the presence of cytokinins, lumichrome, and tryptophan, a precursor of indoleacetic acid (Table 2). Some other factors, such as vitamin B_{12} (14.5% N), can be observed in TLC analyses of some bacterial cultures without any staining, and amino acids, such as tryptophan and histidine, can be detected easily after ninhydrin treatments.

A notable portion of the N-containing compounds released by endophytic cells in this study were lipophilic, rather than hydrophilic, as one would predict if ammonia or certain amino acids were the only N-transfer molecules (Table 1). In most cases, the lipophilic fraction contained ninhydrin-positive materials, but direct ex-

Table 4. Checkpoints for selecting agronomically useful N_2-fixing bacteria.

Step	Protocol	Potential problems
1. Plant collection	Find vigorous plants growing in low N soil.	Localized N sources stimulate plant growth without BNF.
2. Isolation of bacteria	a. Sterilize stem or leaf; collect internal cells	a. Surface contaminants or pathogens may be isolated.
	b. Recover bacteria from the root surface or cortex.	b. Nonspecific soil bacteria or pathogens may be isolated.
3. Growth of bacteria	Supply complete medium.	Mutualists requiring specific plant products may not grow.
4. Primary BNF test	Grow in N-free medium.	N-scavenging bacteria grow.
5. a. Current secondary BNF tests	a. Assay nitrogenase activity.	a. Acetylene is explosive.
	b. Amplify *nif* sequences.	b. Alternative or novel *nif* genes may be present.
b. Alternative secondary BNF tests	Measure riboflavin released in N-free medium.	Riboflavin degrades in light.
6. Establish an association	Show bacteria colonize roots or reinfect the plant.	Nonspecific bacteria may have been isolated originally.
7. Plant tests	Measure N_2 inputs to plant.	Technical issues can limit accuracy of BNF estimates.

traction of bacterial cells to examine internal pools of soluble lipophilic molecules detected much larger amounts of comparable materials. The problems involved in determining whether N-containing molecules in bacterial cultures reflect BNF or mineralization of cellular contents were thoroughly documented in an early review on the origin and significance of ammonia formed by *Azotobacter* cultures (Burk and Horner 1936). Identification of the exact lipophilic molecules released from any particular bacterial isolate will require detailed analytical studies. The compounds in Table 2 offer a list of possibilities, but some bacteria may produce other specialized molecules.

Most N-transfer molecules reported as carriers of N_2-derived N in biological systetms are hydrophilic (Table 2), and thus do not account for all of the N released by *P. agglomerans* AE12 (Table 1). For example, symbiotic cyanobacteria release about 40% of N_2-derived N as ammonium (Peters and Meeks 1989), and one early study concluded that ammonia was released by N_2-fixing *Bradyrhizobium* bacteroids (Bergersen and Turner 1967). More recent data, however, report that alanine is a major carrier of N_2-derived N for N_2-fixing *Bradyrhizobium* bacteroids (Waters et al 1998). Legumes metabolize N_2-derived N from rhizobia into the amides, asparagine and glutamine, or the ureides, allantoin and allantoic acid, for transport to the shoot (Atkins 1991). Bacteria are very good at synthesizing two groups of strongly lipophilic N-rich compounds that are not normally viewed as N-transfer molecules: purines (38–52%N) and pyrimidines (21–25%N). Whether purines or pyrimidines are released by N_2-fixing endophytes is unknown but remains as one possibility.

Another approach to measuring BNF-dependent release of N has been described for *Acetobacter diazotrophicus* (Cojho et al 1993). In that study, *A. diazotrophicus* was cultured with the yeast *Lipomyces kononenkoae* on N-free medium, and yeast growth was related to BNF. In addition, however. other strong interactions between the organisms were observed. The existence of those undefined interactions may complicate the screening of unknown bacteria for release of N in such a coculture system. Direct measurement of N-rich molecules, such as riboflavin, in pure cultures of unknown BNF bacteria is simpler and avoids that potential problem.

E agglomerans apparently is a widespread plant endophyte. It was isolated previously as a dominant endophyte in red clover (*Trifolium prartense* L.) growing in eastern Canadian soils (Sturz et al 1997), and it was identified here in alfalfa seedlings developing under sterile conditions from seeds produced in California. Under an earlier nomenclature, this microorganism was studied as *Ewinia herbicola,* a persistent endophyte in alfalfa (Handelsman and Brill 1985). Isolate *P. agglomerans* AE12 showed only slight promotive effects on the growth of *Sinorhizobium* -free alfalfa, but the seedlings were visibly greener than uninoculated controls (data not shown).

It is doubtful that riboflavin measurements alone will locate superior endophytes that transfer N_2-derived N to rice or other plants. Subsequent tests for plant infectivity and growth are always necessary (Table 4). In our hands, however, assays for riboflavin production have served as rapid, reproducible tests for locating superior BNF capacity in endophytic bacteria (E. Martinez-Romero, in preparation). One isolate chosen for riboflavin production in an N-free medium contained *nif* genes according to subsequent PCR tests and was identified by 16S-rDNA sequence as being in the rRNA superfamily V, referred to as the *Cytophaga-Flavobacterium-Bacteroides,* a group not previously known to have BNF bacteria (Young 1991).

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Critical parameters in facilitating the evolution of N_2-fixing symbiosis between diazotrophs and cereals

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The key features of an experimental model for achieving significant biological N_2 fixation by associations between *Azospirillum* and cereals are suggested to include adequate colonization, endophytic in nature to ensure both access to carbon substrates and suitable microaerobic oxygen conditions, and a means of ensuring adequate transfer of newly fixed nitrogento the host plant. In our research program, we have exploited the property of synthetic plant hormones such as 2,4-dichlorophenoxyacetic acid to enhance access of azospirilla to protected niches such as the base of modified lateral roots (*para*-nodules) and channels between cortical cells. This approach has demonstrated the possibility of achieving such colonization. By using *nifH-lacZ* fusions of *A. brasilense* strains, we have been able to show a relationship between *nifH* expression, oxygen pressure, and the magnitude of acetylene reduction rates in such associations. In addition, the significance of the *flcA* (controlling flocculation) gene in effective colonization by regulating the expression of exopolysaccharides and the conversion of vegetative cells of azospirilla to cysts has been shown by the use of flcA$^-$ mutants.

We have studied the membrane potential and permeability properties of *A. brasilense* Sp7-S with a view to establishing the mechanism of retaining ammonia in free-living cells and the possibility of excretion in association with plants. Using an ammonia-excreting mutant (HM53) with adequate carbon substrate supplied as malate, the transfer of substantial quantities of newly fixed $^{15}N_2$ from the diazotroph to model wheat seedlings, adequate to support significant growth, has been shown. Furthermore, a Tn5-site-directed *flcA*$^-$ mutant of HM53 supported solely by photosynthate from wheat seedlings grown in sand culture in air was able to accumulate significantly more dry weight in the roots and shoots than the ammonia-excreting mutant alone, We suggest that this result vindicates continuing our stepwise approach to achieving a genotype × environment interaction that may eventually provide a symbiosis between *Azospirillum* and cereals such as wheat and rice.

Achieving a rate of biological nitrogen fixation that can directly contribute to the growth of wheat crops is a significant challenge, just as it is with rice crops. Such an ambitious goal can be predicated on either of two assumptions. First, that there are already isolated cases of significant nitrogen fixation in symbioses between existing cultivars of wheat and strains of diazotrophs; therefore. the research task involves no more than finding these successful candidates and then applying them under suitable field conditions. Second, that there may be no such natural symbiotic systems and it will therefore be necessary to engineer or genetically evolve the new system that is required. Such a process would need major improvements in the current associative systems (Day and Döbereiner 1976), which appear to proceed at rates much too low to contribute more than a small fraction of the crop plant's nitrogen requirements (Baldani et al 1983, Bashan and Levanony 1990, Okon and Labandera-Gonzalez 1994).

In our viewpoint, this requirement for innovation is the more likely scenario and the likelihood of finding one or more natural systems adequate for crop production is extremely small. A further feature of this task is that, since the need can now be considered as urgent because of world population trends and environmental concerns regarding nitrogen pollution from fertilizer nitrogen (Kennedy and Cocking 1997), only bold approaches are likely to prove adequate. Nevertheless, the study of natural systems such as those involving bacteria such as *Azoarcus* in Kallar grass (Reinhold-Hurek and Hurek 1998) and *Herbaspirillum seropedicae* and *Acetobacter diazotrophicus* in sugarcane (Boddey et al 1995) is likely to provide the essential clues allowing new systems with rice and wheat to be achieved.

We have also suggested (Kennedy and Tchan 1992) that an adequate system may be achieved sooner by exploiting the ability of some diazotrophs to colonize the roots of grasses and by using a combination of genetic and cultural improvements of these associations that will allow adequate nitrogen fixation and transfer to the plant to occur. It is concluded in this approach that this goal would be more readily achieved than any attempt to genetically engineer the wheat or rice plant because of the greater complexity involved in completely transferring the nitrogen-fixing genetic apparatus to the plant genome and expressing it there.

The significance of the form of colonization of wheat roots by *Azospirillum* has been well documented elsewhere (Tchan et al 1991, Kennedy and Tchan 1992, Kennedy et al 1997). The quantitative measurement of the pattern of colonization by using reporter gene (*lacZ*) fusion techniques has now allowed the recognition of important features in diazotroph:wheat associations. It has been reported that *A. brasilense* Sp7-S, a spontaneous mutant of wild-type Sp7 that we found several years ago, had reduced exopolysaccharide production with consequent impaired flocculation, and performed better in associating with the *para*-nodule (Katupitiya et al 1995). We have since refined our knowledge of this relationship between exopolysaccharide production and the pattern of colonization while isolating the *flcA* gene (Pereg-Gerk et al 1998) and have developed genetic tools allowing the preparation of mutants with an altered colonization pattern (L. Pereg-Gerk, K. Gilchrist, and I.R. Kennedy, in preparation).

This observation regarding variation in flocculation was coupled with the recognition of an altered colonization pattern in para-nodulated wheat roots. This colonization pattern typified by localization around *para* -nodules and less colonization of the root epidermal surface, the main site colonized by wild-type *A. brasilense* Sp7, is predicted to have significant advantages with respect to both carbon or energy supply and improved protection from atmospheric oxygen. It has been possible to examine the relationship between colonization pattern, oxygen concentration, and nitrogenase expression of *nifH-lacZ* as a reporter (see also Vande Broek et a1 1993, Kennedy et a1 1997).

A potential drawback of associations between nonlegume crops and diazotrophs is that the bacteria are expected to use a major portion of their fixed nitrogen for their own metabolism and growth (Kleiner 1985). This leaves little if any nitrogen to directly support plant growth and perhaps only then on the catabolic breakdown of the bacterial cells. To solve this problem, several scientists have made efforts to identify and isolate bacterial mutants that excrete ammonia for absorption by plants (Pedrosa et a1 1989, Christiansen-Weniger and Van Veen 1991). Wood and Kennedy (1996) also reported the existence of a stable ammonia-excreting mutant of Sp7-S (Sp7-SA). An initial trial with this ammonia-excreting mutant in McCartney bottles showed a significant transfer of newly fixed N after 72 h of exposure to $^{15}N_2$ (Kennedy et al 1997); however, there was only a small transfer of newly fixed nitrogen to the tops of the wheat plant. Using this laboratory model, preliminary information regarding the association and conditions for its improvement with respect to bacterial genotype and the potential role of ammonia excretion in wheat roots has been achieved.

It must be stressed, however, that a significant multifactorial improvement is essential before a significant functionality in the system can be claimed. Indeed, although the significance of factors such as colonization pattern, supply of carbon substrates, optimal oxygen, and effective ammonia transport may be established separately, an adequate solution to the problem may appear if all these requirements can be satisfied at once. Furthermore, sustaining a successful technology under farming conditions will be far more demanding, so that a substantial process of further optimization of such diazotroph-crop plant associations has been considered as essential.

In this chapter, we describe our experiments designed to test the hypothesis that genetic modification of colonization pattern can affect the rate of nitrogenase activity in para-nodules by influencing factors such as oxygen concentration. Furthermore, by introducing the property of ammonia excretion together with this genetic modification *(flcA-)* of colonization pattern, increases in the rate of dry weight accumulation in wheat seedlings can be achieved. This integrated review of our recent research in this area is designed to illustrate our approach to the design of a possible symbiosis between azospirilla or similar diazotrophs and cereals.

Methods

Bacterial strains, plasmids, and media

The bacterial strains and plasmids (Pereg-Gerk 1997) used in this work are listed in Table 1. The complete medium was nutrient broth (Difco) for *A. brasilense* and Luria-Bertani (LB) for *Escherichia coli.* Otherwise, *Azospirillum* strains were grown on minimal lactate medium (Galimand et al 1989) or nitrogen-free malate medium (Nfb, Sriskandarajah et a1 1993) supplemented with 40 μg mL^{-1} Congo red. Antibiotic concentrations used for *Azospirillum* were tetracycline, 5 μg mL^{-1}, and kanamycin, 20 μg mL^{-1}.

Examination of flocculation

Flocculation in minimal medium in the presence of 8 mM fructose and 0.5 mM KNO_3 (flocculation medium) was examined (L. Pereg-Gerk et al, unpublished) using a modi-

Table 1. Bacterial strains and plasmids used in this work.

Strain or plasmid	Genotype or phenotype[a]	Reference
Escherichia coli		
S17-1	*pro, thi, hsdR, recA, Tra*$^+$*, IncP*	Simon et al (1983)
Azospirillum brasilense		
SP7	Wild type	Tarrand et al (1978)
Sp7-S	Spontaneous mutant of Sp7, CR$^-$, Floc$^-$	Katupitiya et al (1995)
Sp245	Wild type	Baidani et al (1983)
Sp7-PM23	Spontaneous mutant of Sp7, CR$^+$, Floc$^+$, Swarm$^-$	Pereg-Gerk (1997)
Sp7-PM35	Spontaneous mutant of Sp7, CR$^\pm$, Floc$^-$, Swarm$^+$	Pereg-Gerk (1997)
Sp245-M1-M60	Spontaneous mutants of Sp245, CR$^-$, Floc$^-$,	Pereg-Gerk (1997)
Sp72001/4	*flcA*$^-$, Tn5-induced mutants of Sp7, CR$^-$, Floc$^-$ Swarm+	Pereg-Gerk et al (1998)
FP2	Wild type	Pedrosa et al (1989)
HM53	Ammonia-excreting mutant of FP2	Pedrosa et al (1989)
HM53.1	*flcA*$^-$, Tn5-induced mutant of HM53	McFadden (1998)
Plasmids		
pAB1220-9	pVK100[b] with *flcA* on 9-kb *Hind*III fragment, Tc^r	Katupitiya et al (1995)
pAB2001	pAB2000 containing *flcA* with a Tn5 Insertion	
pAB2051[c]	pLA29.17 derivative[b] containing *flcA* on a 1.1-kb *Pst*I fragment, Tc^r	Pereg-Gerket al (1998) Pereg-Gerk et al (1998)
pAB2053[c]	pLA29.17 derivative containing *flcA* on a 4.6-kb *BamHI-Bgl*II fragment, Tc^r	Pereg-Gerk et al (1998)
pAB2053Z	*lacZ* cartridge cloned into pAB2053, Tc^r, Km^r, lac$^+$	Pereg-Gerk et al (1998)
pLA-lacZ	pLA29.17 derivative[b], *lacZ* constitutive fusion, Tc^r, Km^r, Lac$^+$	Arsène et al (1994)
pAB358	pVK100 derivative, *nifH-lacZ* fusion	Liang et al (1991)

[a]Km^r and Tc^r Indicate resistance to kanamycin and tetracycline, respectively; kb = kilobase, CR = Congo red binding, Floc = flocculation, Swarm = swarming ability, lac = /acZ. [b]pVK100 and pLA29.17 are low-copy, broad-host-range cloning vectors, derivatives of RH2 that are stable in *Azospirillum* strains. [c]In pAB2051, a truncate form of *flcA* is cloned under a constitutive promoter, while in pAB2053 *flcA* is cloned under its own promoter.

fication of the procedure outlined by Sadasivan and Neyra (1987). The inoculum was harvested from a log-phase culture (2 mL) grown in nutrient broth (NB) by centrifugation at 5,000 rpm for 10 min at room temperature. The pellet was washed with minimal medium and inoculated into the flocculation medium to an absorbance of 0.3–0.4at 600 nm. Experiments were conducted in 50-mL flasks containing 10 mL of flocculation medium. which were incubated at 30 °C on a shaker at 200 rpm.

Mutagenesis of *Azospirillum* strains

Spontaneous mutant strains of *Azospirillum brarsilernse* Sp7 and Sp245 impaired in flocculation were isolated (Pereg-Gerk 1997) from the supernatant of flocculated cultures. The number of cells per mL of supernatant was estimated using a counting slide with an Olympus BHA light microscope. The supernatant was diluted to a final concentration of 2,000–3,000bacteria per mL. Because in previous cases the ability to flocculate was positively correlated with the ability to bind Congo red, nonflocculating mutants were selected on minimal lactate agar plates containing 40 μg mL^{-1} Congo red. Fractions of 100 μL of the diluted supernatant were spread on each plate of the selective medium and were then incubated over 2–3 nights at 37 °C. Colonies that failed to bind Congo red and appeared white or light pink were tested further for flocculation. The frequency of the mutation was calculated and the stability of the mutation was estimated following several reisolations.

The floc- phenotype of *A. brarsilense* HM53 was prepared (D. McFadden and I.R. Kennedy 1998, unpublished) by TnS-induced mutagenesis (Pereg-Gerk et al 1998), yielding HM53.1.

Complementation analysis

Complementation of several mutants was examined (Pereg-Gerk 1997) with plasmid clones pAB20S1 and pAB20S3 containing the *flcA* gene of strain Sp7, which complements flocculation and Congo red binding in strain Sp7-S (Pereg-Gerk et al 1998). Transfer of the plasmids into the *Azospirillum* recipient was performed by conjugation using *E. coli* S17-l as a donor. Transconjugants were selected on a minimal lactate medium containing 20 mM ammonia, 5 μg mL^{-1} tetracycline, and, in some cases, 20 μg mL^{-1} kanamycin (Galimand et al 1989).

Plant assays

Wheat *(Triticum aestivum)* cultivars Miskle. Sunbri, and Sunelg, obtained from the Plant Breeding Institute, Narrabri, were grown in hydroponic growth solution under sterile conditions as described by Zeman et al (1992). Seeds were inoculated with bacteria containing a *lacZ* fusion prepared as described by Arséne et al (1994). In some cases, 2,4-dichlorophenoxyacetic acid (2,4-D) was added at the time of inoculation to a final concentration of 0.7 ppm. Ten days after inoculation, the plants were assayed for nitrogenase activity (ARA) and were visualized by staining for β-galactosidase activity (*in situ* X-Gal staining of bacteria) (Katupitiya et al 1995) or examined under the scanning electron microscope (SEM).

Detection of b-galactosidase activity in wheat roots inoculated with *Azospirillum*

The colonization pattern, which was investigated by in situ staining of inoculated roots with 5-bromo-4-chloro-3-indolyl- b-D-galactosidase (X-Gal), as well as the quantification of the b-galactosidase activity of strains carrying *lacZ* fusions in association with wheat, was established as described by Arskne et al (1994). Several *lacZ* fusions were used with different strains of *Azospirillum:* (1) pAB358, a *nifH-lacZ* transcriptional fusion (Liang et al 1991), was used for estimating the potential for nitrogenase activity; (2) pLA-*lacZ,* containing a constitutive *lacZ* fusion (Ardne et al 1994), was used for quantification and detection of bacteria; (3) pAB2053Z, containing a constitutive *lacZ* fusion and the *flcA* gene of strain Sp7 (Pereg-Gerk et al 1998), was used for complementation analysis. Where the effect of oxygen concentration on *nifH-lacZ* expression was observed, colonized wheat plants were grown in an aerated hydroponic system (Deaker 1997) to repress the initial *nifH* expression. Plants were then placed in sealed McCartney bottles containing 0.5 mL of Winogradsky's N-free medium, flushed with N_2 and adjusted to a range of oxygen pressures. The bottles were incubated overnight with shaking (160 rpm) in a 30 °C waterbath. b-galactosi dase activity was determined by the quantitative method (Arshe et al 1994).

Ammonia transfer to wheat from ammonia-excreting mutants

Biological material and culture conditions. *A. brasilense* strain FP2 and its ammonia-excreting mutant HM53 were kindly provided by Dr. E Pedrosa (Pedrosa et al 1989). Bacteria and wheat were grown in media derived from soil medium (SM) (Wood et al 1998), a growth medium based on an "average" ionic soil solution and buffered at pH 6.0. Bacteria were grown overnight in SM with 10 mM ammonia and 10 mM malate, and were conditioned prior to use in carbon- and nitrogen-free SM for 3 h. These conditioned cells were totally deficient in nitrogen sources. All bacterial broths were grown at 25°C in vigorously shaken Schott bottles.

McCartney bottle growth system. Wheat seeds (Sunbri) were surface-sterilized with 0.5% $HgCl_2$, germinated, and handled aseptically according to Zeman et al (1992). Wheat seedlings were grown in 130-mL McCartney bottles containing 10 mL SM free of added carbon or nitrogen and solidified with 0.2% w/v agar (Wood 1999). The seedlings were inoculated with 1 mL *A. brasilense* 2 wk after germination using cultures adjusted to an absorbance of 1.0 at 600 nm in SM-based media free of added carbon and nitrogen. When required, malate (10 mM) was added as a filter-sterilized solution (100 mM). The bottles were sealed tightly with serum stoppers (Thomas Scientific, NJ, USA) and the gas mixture was amended with 1 ± 0.1% enriched $^{15}N_2$. After 70 h of incubation without shaking at 25 ºC with constant light, 13% v/v C_2H_2 was introduced into the bottles to effectively stop the reduction of $^{15}N_2$ and the acetylene reduction rate was assayed (Yu and Kennedy 1995). Ammonia in the semisolid agar was determined by spinning agar extracts at 6,000 g for 5 min and the supernatant analyzed using the indophenol reaction (Chaney and Marbach 1962).

Shoots were cut from the stopped plants about 1 cm from the spent seed, dried overnight at 80 °C, and subjected to Kjeldhal digestion and distillation into boric acid

to determine total N content and isotopic analysis (Bergersen 1980). Isotopic ^{15}N analysis was performed using a high-vacuum triple collector mass spectrometer (VG Micromass 903E) transferred from F.J. Bergersen's laboratory (CSIRO, Plant Industry, Canberra, Australia), upgraded with a linear source control and computerized output interface (Europa, UK). Samples were analyzed with the mass spectrometer using Rittenburg tubes (Bergersen 1980) for measuring enrichment.

Ammonia-excreting FlcA$^-$ *mutants in diazotroph-wheat association.* In further experiments using the ammonia-excreting mutant HM53, a *flcA*$^-$ derivative (HM53.1) was prepared by site-directed transposon mutagenesis (Pereg-Gerk et al 1998). The Sunelg wheat cultivar was used with the following treatments:

1. Controls (plants only)
2. Bacteria (HM53.1-pLA, HMS3-pLA, and Sp7-S-pLA) with N
3. Bacteria (HMS3.1-pLA, HMS3-pLA, and Sp7-S-pLA) without N

All plants received 2,4-D treatment and were grown in sand or sloppy agar in nitrogen-free nutrient solution.

Seeds were wrapped in cloth and placed in a vacuum flask for surface sterilization with 0.5% $HgCl_2$ for 75 s and immediately washed with distilled water several times. The sterile seeds were then transferred to yeast manitol agar (YMA) and germinated at 35 °C. The germinated seeds with no trace of infection were transferred to sterile 500-mL measuring cylinders containing either 100 mL of sloppy agar (0.4%) or 100 g of sand. One week after transplanting, the seedlings were inoculated with 1 mL of bacteria (A_{600}=1). Half of the pots received 10 mg of N as ammonium nitrate about 1 wk after transplanting. Plants grown in sand were watered with hydroponic solution every 2–3 d. The control treatment received hydroponic solution only (without bacteria and without nitrogen).

The cylinders containing three seedlings were placed in water baths to maintain a constant temperature of 28 °C. To minimize contamination and allow air flow, aluminum foil covers were placed loosely on the top of each cylinder. Plants were grown in a glasshouse for a total of 3 wk and separated into roots and shoots and dried in an oven (60 °C for 3 d). The relative dry weight is calculated by taking control data as 100.

Results and discussion

Flocculation mutants and the effect of cyst formation on nitrogenase activity

A spontaneous mutant of strain Sp7 impaired in flocculation, Sp7-S, showed higher nitrogenase activity rates in association with wheat than did the wild type (Katupitiya et al 1995). This observation has led to the investigation of several aspects of the mutation that causes a lack of flocculation: phenotype, genotype, and interaction with plants. Katupitiya and colleagues (1995) suggested a connection between the lack of exopolysaccharide (EPS) and encystment (Lamm and Neyra 1981, Sadasivan and Neyra 1985a,b, 1987, Papen and Werner 1982) in Sp7-S and the higher nitrogenase activity rates in association with plants. This theory was examined in this work (Pereg-

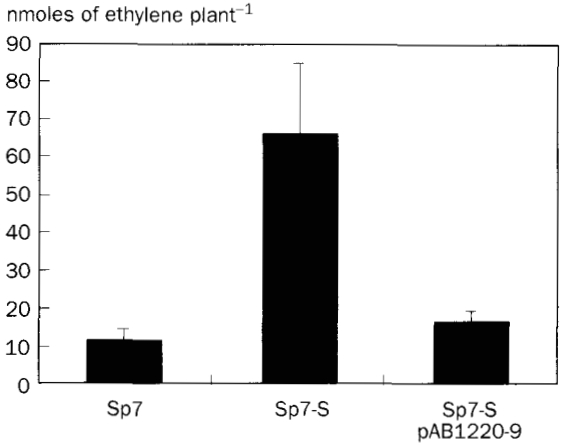

Fig. 1. Acetylene reduction activity associated with 2,4-D-treated plants inoculated with *A. brasilense* Sp7, Sp7-S or Sp7-S complemented for flocculation with plasmid clone pAB1220-9. The assays (Pereg-Gerk 1997) were carried out in a modified atmosphere containing 2.5% oxygen and lasted for 10 h from the injection of acetylene. Data shown are the average of five plants, grown for 10 d after inoculation. Bars indicate standard errors. One-way ANOVA analysis showed significant differences between strains Sp7-S and Sp7 (P = 0.011) and between strains Sp7-S and Sp7-S pAB1220-9 (P = 0.027), but not between Sp7 and Sp7-S pAB1220-9 (P > 0.05).

Gerk 1997) by comparing acetylene reduction rates of the mutant strain Sp7-S with and without the introduction of the plasmid pAB1220-9 containing the *flcA* gene (Table 1). First, transmission electron microscopy showed that this plasmid complemented the capsular material that is absent in strain Sp7-S (L. Pereg-Gerk et al, unpublished). Indeed, as expected, acetylene reduction rates of strain Sp7-S complemented for EPS production by pAB1220-9, which restored flocculation, were reduced (5-fold) compared with those of strain Sp7-S (Fig. 1). Moreover, the complemented strain also formed cyst-like cells on the surface of wheat roots, similar to those formed by the wild-type strain Sp7.

Flocculation mutants of Sp245. The connection between the inability to flocculate and altered colonization pattern of roots in other strains of *Azospirillum* was examined (Pereg-Gerk 1997) by applying the same procedure to *A. brasilense* Sp245, which was reported to be a good colonizer (Jain and Patriquin 1984, Assmus et al 1995). *A. brasilense* Sp245 appeared drier than Sp7 on Nfb plates and also showed a higher degree of flocculation, leaving the supernatant visually very clear. This probably explained the higher frequency of the mutation that was found for Sp245, in which 1.36% of the colonies (60 out of 4,400) did not bind Congo red (named Sp245-M1, M2, M3, ... M60). Following five reisolations of the 60 mutants, only five of the cultures reappeared red (Sp245-M36, M40, M41, M42, and M50) and, therefore, the stability of the mutation from Sp245 was estimated as 92%.

Characterization of several mutants of A. brasilense Sp7 and Sp245. Several mutant strains of interest were further investigated and characterized, among them Sp7-PM23, which regained its ability to bind Congo red but was impaired in swarming in semisolid medium (both NB and minimal lactate); Sp7-PM35, which partially bound Congo red but lost its ability to flocculate; and Sp245-M4/M5/M6, which did not bind Congo red and showed higher rates of nitrogenase activity than the wild-type Sp245 in association with plants (Fig. 2). Similar to Sp7-S, the mutant strains Sp7-PM3S and Sp245-M4/M5/M6 did not go through the stages of flocculation of' the wild-types Sp7 and Sp245 (Bastarrachea et a1 1988). In contrast to Sp7-S, however. they did not also lose their ability to swarm on semisolid media. Colonies of these mutant strains grown on solid agar plates (NB or minimal lactate) had the same mucoid appearance of the wild type in the first 1–2d at 30 °C. Longer incubation, however, resulted in red, dry-looking colonies of the wild types, while the mutants remained in the mucoid form. suggesting a defect in the differentiation into cysts. Strains Sp245-M4/M5/M6 did not stain red at any stage, whereas Sp7-PM35 partially did. Similar to the wild types, the mutant strains Sp7-PM35 and Sp24S-M4/M5/M6 were able to fix nitrogen (as measured by acetylene reduction activity, ARA) in pure cultures (acetylene reduction rates were similar to those of the wild type, data not shown), were motile in liquid media (NB or minimal lactate), and were able to use both nitrate and ammonium for growth.

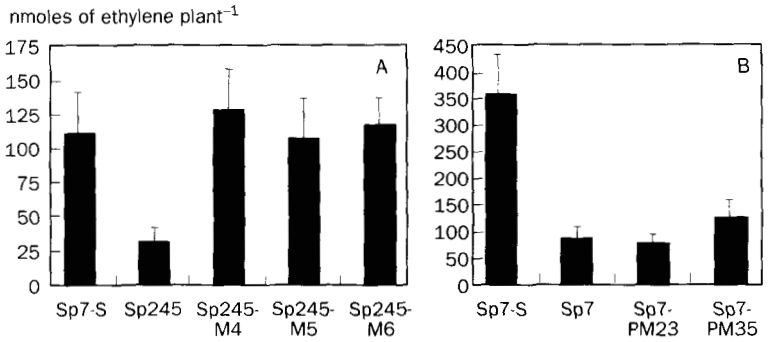

Fig. 2. Acetylene reduction activity of *A. brasilense* Sp7 and Sp245 strains associated with 2,4-D-treated plants (Pereg-Gerk 1997). Plants were inoculated with wild-type and spontaneous mutants of strain Sp245, Sp245-M4/M5/M6 (A), or with wild-type and spontaneous mutants of strain Sp7, Sp7-PM23/PM35 (B). Strain Sp7-S, in which increased rates of acetylene reduction were reported (Katupitiya et al 1995), was used as a control. The assays were carried out in a modified atmosphere containing 2.5% oxygen, and lasted for 20–24 h (a day) from the injection of acetylene. Data shown are the average of 10 plants, assayed 10 d after inoculation. Bars indicate standard errors. One-way ANOVA analysis showed significant differences between strains Sp7-S and Sp245 ($P = 0.042$) and between strain Sp245 and the mutants (Sp245-M4, $P = 0.022$; Sp245-M5, $P = 0.027$; Sp245-M6, $P = 0{,}011$). In (B), the control strain Sp7-S showed significant differences from all of the other strains (in all cases $P < 0.01$). There was no significant difference ($P > 0.05$) among the mutant strains Sp7-S and Sp245-M4/M5/M6 in (A) and among Sp7, Sp7-PM23, and Sp7-PM35 in (B).

The appearance of *para*-nodules on wheat following treatment with 2,4-D was reported to be highly correlated with increased rates of nitrogenase activity by *Azospirillum* on plants (Tchan et al 1991, Christiansen-Weniger 1992, Zeman et a1 1992). The nitrogenase activity was even higher when the roots were inoculated with the mutant strain Sp7-S rather than with the wild-type Sp7 (Katupitiya et al 1995). Similarly, nitrogenase activity (acetylene reduction) observed with wheat roots inoculated with the mutants Sp245-M4/M5/M6 was significantly higher (between 4- and 5-fold) than with roots inoculated with the wild-type Sp245 (Fig. 2A). The acetylene reduction rates in these mutant strains were as high as in the mutant Sp7-S on 2P-D-treated plants.

There was no difference in acetylene reduction rates between plants inoculated with the wild-type Sp7 and plants inoculated with the nonswarming mutant Sp7-PM23 (Fig. 2B) that retained the ability to flocculate. Plants inoculated with the mutant Sp7-PM35 showed slightly higher (1.5-fold) rates than the wild type, but not as high as the mutant strain Sp7-S (Fig. 2B). The 3-fold difference in activity with Sp7-S in Figure 2 is typical of different experiments, probably as a result of the extent of colonization. These results are all consistent with the hypothesis that deletion of the flocculation character, also involving loss of the ability to encyst, leads to strains that colonize wheat roots in a manner better able to carry out nitrogen fixation activity in *para*-nodulated seedlings.

Effect of 2,4D on *nifH* expression of *A. brasilense*

Figure 3 demonstrates the effect of 2,4-D treatment on *nifH* expression of *A. brasilense* over a range of oxygen concentrations (Deaker and Kennedy 1996). The expression of this gene, coding for the iron-protein of nitrogenase most highly sensitive to oxygen, is indicative of nitrogen-fixing conditions (Liang et a1 1991) and is one prerequisite for nitrogenase activity. There was no significant effect of 2,4-D treatment on the expression of Sp7 *nifH* (Fig. 3A). In each case, *nifH* expression is low at 0% oxygen, then increases and reaches an optimum between 1% and 2.5% oxygen. *nifH* expression then decreases at 5% oxygen, after which expression remains relatively constant. There was a significant effect of 2,4-D treatment, however, on plants inoculated with Sp7-S (Fig. 3B). These results are consistent with those obtained earlier for rates of acetylene reduction with oxygen concentration with and without 2,4-D treatment (Kennedy and Tchan 1992). They also agree with published data on acetylene reduction activity and with bacterial cell numbers obtained by Katupitiya et al (1995), indicating that the number of Sp7-S colonizing the root is significantly increased by the addition of 2,4-D when para-nodules are formed. The difference in numbers of cells of Sp7, however, was less pronounced. Whole plants were exposed to the different oxygen concentrations and, as mentioned previously, Yu et al (l993) demonstrated that there was no significant change in oxygen concentration over the course of the incubation in this system.

Figure 4 shows the *nifH* expression of *para*-nodulated wheat inoculated with *A. brasilense* Sp7 and Sp7-S as a function of oxygen concentration. At 0% oxygen, *nifH* expression is low. It increases to an optimum expression when the oxygen concentra-

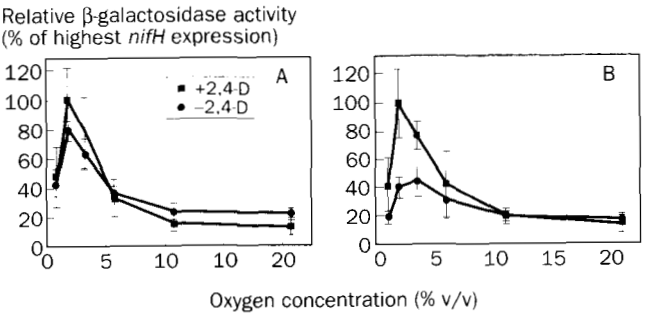

Fig. 3. Relative *nifH* expression as a function of oxygen concentration in wheat inoculated with *A. brasilense* Sp7 and Sp7-S with and without 2,4-D (R. Deaker and I.R. Kennedy, in preparation). (A) Sp7. (B) Sp7-S. Results are relative to the highest nifH expression, indicated as 100%. Data are the averages of 5 plants, with 95% confidence Intervals shown.

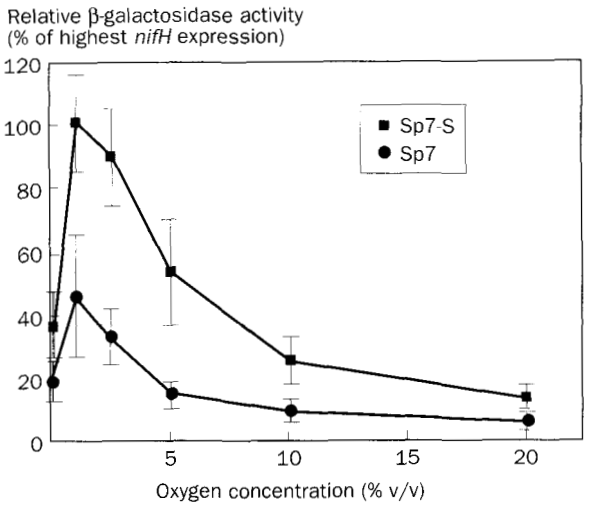

Fig. 4. Expression of *nifH-lacZ* in para-nodulated wheat inoculated with *A. brasilense* as a function of oxygen concentration (R. Deaker and I.R. Kennedy, in preparation). In the comparison of Sp7 and Sp7-S, the expression is relative to the highest average *nifH* expression as ß-galactosidase activity. Results shown are the averages of 5 plants, with 95% confidence intervals shown.

tion is between 1% and 2.5%. The expression of Sp7 *nifH* then decreases rapidly to almost a minimum value at 5% oxygen. In contrast, Sp7-S *nifH* is still expressed at a relatively high rate at 5% and does not reach a minimum until the oxygen concentration is between 10% and 20%. At its optimum expression, the *nifH* of Sp7-S is more than twice as high as Sp7 *nifH*. This higher expression with Sp7-S is consistent with a mode of colonization allowing for greater protection from oxygen than surface colo-

nization noted elsewhere (Katupitiya et al 1995, Kennedy et al 1997). The relative expression of *nifH* was calculated as a percentage of the highest *nifH* expression, which was exhibited by Sp7-S in each experiment. The results illustrate a significantly higher relative expression of Sp7-S over a wider range of oxygen concentrations.

Transfer of ammonia from an excreting mutant to wheat seedlings using ^{15}N labeling

Apart from symptoms of N deficiency, wheat seedlings grew well for up to 6 wk in semisolid C- and N-free agar enclosed within McCartney bottles. In the cocultures containing 10 mM malate, strains FP2 and HMS3 formed characteristic pellicles or veils close to the surface of the agar (Wood 1999). In pure cultures (without wheat and amended with malate), only HM53 excreted ammonia into its medium. consistent with earlier reports (Pedrosa et al 1989). In coculture with wheat, however, no free ammonia in the medium could be detected, indicating the capacity of wheat seedlings to completely scavenge this form of inorganic nitrogen using their roots. In this closed system, *A. brasilense* actually fixed more N_2 (ARA) with wheat than in pure cultures alone (Wood 1999). presumably as a result of complete de-repression of nitrogenase genes. Natural variation in seedlings, however, resulted in no statistically significant differences in shoot dry weight or shoot N content for any treatment (Table 2) in the 70-h assay period. In the absence of added malate, all bacteria failed to fix N_2 (ARA) or to excrete ammonia.

The isotopic ^{15}N analysis allowed the transfer of added $^{15}N_2$ to be directly traced from *A. brasilense* to the plant tissue. The leaves of seedlings in control bottles inoculated with a non-N_2-fixing deletion mutant were not significantly enriched above background levels (0.3663 atom % ^{15}N; Table 2).

The combination of 10 mM malate and ammonia-excreting mutant HM53 enriched the shoot tissue with ^{15}N added as $^{15}N_2$. About 20% of the nitrogen in the wheat shoot tissue was derived from $^{15}N_2$ newly fixed by *A. brasilense* at the end of the 70-h exposure period (Wood 1999). The wild-type FP2 did not enrich the shoot tissue with any significant amounts of ^{15}N with or without malate over this period (Table 2). The difference between the enriched and nonenriched shoot material was approximately 10 times more than the least significant difference. This statistical significance was achieved despite the low number of replicates and the low initial $^{15}N_2$ enrichment of the atmosphere.

Effect of combining *flcA*⁻ and ammonia excretion on growth of wheat seedlings

In an initial experiment where the dry weight accumulation in wheat seedlings inoculated with different strains of modified azospirilla was measured, a trend indicating increased growth rate with a flocculation mutant of the ammonia-excreting strain (HMS3.1) compared with the ammonia-excreting strain itself (HMS3) and an uninoculated plant control was observed (Fig. 5). In the absence of added nitrogen other than that in seeds, dry matter accumulation can be considered as an excellent

Table 2. Data set for $^{15}N_2$ transfer experiment (Wood 1999).

Strain	mM malate	Pure culture[a] ARA[b]	Co-culture[a] NH_4^{+c}	ARA[b]	Shoot DW[d]	Shoot N[d]	Atom%^{15}N[e]	% Ndfa[f]
Control	0	0	0	0	70	2.70	0.36672 ± 0.05195	0
	10	0	0	0	72	2.53	0.36698 ± 0.04842	0
FP2	0	0	0	3	92	2.88	0.36707 ± 0.0090	0.03
	10	150	0	192	97	2.77	0.37084 ± 0.0133	0.38
HM53	0	0	0	5	85	2.79	0.37125 ± 0.0296	0.45
	10	320	24	468	90	3.06	0.58281 ± 0.0266	20.0
LSD[g]		56	4	70	15	1.2	0.024	

[a]Determined after 70 h. [b]nmoles h^{-1} $bottle^{-1}$. [c]µmoles $bottle^{-1}$. [d]Dry weight in mg. [e]Headspace = = 1.366% atom%^{15}N as N_2. Shoot samples grown without ^{15}N enrichment had ^{15}N content of 0.366234 ± 0.006 atom%^{15}N. [f]Subtracted from the relevant "control" value. Each average was calculated from 3 or 4 Independent replicates. [g]LSD = least significant difference.

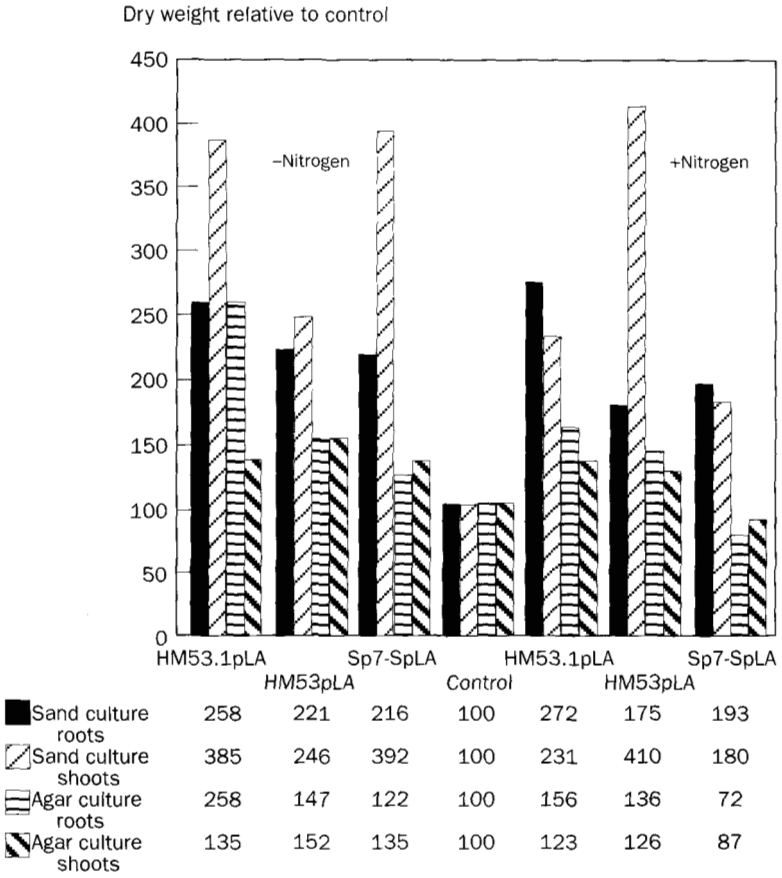

Fig. 5. Shoot and root dry weight of wheat seedlings as affected by treatment with *flcA* and ammonia-excreting mutants of *A. brasilense.* See Table 1 for strain descriptions (all strains were labeled with the plasmid-borne constitutive *lacZ* gene for microscopy). Most treatments used 5 seedlings with the coefficient of variation of seedling dry weights (SE as a percentage of the mean value) lying in the range of 5–25%. The minus-nitrogen control seedlings in sand and agar had a dry weight totaling a mean of 6.4 and 13.4 mg, respectively.

measure of total nitrogen fixation (Pinchbeck et al 1980). These results indicating significant nitrogen fixation are consistent with the general hypotheses concerning improved colonization and nitrogen transport being examined in this paper. The increased growth rate, however, was matched in this experiment by inoculation with Sp7-S itself, though not in tests where "starter-N" was added to sand or sloppy agar (Fig. 5). Because the Sp7-S strain is not known to be an ammonia excreter, the exact mechanism of increased growth in these wheat seedlings seems unclear. Perhaps the azospirilla can be mineralized in the system over the growth period after inoculatison of 2 wk. Where starter-N was added to the treatments, the superiority of the *flcA*$^-$ mutant of the ammonia excreter was not evident.

These results on the increased growth of wheat using mutants of *A. brasilense* can be rationalized in terms of factors such as control of nitrogenase expression. oxygen inhibition, and rate of nitrogen transport. These laboratory data. however, are preliminary in nature. The trial serves to illustrate how the experimental model of the para-nodule can be employed to generate information relevant to the overall goal of symbiosis and the means to achieve this.

Conclusions: the need for an appropriate model to gauge quantitative improvements

The objective of this work has been to seek better definition of the key factors that control the expression of a potential N_2-fixing symbiosis between *Azospirillum* and wheat. Such an ambitious objective, involving a genotype-environment interaction that does not exist now, can only be achieved using an experimental model allowing quantitative assessments of relative functionality to be made. It is extremely unlikely that this objective can be reached efficiently by random trials of cultivars and strains, simply hoping that the correct combination can be found by good fortune without an appropriate intellectual strategy to gauge real advances. It is suggested that such a strategy must involve a series of step-wise improvements in the experimental model that can be quantitatively measured. The model of the para-nodule (Kennedy and Tchan 1992, Kennedy et al 1997) is the intellectual strategy being employed in this chapter. The use of 2,4-D is justified by the need to have adequate endophytic or protected colonization to allow a statistically valid assessment of the system. In our opinion, significant progress has already been achieved, although there are at least as many difficulties to be overcome. In this chapter, we make further progress by combining the dual needs of better colonization with that of significant ammonia transport from the bacterium to the plant.

A book soon to be published (Kennedy 1999) proposes a novel theory that all action in ecosystems is derived from the resonant impulses of energy quanta reflected between the elementary particles making up molecules, acting as positive forceful interactions that provide the basis of the principle of conservation of momentum. These impulses from quanta cause a perceptible recoil in the molecule receiving the impulse, altering its action or quantum state. Although thermodynamics is usually applied to systems or ensembles of molecules, action biothermodynamics relates energy to action, providing a means of relating specific dynamic processes involving individual molecules and their interactive properties to the properties of whole systems.

At temperature equilibrium, the average rotational torques exerted on each set of molecules by the forces of action resonance are equal, corresponding to equal intensity of their Brownian motion. But the resonant frequencies of the quanta are characteristic for each set of molecules, conferring specificity on these forces or torques. The intensity of the specific action exchange forces between the members of a set of molecules corresponds to the chemical potential of each different chemical species. Although these action dispersion forces operate randomly, all molecules will natu-

rally influence the action exchange field in their vicinity, depending on their material shapes and the dynamic modes of their reflections of quanta.

For example, in the interaction between *Azospirillum* and wheat, action resonance forces, or rather their absence, dictate the pattern of colonization possible for particular strains and their mutants. Thus, the exopolysaccharide of the Sp7 strain confers an affinity between these cells and the epidermis of wheat roots that is absent with Sp7-S and other *flcA*$^-$ mutants. The latter are dynamically repelled by the exchange momentum of quanta from the root surface and are unable to bond. This dispersive property with respect to the root surface, however, also allows them more mobility and they are able to achieve a more endophytic colonization where they can find protected niches inside the roots or in crevices in the surface.

In biological systems and ecosystems, these torques or turning forces represent the efficient cause of the genotype-environment interaction; the environment modulates the expression of genotypes by controlling the motion of molecules and directs their transport or interconversion, impulse by impulse. The genotype in turn generates products or coupling agents that modulate the action resonance field in their vicinity. The action field is then involved in a two-way control of the expression, regulation, and evolution of each genotype in a complete ecosystem or microecosystem such as a nitrogen-fixing symbiotic system. In symbiosis, there must exist a complementarity of action forces between the two organisms that is expressed in their ability to cooperate.

The action resonance theory was designed as a global explanatory theory for biology, from a consideration of numerous biological phenomena (Kennedy 1999) against a background of orthodox physics and chemistry. The only current alternative theory, statistical mechanics, is often inadequate and has little explanatory power for complex systems such as ecosystems. Action resonance theory provides a model based on a view of microscopic physical reality that can play a positive role in experimentation. This arises as a result of its capacity to suggest worthwhile approaches and subsidiary hypotheses for experimental testing.

The action processes, driven by the impulses from energy, that are significant for putative symbiosis between diazotrophs and cereals can be identified. These processes require modulation by genetically specified coupling agents that are synthesized as the response of genes to their interaction with the environment. A list of these genetically coupled processes could include:

1. *Chemical recognition by azospirilla of the rhizosphere or endorhizosphere habitat.* This will involve the appropriate chemotactic response controlling the flagellar motor, under genetic control (see Zhulin and Armitage 1992, Zhulin et al 1996), allowing bacteria to move purposively. These chemical gradients also flow from energy substrates for the bacterium, allowing the performance of biochemical work by such motors or coupling agents.
2. *Binding interactions with the root surface and entry of endophytes.* Binding of azospirilla to the root surface (Jain and Patriquin 1984) may involve action of flagellae and interactions of exopolysaccharides (Michiels et al 1990, 1991, Croes et al 1993, Vande Broek and Vanderleyden 1995), but this bind-

ing material or stickiness may be inimical to colonization elsewhere in the system. Endophytes will require entry through an appropriate portal, possibly through cracks or during epidermal extension. The process of entry also involves dispersive forces exerted by the bacterium (e.g., flagellar action, etc.), thus separating cells, and may even require the extra dispersive action forces involved in bacterial and plant cell division. Such a process may involve the action of enzymes such as cellulases or polygalacturonases, allowing bonds between cells to be broken and entry of bacteria. In *para*-nodulation, the synthetic auxin 2,4-D provides the stimulus for such activity. Companion bacteria can sometimes perform a similar task.

3. *Access to carbon substrate from the plant.* This is essential on a continuous basis to allow adequate colonization and the establishment of an adequate density of bacterial respiratory activity to reduce oxygen activity to a point where the *nif* genes can be expressed through the mode of expression of the *nifA* regulatory codon.
4. *Ammonia release from daizotroph cells.* A regulated (or deregulated) release of ammonia from N_2-fixing bacterial cells into plant tissue will be required, leading to maximal expression of nitrogenase activity. This must involve a quantitative understanding of the bioenergetics of the bacterial cell membrane, which is now available for *A. brasilense* Sp7-S (Wood et al 1998), and of the control of movement of ammonia across these membranes (see Wood 1999, for a specific mathematical model).
5. *Ammonia assimilation as organic nitrogen products* (Schubert 1986). Because ammonia is regarded as a suitable source of nitrogen for plant roots, no special genetic apparatus should be necessary for the transport and assimilation of ammonia. The enzymic processes of ammonia assimilation provide the means by which the direction of ammonia transport can be controlled, provided the bacterial cell membrane is sufficiently permeable.
6. *Sustained symbiosis during the plant growth cycle.* This requires extended colonization with diazotrophs as the plant grows to maturity. The sugarcane model where colonization of the xylem seems to be involved would seem to have advantages with respect to proliferation of colonized tissue. The application of synthetic auxin to induce para-nodules at one stage of the growth cycle seems inadequate in this respect.

Identification of these and other relevant action processes can provide the logical basis for selection of plant and microbial genotypes and their genetic modification to meet the needs of critical parameters for symbiosis. It must be recognized that genetic manipulation will only succeed if it leads to gene products consonant with the environment and provided the outcome sought can be thermodynamically spontaneous in that environment. In general, processes that generate action also increase the entropy of molecular systems.

These considerations may at first sight seem to impose a burdensome constraint on the range of possibilities, but action biothermodynamics can also serve as a means of drastically reducing the range of approaches worth trying, indicating those bound

to fail. Applying a strategy governed by action resonance theory would result in much greater economy in the use of resources, compared with the common method of rather speculative hypothesis (hunches) followed by experimental trial and error. By identifying these separate phases above. methods for studying each of the critical parameters can then be developed. For example, a modified Fåhreus technique for studying plant roots by (confocal) microscopy could well be beneficial for optimizing some of the biodynamic parameters related to colonization.

Ideally, in the search for spontaneity, the developing model symbiotic system itself (para-nodules or alternative models such as xylem colonization) would be given a role in this selection process; these *para*-nodules/xylem could be exposed to colonization by competing strains and mutants. Obviously, the genotype-environment interaction must include the plant genome. Genetic variation in cereals or other nonlegume crops could be explored, as well as that in bacterial strains. Because the most successful associations would yield the most vigorous systems (i.e., the greenest), these better performing systems could be used for reisolation of the most successful candidates for future inoculation.

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Notes

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Realizing the genetic predisposition of rice for symbiotic nitrogen fixation

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A long-standing goal of biological nitrogen fixation research has been to extend the nitrogen-fixing rhizobial symbioses of legumes to nonnodulated cereal plants such as rice. To formulate strategies for developing rice-rhizobia symbioses, adopting a systematic approach, we initially studied the extent of predisposition of rice to form an intimate association with rhizobia.

The induction of Rhizobium nod genes by plant-produced flavonoids is essential for the infection of legume roots. Our studies indicated that the roots of certain rice cultivars exude compounds that are able to induce, albeit to a low extent, transcription of the *nod* genes of *Rhizobium* sp. NGR234. Neither rhizobia nor purified Nod factors, however, could elicit root hair deformation or cortical cell divisions leading to true nodule development in rice. Rhizobia primarily invade rice roots through cracks in the epidermis and fissures created during the emergence of lateral roots. This infection process, unlike in legumes, is *nod*-gene independent, and does not involve the formation of infection threads. Moreover, rhizobial invasion provokes a mild defense response localized in the vicinity of the colonization site.

During legume nodulation, specific plant genes, known as early nodulin *(ENOD)* genes, are induced and are required for normal development of the nodule. We found that several legume *ENOD* genes hybridized to DNA from a variety of rice genotypes. This work was subsequently confirmed by the isolation of rice cDNAs showing considerable sequence homology to legume *ENOD93* and *ENOD40.* Legume nodulation also requires the ability to respond to the rhizobial Nod signal, and the consequent signal-induced expression of the *ENOD* genes. Studies revealed that rhizobial Nod factors can induce the expression of legume *ENOD12* promoter in rice, thus demonstrating that rice has a mechanism to perceive Nod factors and possesses a signal transduction chain that links such recognition to *ENOD* gene transcription. In addition, similar to the situation in legumes, the expression of the legume *ENOD40* promoter is induced only in the vascular tissues in rice. Likewise, the expression of rice *ENOD40* in soybean nodules is also restricted to the vascular bundles. These findings clearly suggest that legume and rice *ENOD40s* share a similar regulatory mechanism(s).

Research makes it evident that rice possesses some developmental subprograms in its genome, which are similar to those that lead to the development of symbioses in legumes. It is therefore essential that studies be extended at the cellular and molecular levels to identify why symbiotic responses do not occur fully in rice in order to contemplate genetically engineering this major cereal crop to form a more intimate endosymbiotic association with rhizobia.

The demand for fixed nitrogen as a nutrient for crop production has increased dramatically during the second half of the 20th century. The increased requirement of fixed nitrogen for enhancing crop production is met by industrial nitrogen fixation (through the Haber-Bosch process) in complementation with biological nitrogen fixation (BNF). It is estimated that twice as much fixed nitrogen will be required to raise rice production by 2020 to supplement the food requirements of the increasing human population (IRRI 1993). Both biologically and industrially fixed nitrogen are needed to meet the demand for food production, but BNF has advantages of lower cost and reduced environmental hazards and is more consistent with the development of sustainable agriculture. To achieve food security through sustainable agriculture, the requirement for fixed nitrogen will need to be increasingly satisfied by BNF rather than by industrial nitrogen fixation. Because of the importance of BNF in sustainable agriculture, we must improve existing biological nitrogen-fixing systems and develop nitrogen-fixing nonlegume crops, particularly for important cereal crops such as rice.

Among the conventional BNF systems, free-living and associative diazotrophs have a low to moderate potential to supply N to rice because nitrogen fixed outside the plant is subject to loss. Green-manure crops such as *Azolla* and *Sesbania* have a high potential for nitrogen supply to support higher grain yields of rice, but farmers are reluctant to use them as there is no economic advantage over chemical nitrogen fertilizer (see Ladha and Reddy 1995, Ladha et al 1998). If a BNF system could be assembled in the rice plant itself, it could enhance the potential for nitrogen supply because fixed nitrogen would be available directly to the plant, with little or no loss, besides ensuring no additional economic burden to farmers. To address this challenge, different approaches toward developing rice capable of autonomous nitrogen fixation are now being contemplated (Khush and Bennett 1992, Ladha and Reddy 1995, Reddy and Ladha 1995). The strategies for developing nitrogen fixation in rice include establishing effective endophytic associations, developing legume-like nodulation, and expressing nitrogen fixation *(nif)* genes. In this chapter, we review the status of research aimed at determining the genetic potential of rice for nodulation and highlight the areas that need to be explored to overcome the inherent constraints in rice to developing symbioses with rhizobia.

Legume-*Rhizobium* symbiosis: a model for developing nitrogen-fixing symbiosis in rice

Leguminous plants are capable of forming a symbiotic association with soil bacteria known as *Rhizobium. Mesorhizobium, Sinorhizobium, Bradyrhizobium,* and *Azorhizobium* (collectively referred to as rhizobia). Reciprocal communication between the legume host and its bacterial partner leads to the formation of a specialized plant organ, the nodule. in which rhizobia carry out nitrogen fixation. Nodule development in legumes results from a series of interactions between the host plant and rhizobia, such as attachment and recognition of bacteria, root hair curling, infection, and the induction of cortical cell divisions leading to nodule organogenesis (Fig. 1;

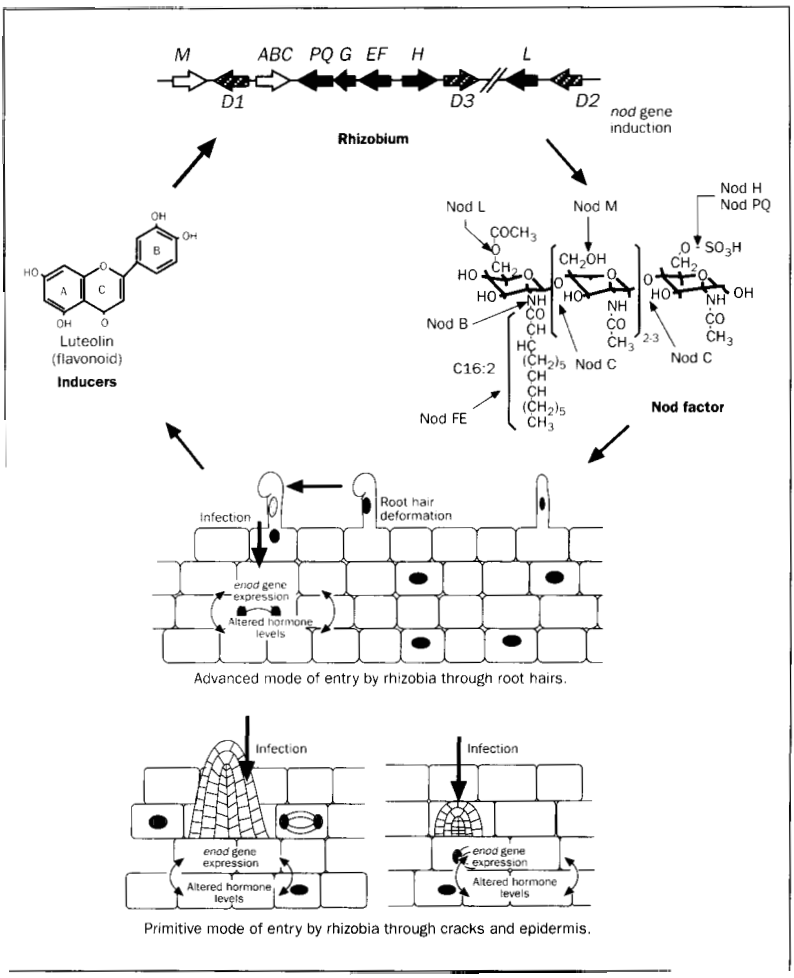

Fig. 1. Initial stages in the legume-*Rhizobium* symbiosis. (Modified from Hirsch 1992.)

see Denarie et al 1996, Pawloski and Bisseling 1996). The molecular interactions between the symbiotic partners are invoked by plant-secreted flavonoids and rhizobia-produced lipo-chitooligosaccharide signal molecules known as Nod factors (Denarie et al 1996, Long 1996). The process begins with the secretion of flavonoids from roots and consequent flavonoid-triggered *nod* gene expression in the microsymbiont, leading to the production of Nod factors. Nod factors, in turn, activate early nodulin *(ENOD)* genes governing the processes involved in root hair deformation and curling, initiation of infection threads, and cortical cell division during the early steps of nodulation in legumes. To establish symbiosis between rhizobia and rice, it is first essential to methodically evaluate interactions of rice with rhizobia to identify similarities and differences in rice-rhizobia1 interactions vis-2-vis those occurring in legume-rhizobia1 interactions.

Rice root exudate has limited ability to induce *nod* gene expression in rhizobia

For rhizobia to infect the legume root, the bacterial *nod* genes must be induced by plant-produced flavonoids. To ascertain whether rice root exudates have such an ability to promote *nod* gene expression, we employed different rhizobial strains carrying *nod-lacZ* fusion reporter genes to examine whether axenically generated rice root exudates could activate the expression of *nodSU* in *Rhizobium* NGR234, *nodY* in *B. japonicum* USDA 110, and *nodA* in *R. leguminosarum* bv. *trjfolii* ANU843. Earlier, we reported no *nod* gene activation in any rhizobial strain with the root exudates from several lowland rice varieties (Reddy et al 1997). Our recent studies, however, showed that the root exudates derived from the upland rice cultivars Palawan, Moroberekan, and IRAT225, and lowland variety IR20 were able to induce, albeit to a very low extent, the transcription of *nod* genes of *Rhizobium* NGR234, a rhizobial species that has an extraordinarily large host range (Table 1). In plate assays with *B. japonicum*

Table 1. Induction of *nod* gene expression in *Rhizobium* NGR234 *(nodSU-lacZ)* in response to rice root exudates.

Rice variety	ß-galactosidase activity (Miller units) with root exudate[a]			
	Water soluble fraction		Methanol soluble fraction[b]	
Control	86.2 [c] a		86.2 [c] a	
IR20	99.6 d	(15.5)	92.5 bc	(7.3)
IRAT225	92.1 c	(6.8)	91.6 b	(6.3)
Kinandang Patong	86.3 a	(0.1)	88.7 a	(2.9)
Moroberekan	88.9 b	(3.1)	96.2 d	(11.6)
Palawan	98.4 d	(14.6)	95.4 cd	(10.7)

[a]Each value represents an average of five replicates. [b]Methanol:water (1:1) soluble fraction. [c]Basal activity obtained without the addition of root exudate fraction. Values in a column followed by a common letter are not statistically different according to Duncan's multiple range test (P= 0.05). Values in parentheses indicate percent increase in ß-galactosidase activity.

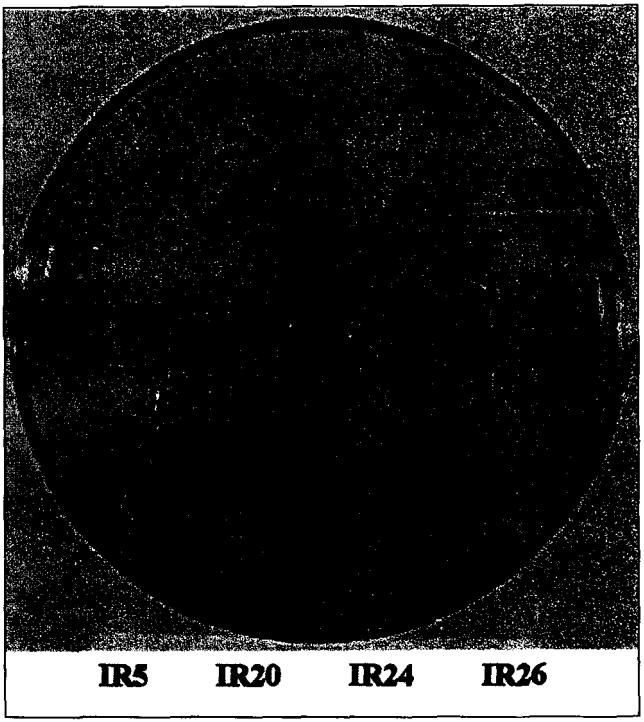

Fig. 2. Plate assay of ß-galactosidase activity depicting nod gene induction in *Bradyrhizobium japonicum* USDA110 ZB977(nodY-lacZ) in the root environment of rice variety IR20.

USDA110, *nod* gene induction was observed occasionally in the rhizobial cells growing in a zone around the root tip of young seedlings of rice variety IR20 (Fig. 2). This provides a clue that the production of *nod* gene inducers is probably restricted to a particular developmental stage of the rice root. Results available so far seem to indicate that rice root exudate either lacks or contains only very low concentrations of the appropriate activators of nod gene expression and can include antagonistic substances that inhibit activation of the rhizobial *nod* genes. Studies are now under way to identify the reason(s) for the absence or reduced level of *nod* gene expression in rhizobia in the presence of rice root exudate.

Response of rice root hairs to rhizobia and Nod factors

Like in legumes, most rhizobia stimulated root hair formation in several rice cultivars, and, shortly after inoculation, the rhizobial cells attached themselves, frequently in a polar fashion, to the root surface, including the rook hairs (Reddy et al 1997, Terouchi and Syono 1990). In the subsequent stages of colonization, however, the rhizobial cells gradually lost the ability to attach preferentially in polar orientation to

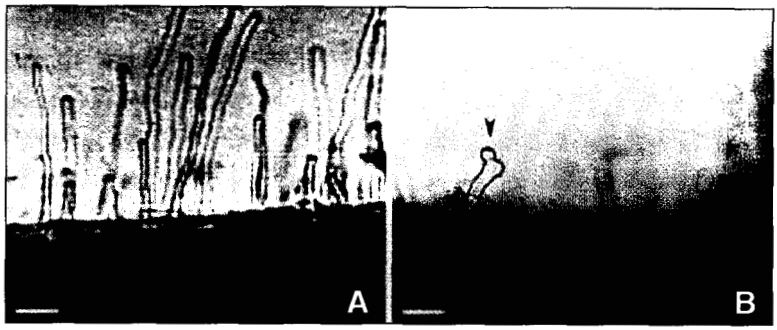

Fig. 3. Root hair deformation in rice variety Milyang 54. (A) Control and (B) inoculated with *Bradyrhizobium* ORS322. Note deformed root hairs (arrowheads). Bars = 100 μm.

the root surface, and did not elaborate cellulose microfibrils (Reddy et a1 1997). These later events in the rice-*Rhizobium* association contrast with the discrete *nodD*-dependent bacterial aggregation at root hair tips and extensive elaboration of cellulose microfibrils characteristic of phase 1 attachment and phase 2 adhesion in the legume-*Rhizobium* symbiosis (Dazzo et a1 1984, 1988, Mateos et a1 1995).

Although most rhizobia were able to stimulate the formation of root hairs in the rice cultivars, they failed to induce deformation or curling of root hairs (Reddy et a1 1997). The only exception was a marginal ability of *Bradyrhizobium* ORS322 to induce root hair deformation on rice variety Milyang 54 (Fig. 3; Reddy et al 1995). In fact, the rhizobial Nod factors, whose synthesis is specified by rhizobial *nod* genes, are responsible for root hair deformation and curling and triggering a nodule organogenic program in legumes. In rice, even the addition of purified Nod factors (10^{-6} - 10^{-9} M, from *Rhizobium* sp. NGR234) failed to promote root hair deformation (Reddy et a1 1997). This result contrasts with an earlier report of rice root hair deformation observed using a recombinant strain of *R. leguminosarum* bv. *trifolii* ANU843 harboring multiple copies of pSym-borne *nodDABC* genes (Plazinski et a1 1985). Similarly, NodNGR factors also failed to activate the *MtENOD12* promoter in the root hairs of transgenic rice (Reddy et a1 1998b, see below). The apparent inability of rice root hairs to respond to Nod factors may be due to the absence of the appropriate Nod factor structures required for induction, the putative plant receptor(s) that perceive them, or some other element(s) in the Nod factor signal transduction pathway required for induction of root hair deformation (Reddy et al 1997).

Rhizobia promote the formation of thick, short lateral roots in rice

In legumes, rhizobia induce an organogenic program leading to the formation of nitrogen-fixing nodules having a "stem-like'' anatomy. In the nonlegume *Parasponia,* however, the *Rhizobium*-induced nodules are modified lateral roots. There are reports that nodule-like structures or hypertrophies are induced in rice, albeit at very low frequencies (in 0.1–0.2% of the plants), in response to the application of rhizobia to

either normal roots (Bender et al 1990, de Bruijn et al 1995, Jing et al 1990. 1992, Li et al 1991, Rolfe and Bender 1990, Rolfe et a1 1992, Shizhen and Dongwei 1994) or enzyme-treated roots in the presence of polyethylene glycol and calcium chloride (Al-Mallah et al 1989). These nodule-like structures or hypertrophies appeared to originate from lateral root primordia (Cocking et al 1994, de Bruijn et al 1995). During our studies with more than 70 rice varieties inoculated with 25 different broad and narrow host range strains of rhizobia, we failed to observe the development of nodule-like structures or hypertrophies on rice roots, although rhizobia promoted the formation of thick, short lateral roots (TSLR, Fig. 4; Reddy et al 1997, Webster et al 1997).

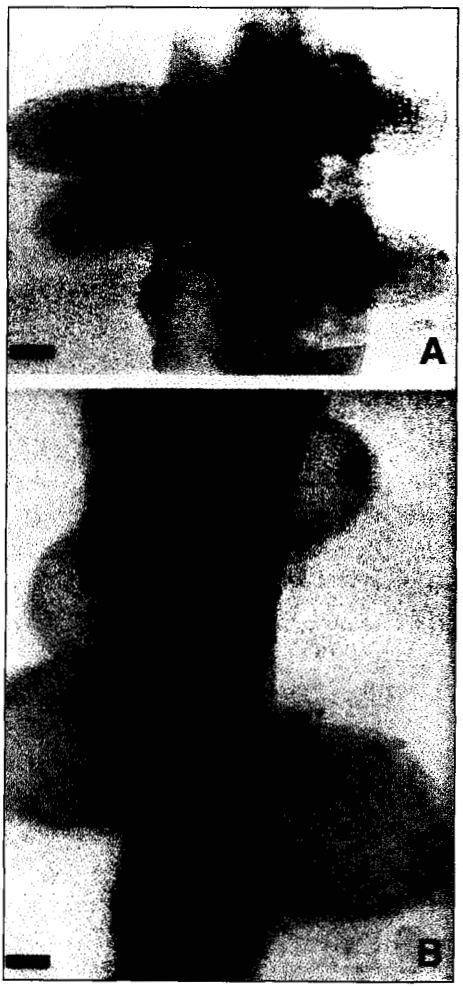

Fig. 4. Morphology of thick, short lateral roots in rice variety Calrose. (A) Control and (B) inoculated with *Rhizobium* IRBG353. Bars = 250 pm in A and 150 pm in B.

Rhizobia-produced IAA, but not *trans*-zeatin or Nod factors, is responsible for the development of thick, short lateral roots

Nod factors produced by rhizobia are clearly responsible for promoting nodule formation in legumes (see Denarie et al 1996). In addition, indole-3-acetic acid (IAA) generated by rhizobia has also been implicated in nodule development (Fukuhara et al 1994, Yuhashi et a1 1995). Because wild-type strains of rhizobia that normally induce TSLR formation in rice have the ability to produce Nod factors as well as IAA, Reddy et al (1997) examined whether rhizobia defective in the production of these bioactive molecules were capable of inducing any phenotypic changes in rice roots. Studies showed that Nod- mutants of *R. meliloti* 102 1 and *B. elkanii* USDA94 induced TSLR in rice plants with the same efficiency as their parent wild-type strains. These results indicated that *nod* genes, required for Nod signal production, do not play a key role in the induction of TSLR in rice. These findings were confirmed by showing that the addition of Nod factors (10^{-9} M) from *Rhizobium* NGR234, supplied to the growth medium every alternate day for 15 d, did not induce TSLR (Reddy et al 1997). Contrary to the above results, *B. elkunii* TN3, a mutant of USDA31, deficient in IAA production but not in Nod factor synthesis, induced TSLR with lower frequency than the parent wild-type strain, suggesting a role for IAA in the promotion of TSLR formation in rice (Reddy et a1 1997).

Along with IAA, rhizobia also produce cytokinins (see Taller and Sturtevant 1991, Torrey 1986). In alfalfa roots, localized *trans*-zeatin production by *R. meliloti* GMI225 (pTZS) induced the formation of nodule-like structures (Cooper and Long 1994). To explore the potential role of cytokinins in the induction of TSLR formation or other changes in root morphology, Reddy et a1 (1997) tested rice plants with a Nod- mutant strain of *R. meliloti* GMI225 with or without the plasmid pTZS that constitutively expresses an isopentenyl transferase gene, enabling continuous *trans*-zeatin secretion (Cooper and Long 1994). The results showed that the presence or absence of plasmid pTZS did not influence the frequency of TSLR formation by *R. meliloti* in rice, suggesting a probable lack of *trans*-zeatin involvement in TSLR formation. Taken together, the above results suggest that rhizobia-produced IAA, but neither *trans*-zeatin nor Nod factors, seems to promote the formation of TSLR in rice (Reddy et al 1997).

Rhizobial invasion and colonization of rice roots are *nod* gene-independent phenomena

Rhizobia are able to invade rice roots through cracks in the epidermis and fissures created during the emergence of lateral roots and colonize intercellular spaces in the epidermis, exodermis, and cortex (Reddy et a1 1997, Webster et a1 1997). No evidence of infection threads was observed (Reddy et a1 1997). This contrasts with the mode of rhizobial infection in legumes, where bona fide infection threads ultimately develop as a route for rhizobia1 intercellular dissemination. Reddy et a1 (1997) found that the wild-type *A. caulinodans* ORS571 and its Nod- derivatives behaved similarly

with regard to the patterns of invasion and colonization within the rice root, indicating a lack of *nod* gene involvement in the processes of infection and colonization. It is intriguing that the application of the flavonone naringenin stimulated significantly the colonization of the cracks at the junction of lateral roots by *A. caulinodans* (Webster et al 1997). It has been suggested that naringenin may be acting as a signal molecule for intercellular colonization by *A. caulinodans* by a mechanism that is *nod* gene-independent, unlike in the legume *Sesbania rostrata.*

Rhizobial colonization evokes a mild defense response in rice roots

Rhizobial infection and colonization of legume nodules do not seem to elicit a significant defense response in the host plant. This may be because of the development of a plant-derived membrane interface between the cells of the host and the infecting or colonizing microsymbiont. Moreover, several sets of bacterial genes (*exo, lps,* and *ndv*) are involved in modifying the chemistry of the bacterial cell wall to disguise the invader and promote establishment within the host (Vance 1990). It is important to understand more about factors that trigger defense responses in rice and to ensure that this response is not elicited by a putative rhizobial symbiont (Bennett and Ladha 1992). In this regard, it is important to mention that, although rhizobia have the ability to invade and colonize rice roots (Reddy et al 1997, Webster et al 1997, Yanni et al 1997), they appear to evoke a localized plant defense response culminating in the dissolution of the lamellar matrix during intercellular colonization or in host cell death upon intracellular infection by the bacterial cells (Reddy et al 1997). Unlike in *Rhizobium*-legume symbiosis, no membrane-enclosed (or compartmentalized) rhizobial cells were found in rice root tissues, and the lack of a membrane interface between plant and rhizobial cells might be responsible for the observed defense responses in the host, as well as in rhizobial cells. Such a defense response could probably be curtailed if rice were programmed to synthesize a membrane interface around the colonizing bacterial cells, akin to the membrane sequestering the bacteroids in legumes.

Rice harbors homologues of legume *ENOD* genes

During legume nodulation, specific plant genes, known as early nodulin *(ENOD)* genes, are induced and are required for the normal development of nodules. If rice can be engineered to interact with rhizobia, one requirement will be the presence of many of the proteins known to be required for legume nodule development. Recently, Reddy et al (l999) showed that cDNAs of several legume *ENOD* genes hybridized to DNA from a wide variety of rice genotypes (Table 2; Fig. 5). Characterization of two of these homologues from rice, *OsENOD40* (Kouchi et al 1999) and *OsENOD93a* (Reddy et al 1998a), revealed open reading frames encoding peptides having considerable homologies to legume *ENOD40* and *ENOD93,* respectively. Although the actual functions of the *ENOD* genes in nodule organogenesis are yet to be deciphered, *ENOD40* has been proposed as playing a pivotal role in the initiation of nodule formation (Mylona et al 1995). Considering the widespread occurrence of *ENOD40* and

Table 2. Semiquantification of hybridization signals obtained in various genomes of *Oryza* species and related monocots after probing with legume *ENOD* cDNAs.

Species	Genome	Acc.	% Hybridization signal[a] (relative to soybean)							
			ENOD2	*ENOD5*	*ENOD12*	*ENOD14*	*ENOD40*	*ENOD55*	*ENOD70*	*ENOD93*
0. sativa	AA	3								
0. rufipogon	AA	5								
0. longistaminata	AA	3								
0. barthii	AA	1								
0. nivara	AA	3								
0. glumaepatula	AA	1								
0. perennis	AA	1	*							
0. punctata	BB	4								
0. punctata	BBCC	3								
0. minuta	BBCC	4								
0. malamphuzhaensis	BBCC?	3								
0. officinalis	CC	6								
0. rhizomatis	CC	3								
0. eichingeri	CC	5		*						*
0. alta	CCDD	4								
0. latifolia	CCDD	4								
0. grandiglumis	CCDD	4								
0. ridleyi	HHJJ	4								
0. longiglumis	HHJJ	4								
0. australiensis	EE	4			*				*	
0. brachyantha	FF	2				*	*	*		
0. granulata	GG	5								
0. meyeriana	GG	3								
0. indandamanica	GG	1								
Porteresia coarctata	.....	1								
Leersia tisseranti	.....	1	*	*		*				
Leersia perrieri	.....	1								*
Rhynchoryza subulata	.....	1					*	*		
Hygroryza aristata	.....	1							*	
Chikusichloa aquatica	.....	1			*					
Maize	.....	1								
Sugarcane	.....	1								
Soybean										

[a]Hybridization signal strengths. 100% (■), 51–75% (▨), 26–50% (▥), less than 25% (□). Asterisks depict the plant species in the *Oryza* complex and in the related genera that gave relatively strongest hybridization signals with respective probes. Denstometric readings were scored from the autoradiographs showing the hybridization signals that did not satulate the denstometer detector response.

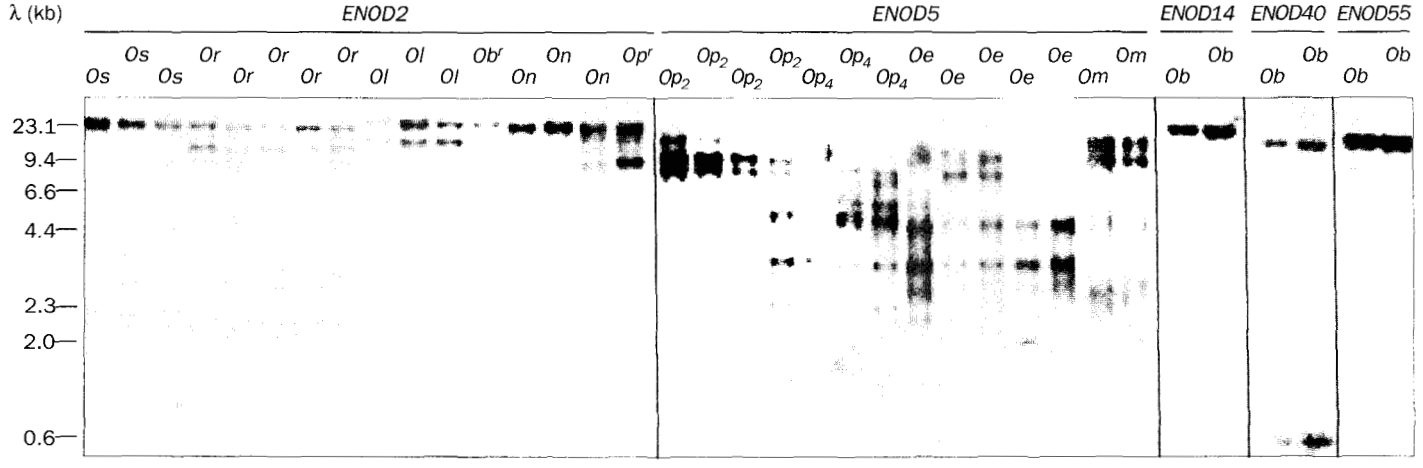

Fig. 5. Southern blots of *Dral*-digested DNA of *Oryza* species that gave strong hybridization signals with ^{32}P-labeled *ENOD* cDNA probes. *Os* = *O. sativa* (AA genome—IR31917, IR56, IR64); *Or* = *O. rufipogon* (AA genome—105908,105909, 105910, 106412, 106423); *Ol* = *O. longistaminata* (AA genome—103886, 103890, 103902); *Ob^r* = *O. barthii* (AA genome—-101937);*On* = *O. nivara* (AA genome—103407,105721, 106185); *Op^r* = *O. perennis* (AA genome—104823);Op2 = *O. punctata* (BB genome—103896, 104064, 105690, 105980); *Op4* = *O. punctata* (BBCC genome—100884,101409. 104975); *Oe* = *O. eichingeri* (CC genome—-101424,105181, 105182, 105408, 105413); *Om* = *O. malamphuzhaensis* (BBCC genome?–105223, 105328); Ob = *O. brachyantha* (FF genome—101232, 94-10482).

the consistent expression of the homologues of this gene in vascular bundles in several plant species, Kouchi et al (1999) suggested that the primary role of *ENOD40* might be related to differentiation or functions of vascular bundles or both.

Rice has a mechanism to perceive Nod factors and possesses a Nod signal transduction system

The demonstration that Nod factors are perceived by rice would be a significant step toward devising strategies to make rice amenable to nodulation. *MtENOD12* gene expression was shown to be an excellent molecular marker for Nod factor perception in alfalfa (Journet et a1 1994). To determine whether Nod factors are recognized by rice, we introduced the *MtENOD12* promoter fused to GUS (b -glucuronidase) in rice and analyzed its responsiveness to Nod factors (Fig. 6A-H; Reddy et a1 1998b). The results of the study showed that the *MtENOD12* promoter was activated in rice root cortical cells, the endodermis, the pericycle, and parenchymatous cells in stele in response to NodNGR factor (10^{-6}–10^{9} M) application (Fig. 6D-F). Werthmuller et a1 (1998) showed that the expression of *MsENOD12* in transgenic rice roots is enhanced by the application of Nod factors in the presence of auxin. These findings imply that rice has a mechanism to perceive Nod factors, and also possesses a signal transduction system to enable subsequent activation of the legume early nodulin promoters. As in legumes, the presence of excess combined nitrogen in the growth medium inhibited the expression of the symbiosis-related gene *ENOD12* in rice in response to Nod factors (Reddy et a1 1998b, Werthmuller et a1 1998). This suggests that Nod factor action on *MtENOD12* expression in rice is controlled by the nitrogen status of the plant, indicating that at least a part of the nitrogen-mediated regulatory mechanism(s) responsible for symbiotic responses in legumes is conserved in rice.

Epidermal cells of rice roots are recalcitrant to Nod factors

NodNGR factors were unable to induce root hair deformation (Reddy et a1 1997). Similarly, NodNGR factors were also ineffective in activating the *MtENOD12* promoter in the epidermal cells, including root hairs of transgenic rice (Fig. 6E; Reddy et al 1998b). The apparent inability of epidermal cells to respond to Nod factors may be due to the absence of the putative cell receptor(s) that perceive them and the absence or repression of Nod signal transduction in these cells. Efforts are now under way to incorporate a putative Nod factor receptor gene into rice to test its ability to induce the response in root hairs and epidermal cells to rhizobia and Nod factors.

Do the epidermis and exodermis form a barrier for Nod factor signal transmission into the interior of rice roots?

In alfalfa, upon inoculation with either *R. meliloti* or purified Nod factors, *MtENOD12* expression occurred specifically in the differentiating root epidermal cells before and during root hair emergence, and its expression gradually shifted to the cortex and

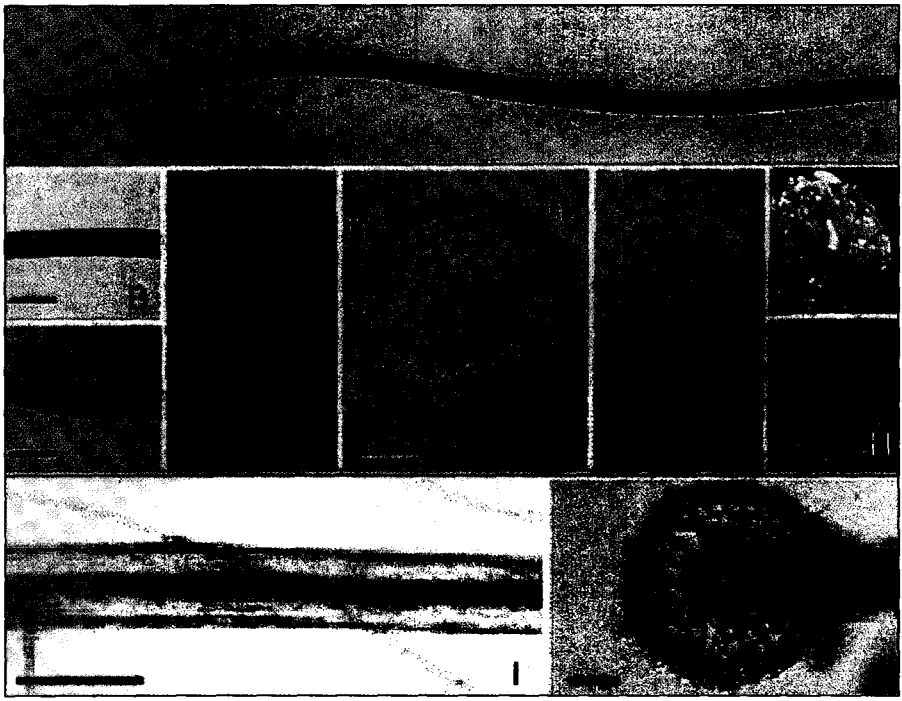

Fig. 6. Histochemical localization of GUS activity in calli and roots of transgenic rice (var. Taipei 309) carrying the (A-H) *MtENOD12*-GUS or (I, J) GmENOD4OGUS fusion.

(A-F) Roots of transgenic rice carrying *MtENOD12*-GUS fusion treated with Nod factors. The Nod factor treatments were with a 10^{-6} M concentration for 24 h unless otherwise stated. (A, B) *MtENOD12*-GUS activity in the elongation zone of roots treated with 10^{-6} M and 10^{-9} M Nod factors, respectively. (C) GUS activity in the vascular trace in the maturation zone of a root. Note enhanced GUS expression at the sites of lateral root emergence. (D) Transverse 80-µm section from the elongation zone of a root showing uneven GUS expression in cortical parenchyma. (E, F) Transverse 60-µm section from the elongation/maturation transition zone of a root showing GUS expression in a cluster of cells in cortical parenchyma, as well as in endodermis, pericycle, and parenchyma cells around vascular elements in the stele. Note the absence of GUS expression in the epidermis, exodermis, and cortical sclerenchymatous cell layer in (E). (F) is a magnification of the stele portion in (E) showing GUS activity in the endodermis, pericycle, and parenchyma cells around the vascular elements in the root. (G, H) Transgenic calli treated (G) without and (H) with 10^{-9} M NodNGR factors for 6 h. (I) Root showing *GmENOD40*-GUS expression in the vascular bundle without Nod factor treatment. (J) Transverse 80-µm section of a root exhibiting *GmENOD40*-GUS expression in the vascular bundle in the absence of Nod factor treatment. Note enhanced GUS expression at the site of lateral root emergence. Symbols: Asterisks = clusters of cortical parenchyma cells; Cs = cortical sclerenchyma; En =endodermis; Ep = epidermis; Ex = exodermis; Pc = pericycle; Vp = parenchyma cells around/adjacent to vascular elements. Bars = 1,000 µm in A. B, C, G, H. and I; 100 µm in D, E, F, and J.

later to mitotically active cortical cell foci as the root matured (Journet et al 1994, Pichon et al 1992, 1994). In rice, on the other hand, NodNGR factors, although they promoted *MtENOD12*-GUS expression in the cortex and stele, they failed to induce *MtENOD12* expression in the exodermal cell layer as well as epidermal cells, including root hairs (Fig. 6E; Reddy et al 1998b). Nevertheless, at any given concentration of Nod factors (10^{-6} – 10^{9} M), the percentage of the roots showing *MtENOD12* expression was much higher when the excised root segments (2-4 cm long) were incubated in the medium containing Nod factors than in the roots still attached to the shoot system (i.e., intact roots) during incubation in the medium supplemented with Nod factors (P.M. Reddy et al, unpublished). In addition, the intensity of the GUS expression was also much higher in the excised roots than in the intact roots.

One of the reasons for the lack of GUS expression in the epidermis and exodermis in response to NodNCR factors may be that the Nod factors are not perceived by the cells of these two outermost layers of the roots, although we cannot rule out the role of other tissue-specific regulatory mechanisms controlling the expression of *MtENOD12*-GUS fusion in these cells. On the other hand, the expression of *MtENOD12*-GUS fusion in cortical parenchymatous cells, the endodermis, and pericycle and its enhancement in parenchymatous tissue surrounding vascular elements clearly demonstrated that the cells in these internal tissues have the ability to perceive Nod factors (Fig. 6A-F; Reddy et al 1998b). In spite of this ability, however, lower GUS intensity in these internal tissues in uncut roots exposed to Nod factors may be because either the Nod factor signal is not effectively transduced through the epidermis and exodermis or these cell layers probably form a barrier for Nod factor diffusion into the internal tissues of the roots. In contrast, intense GUS staining in the internal tissues when the excised root segments were exposed to the Nod factors indicates that this enhanced GUS expression is probably promoted because of the facilitation or establishment of direct contact between the signal molecules and the cells in these tissues due to diffusion of the Nod factors through the cut ends of the roots. Indeed, in alfalfa, Nod factor signal-dependent triggering of the *MtENOD12* gene is not a systemic response and requires direct contact between the signal molecule and the target plant cell (Journet et al 1994).

Auxins and cytokinins also modulate expression of *ENOD12* in rice

In *Medicago truncatula, MtENOD12* is responsive only to Nod factors. Neither auxins nor cytokinins induce its expression (D.G. Barker, personal communication). Nonetheless, like Nod factors, cytokinins also promoted the expression of some early nodulin genes, such as *ENOD2, MsENOD12A,* and *ENOD40,* involved in nodule development in legumes (Bauer et al 1996, Dehio and de Bruijn 1992, Hirsch and Fang 1994, Minami et al 1996a,b). In alfalfa, however, *MsENOD12A* also responded to auxins in addition to cytokinins and Nod factors (Bauer et al 1996). Based on these results, researchers inferred that cytokinins and auxins, besides Nod factors, take part in the signal transduction cascade responsible for nodule organogenesis in legumes. To ascertain whether auxins and cytokinins influence the expression of *MtENOD12*

in rice, Reddy et al (1998b) subjected transgenic plants to treatment with N-naphthaleneacetic acid (NAA, 5 μM), kinetin (10 μM), or 6-benzylaminopurine (10 μM) and analyzed them for GUS expression in roots. NAA mimicked the Nod factor-elicited tissue-specific expression of *MtENOD12* in cortical parenchyma cells, the endodermis, and pericycle in the elongation region of roots except that, unlike with the Nod factor treatment, the NAA-induced expression in cortical parenchyma was uniform and never restricted to clusters of cells. On the other hand, cytokinins did not induce *MtENOD12*-GUS expression in the rice root tissues. Furthermore, even the NodNGR factors (10^{-6} M), irrespective of whether they were supplemented simultaneously with or subsequent to treatment with cytokinins, failed to induce GUS expression in roots treated with cytokinins. These findings suggest that cytokinin-mediated processes interfere with Nod factor-elicited *MtENOD12* expression in rice. Taken together, these results indicate that, in rice, Nod factors, NAA, and cytokinins may act on similar signaling elements that modulate *MtENOD12* expression.

Tissue-specific expression of *ENOD40s* is similar in both rice and legumes

To ascertain whether regulation mechanisms related to the activation of *ENOD40* expression are shared between rice and legumes, we developed transgenic rice carrying the *GmENOD40*(2) promoter fused to GUS and analyzed the activity of the promoter in rice (P.M. Reddy, J.K. Ladha, S.K. Datta, and G. Stacey, unpublished). Similar to the situation in legumes (Corich et al 1998, Kouchi and Hata 1993, Papadopoulou et al 1996, Yang et al 1993), in rice, expression of the *GmENOD40*(2) promoter was confined to the vascular traces of the root and stem, though the induction of *GmENOD40*(2) is greater in the vascular bundles at the sites of lateral root emergence than in the vascular traces away from those sites (Fig. 6I,J). In a separate study, Kouchi et al (1999) examined the induction of rice *ENOD40* in soybean roots and found that its expression was also confined to the vascular tissue of roots and nodules, thus evidencing that the pattern of expression was essentially the same as that of soybean *ENOD40* (Kouchi and Hata 1993, Yang et al 1993). Together these findings indicate that legume and rice *ENOD4Os* share a similar regulatory mechanism(s). In addition, these results substantiate our earlier findings with the *MtENOD12* promoter that the signal transduction machinery essential for legume *ENOD* gene expression is conserved, at least partially, in rice.

Does rice possess a mechanism to maintain a regulated supply of oxygen to sustain nitrogenase activity in rhizobia?

A regulated supply of oxygen to rhizobia is essential for maintaining nitrogenase activity. A low but constant supply of oxygen is maintained in legume nodules via the action of leghemoglobin. Recently, it has been demonstrated that the interior of rice roots is conducive to the expression of nitrogenase genes by endophytic diazotrophs such as *Azoarcus* sp. and *Herbaspirillum seropedicae* (Egener et al 1998, E.K. James et al, personal communication). It is still not known whether the internal environment

of rice roots is also favorable to rhizobial nitrogen fixation. If not, then a leghemoglobin-like system may need to be incorporated in rice to support nitrogen fixation by rhizobia. Actually, globin genes are widespread in nonleguminous plants, including rice (Arredondo-Peter et a1 1997), and encode plant hemoglobins of cryptic function (Landsmann et a1 1986). Interestingly, in *Parasponia* nodules, a class I hemoglobin gene, which has very high homology to nonsymbiotic hemoglobins, is recruited to perform the symbiotic function (Bogusz et al 1988). Recently, a hemoglobin gene that is very closely related to symbiotic leghemoglobin genes has been identified in the nonlegumes *Arabidopsis thaliana* and *Brassica napus,* suggesting that hemoglobin genes homologous to symbiotic hemoglobin genes may be present in a wide range of plant species (see Trevaskis et al 1997). It is yet to be determined whether rice possesses a hemoglobin gene that is closely related to a symbiotic hemoglobin gene. If present, it would be useful to determine whether it is expressed at the site of symbiosis and whether the hemoglobin protein can function as an oxygen regulator. If not, necessary amendments may have to be made to incorporate a leghemoglobin-like system to support symbiotic nitrogen fixation in rice.

Conclusions

In legume-*Rhizobium* symbiosis, the host plant provides the genetic information for the development of nodules. The role of the rhizobia is to trigger the host plant genes to facilitate infection and help create a nodular niche for N_2 fixation (see Denarie et al 1996, Long 1996). A monocot plant such as rice would unlikely possess the complete complement of genes or genetic programs involved in the nodule ontogeny program that could be induced by rhizobial strains. A reason for optimism, however, is that, although rice does not develop a symbiotic association with rhizobia, it is able to enter into symbiotic associations with mycorrhizal fungi (see Khan and Belik 1995). Genetic links between the processes involved in nodulation and arbuscular mycorrhizae have been found in legumes (Albrecht et al 1998,1999, Denarie and Gianinazzi-Pearson 1997, Gianinazzi-Pearson 1996). Studies on nodulation mutants of pea have demonstrated that the early nodulin genes *ENOD2, ENOD11, ENOD12,* and *ENOD40,* which control the initial steps of nodulation, also govern the early stages of mycorrhiza development (van Rhijn et al 1997). Thus, because rice is able to form symbiotic associations with mycorrhizal fungi, and because the formation of such an association of mycorrhizal fungi with legumes is mediated by *ENOD* genes, we can infer that at least some of the genetic machinery required to promote endosymbiosis with rhizobia likely exists and functions in rice. Our studies revealed that rhizobial Nod factors are able to induce expression of the legume *ENOD12* promoter in rice, thus strongly suggesting that at least a portion of the signal transduction machinery important for legume nodulation exists in rice (Reddy et a1 1998b). In addition, we also demonstrated that homologues of *ENOD* genes are conserved, probably to varying extents, in all *Oryza* species (Kouchi et a1 1999, Reddy et a1 1998a, 1999). Among various homologues of *ENOD* genes detected in rice, the *ENOD40* homologue is of particular interest as it was proposed as playing a pivotal role in nodule development

in legumes (Mylona et al 1995). Taken together, these findings suggest that the genetic machinery regulating nodule development in legumes is conserved, at least partially, in rice.

In conclusion, certain critical differences exist between the association of rice and rhizobia relative to the root-nodule symbiosis with legumes. Some of the molecular interactions that occur in these plant-microbe associations, however, may be similar. It is therefore essential that studies be further extended at the cellular and molecular levels to identify why such responses do not occur fully in rice in order to contemplate genetically engineering this major cereal crop to form a more intimate endosymbiotic association with rhizobia.

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Notes

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Characterization of rice *ENOD40*: Do *ENOD40s* accomplish analogous functions in legumes and nonlegumes?

Hiroshi Kouchi, Ken-ichi Tahane, Rolando B. So,
Jagdish K. Ladha, and Pallavolu M. Reddy

We have isolated the *ENOD40* gene homologues *ObENOD40* and *OsENOD40* from the wild and cultivated rice genotypes *Oryza brachyantha* and *O. sativa,* respectively. Rice *ENOD40s* contain two nucleotide sequence domains, regions I and II, which are highly conserved in all legume *ENOD40s.* Region I of rice *ENOD40s* potentially encodes an oligopeptide that is similar to those found in legume *ENOD40s.* The expression of *OsENOD40* was detected only in stem vascular bundles. Detailed *in situ* hybridization studies revealed that transcription of *OsENOD40* is confined to parenchyma cells surrounding the protoxylem during the early stages of development of lateral vascular bundles of the stem. The expression pattern of *OsENOD40* promoter-GUS fusion in nodules developed on transgenic hairy roots of soybean was also restricted to peripheral cells of nodule vascular bundles, thus presenting evidence that the regulation mechanism of *ENOD40* gene expression is well conserved through legumes and the very distantly related monocot, rice. These results strongly suggest that *0sENOD40* and legume *ENOD40s* share common, if not identical, roles in differentiation or function of vascular bundles or both.

The development of legume nodules is accompanied by the activation of a unique set of plant genes termed "nodulin" genes (van Kammen 1984). Nodulin genes are classified into early and late nodulin genes according to the timing of their induction during nodule development (Nap and Bisseling 1990, Venna et al 1986). Early nodulin genes, which are expressed at the very early stages of nodule development, have been postulated to mediate rhizobial infection and nodule organogenesis.

Among early nodulin genes *(ENOD)* that are activated rapidly upon rhizobia1 inoculation, *ENOD40* was expsessed very early during nodule initiation (Kouchi and Hata 1993, Yang et al 1993). In fact, rhizobia-secreted lipochitin signal molecules (Nod factors) alone were sufficient to elicit *ENOD40* expression (Minami et al 1996, Vijn et al 1995). *ENOD40* is first induced in the root pericycle within a few hours after inoculation with Nod factors alone or bacteria, and its expression is subsequently extended to the dividing cortical cells (Kouchi and Hata 1993, Minami et al 1996, Vijn et al 1995, Yang et al 1993). An intriguing feature of *ENOD40* is that, although

its nucleotide sequence is highly conserved in many legumes, only a small oligopeptide of 12–13 amino acids seems to be a possible translation product.

The exact function of *ENOD40* in nodule development has yet to be determined. Charon et a1 (1997) showed that transgenic *Medicago truncatula* overexpressing ENOD40 exhibited extensive induction of spontaneous cortical cell divisions in its roots. They also indicated that micro-injection of *ENOD40* cDNA into *Medicago* roots induces the expression of *ENOD12* that is activated before the formation of nodule primordia. Thus, the involvement of *ENOD40* was implicated in triggering cortical cell divisions during the beginning of nodule morphogenesis. *ENOD40* has also been implicated, however, in the late stages of nodule development and/or symbiotic functioning because its expression persists abundantly in the peripheral cells of vascular bundles in fully mature nodules (Kouchi and Hata 1993, Yang et a1 1993).

Nodulin genes have been defined as plant genes that are induced exclusively during nodule formation in legume plants (van Kammen 1984). Recent studies, however, revealed the existence of several structural and functional homologues of nodulin genes in legumes and nonlegumes, such as those of leghemoglobin (Andersson et a1 1996, Amedondo-Peter et a1 1997, Jacobsen-Lyon et a1 1995, Trevaskis et a1 1997), uricase II (Takane et a1 1997), and *ENOD93* (Reddy et a1 1998). Thus, Andersson et al (1996) and MyIona et al (1995) hypothesized that nodulin genes have arisen as a result of the recruitment of preexisting nonsymbiotic genes, which might have roles common to all plants. Here we describe the isolation and characterization of *ENOD40* homologues from rice. Our findings, together with the previous identification of *ENOD40* from tobacco (van de Sande et al 1996), strongly suggest that functional *ENOD40* genes are common in the plant kingdom.

Regions I and II of legume *ENOD40s* are also conserved in rice *ENOD40s*

To identify prospective rice species for the isolation of *ENOD40* homologues, we first performed a genomic Southern blot analysis of DNA prepared from various rice genotypes using soybean *ENOD40* cDNA as a probe. Among 80 rice genotypes tested, DNA from two wild rice species, *Oryza brachyantha* and *O. australiensis,* gave strong hybridization signals (Reddy et a1 1999). Therefore, a genomic library of *O. brachyantha* was screened with a soybean ENOD40 probe. As a consequence, we isolated *ObENOD40,* in which an *Xba*I fragment (ca. 640 bp) that hybridized with the soybean *ENOD40* cDNA contained a sequence for encoding an oligopeptide that is highly conserved in all legume *ENOD40s.* This fragment was subsequently used to screen a genomic library of *O. sativa* (var. Nipponbare) to isolate *OsENOD40.*

The nucleotide sequences of the *Xba*I fragments of *OsENOD40* and *ObENOD40* are 87% identical (Fig. 1). We generated and cloned *OsENOD40* cDNA using mRNA prepared from rice stems by means of 5'- and 3'-RACE techniques. The sequences of 5'- and 3'-RACE products were identical with those expected from the genomic sequence, indicating that the *OsENOD40* gene, like legume *ENOD40* genes, does not contain introns.

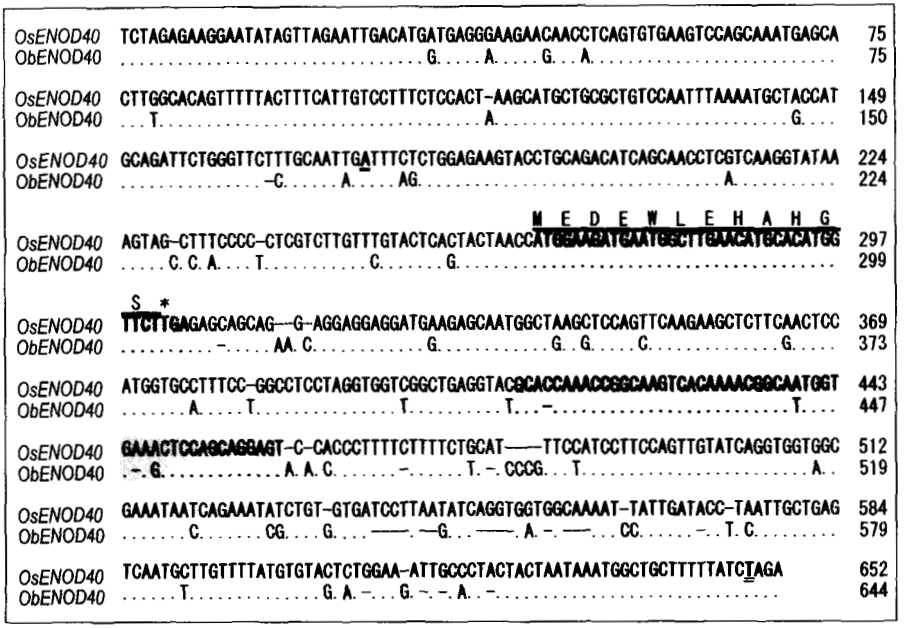

Fig. 1. Nucleotide sequences of XbaI fragments of *OsENOD40* and *ObENOD40.* Region I (with predicted peptide sequence) and region II are shaded. The 5' end and polyadenylation site of mRNA determined by 5'- and 3'-RACE are indicated in boldface with an underline and double underline, respectively.

Southern blot of rice DNA digested with *Eco*RI or *Xba*I and probed with *OsENOD40* cDNA displayed, in each case, only one hybridizing fragment under high-stringency conditions (Fig. 2). On the other hand, DNA digested with either *Dra*I or *Pst*I revealed two prominent hybridizing bands in each case. Analysis of the genomic sequence of *OsENOD40* showed no *Eco*RI site but revealed an internal site for both *Dra*I and *Pst*I restriction enzymes. Thus, Southern blot results in conjunction with sequence data clearly show the existence of only a single copy of *OsENOD40* in the rice genome.

Comparison of the nucleotide sequences of legume and tobacco *ENOD40s* revealed the presence of two highly conserved regions, which were termed regions I and II (van de Sande et al 1996). A remarkable feature of both rice *ENOD40s,* notwithstanding their low homology to legume and tobacco *ENOD40s,* is the presence of two nucleotide stretches in them that are significantly homologous to regions I and II in legume and tobacco *ENOD40s.* Figure 3 compares rice *ENOD40* regions I and II together with their respective deduced amino acid sequences with those in legume and other nonlegume *ENOD40s.* A sequence of *ENOD40* of *Zea mays* (*ZmENOD40*), found in a DNA database as an expression sequence tag, showing high homology to the rice *ENOD40* sequence is also included in the comparison. It is evident from the comparison that region I of *ENOD40s* of all three monocotyledons, *O. sativa, O. brachyantha,* and *Z. mays,* encodes a 12-amino-acid-long peptide that is considerably similar to those encoded by legume and tobacco *ENOD40s.*

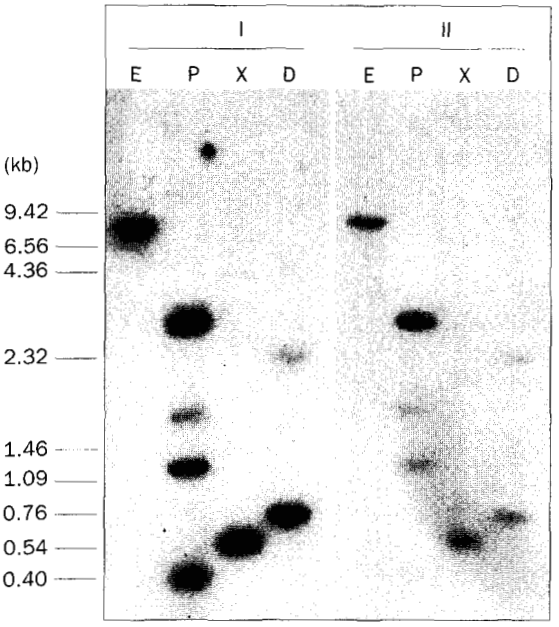

Fig. 2. Southern blot analysis of rice DNA with *OsENOD40.* Genomic DNA (10 mg) from an *O. sativa* callus was digested to completion with *EcoRI* (E), *PstI* (P), *XbaI* (X), or *DraI* (D) subjected to 0.8% agarose gel electrophoresis and probed with *OsENOD40* cDNA. The hybridization filter was washed under low-stringency conditions (panel I) and then under high-stringency conditions (panel II.

ENOD40s carry a second conserved sequence, region 11, located in the central part of the mRNA. In all *ENOD40s,* including those from rice, the nucleotide sequence of this region is more highly conserved than in region I, but it lacks a discernible open reading frame and coding capacity. Although an oligopeptide sequence derived for region II appears to be well conserved in most legumes with indeterminate nodules and in tobacco, the sequences encoding a predicted peptide are interrupted by a stop codon in the middle of region II of rice *ENOD40s.* In addition, rice has no in-frame start codon upstream in region II. In spite of the paucity of coding capacity, expression of the sequence comprising region II of legume *ENOD40* is able to induce cortical cell divisions in alfalfa roots, as can region I, thus suggesting that untranslated *ENOD40* mRNAs from region II exert biological activities (Charon et a1 1997).

Region I of *ENOD40s* potentially encodes an oligopeptide of 12 amino acids in legumes that form determinate nodules, 13 amino acids in those forming indeterminate nodules, 10 amino acids in a nonlegume, tobacco, and 12 amino acids in rice and maize. Obviously, the small peptide encoded by region I is the only possible translation product common to all legume and nonlegume *ENOD40s.* Competition studies with enzyme-linked immunosorbent assays using antibodies against a synthetic oligopeptide suggested the existence of the region I-derived peptide in soybean nodule extract (van de Sande et al 1996, H. Kouchi, unpublished data). Although transient expression of the *ENOD40* fragment spanning region I provoked cortical cell division in alfalfa roots (Charon et a1 1997), there is no direct evidence for biological activity of the region I oligopeptide. A recent study using transgenic white clover carrying an auxin-responsive promoter *(GH3)-GUS* fusion failed to show involve-

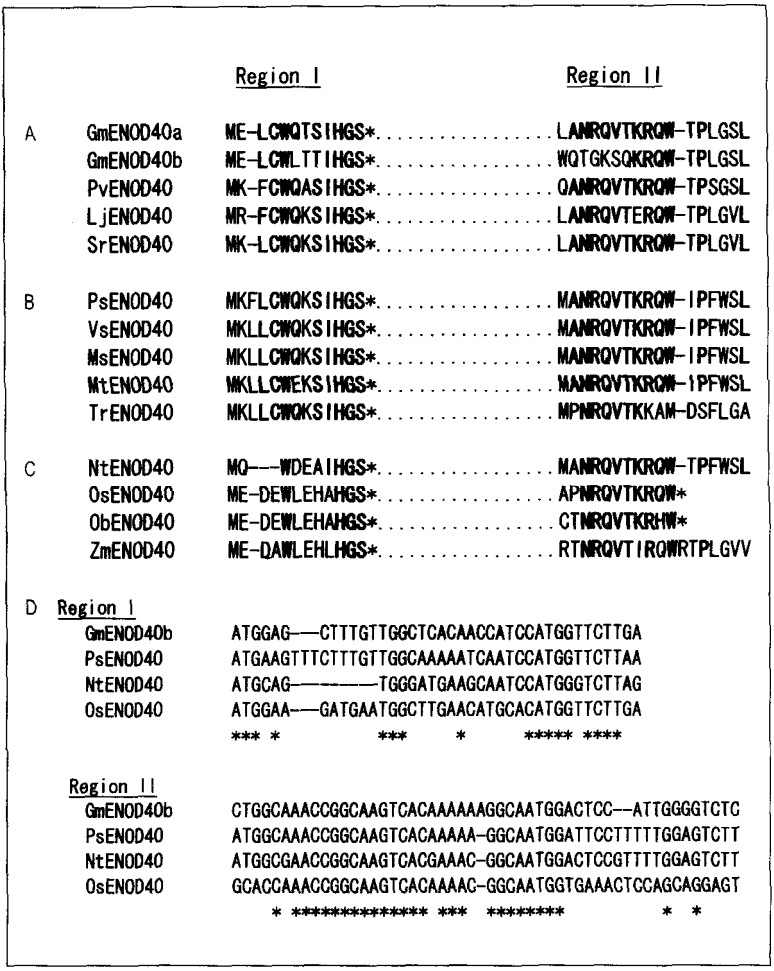

Fig. 3. Comparison of predicted amino acid sequences for regions I and II of *ENOD40s* from various legumes and nonlegumes. (A) Legumes with determinate nodules, (B) legumes with indeterminate nodules, (C) nonlegumes, (D) comparison of nucleotide sequences of regions I and II of legume and nonlegume *ENOD40s.* Gaps (-) were introduced to optimize alignments. Asterisks in (A-C) indicate stop codon, asterisks in (D) depict conserved nucleotides. Plant species and database accession numbers are as follows: Gm, Glycine max (a, D13503, X69155; b, D13504, X69154); Pv, *Phaseolus* vulgaris (X86441); Lj, *Lotus japonicus* (Kouchi, unpublished); Sr, *Sesbania rostrata* (Y12714); Ps, *Pisum sativum* (X81064); Vs, *Vicia sativa* (X83683); Ms, *Medicago sativa* (X80263); Mt, *Medicago truncatula* (X80264); Tr, *Trifolium repens* (AJ000268); Nt, *Nicotiana tabacum* (X98716); Os, *Oryza sativa* (this work); Ob, *O. brachyantha* (this work); Zm, *Zea mays* (W21740, A1001271).

ment of the region I peptide in altering the internal auxin concentration (Mathesius et al 1998). Thus, identification of the oligopeptide encoded by region I in plant tissues and its biological activity are yet to be elucidated.

OsENOD40 expression is localized in xylem parenchyma cells in rice stems

A northern blot analysis of RNAs prepared from various organs of rice plants showed that *OsENOD40* is expressed only in stems through the vegetative to reproductive stages of growth. Therefore, we performed in situ hybridization studies to localize the expression of *OsENOD40* in rice stems. Hybridization of stem sections, derived from 26-d-old rice seedlings that were at the 4th leaf stage, with digoxigenin-labeled antisense *OsENOD40* mRNA showed that *OsENOD40* expression is restricted to lateral (large) vascular bundles (Fig. 4A). In contrast, intermediate (small) vascular bundles and anastomosing bundles (nodal plexus) in the stem as well as incipient vascular traces near the shoot apex exhibited no hybridization signals at all. Observa-

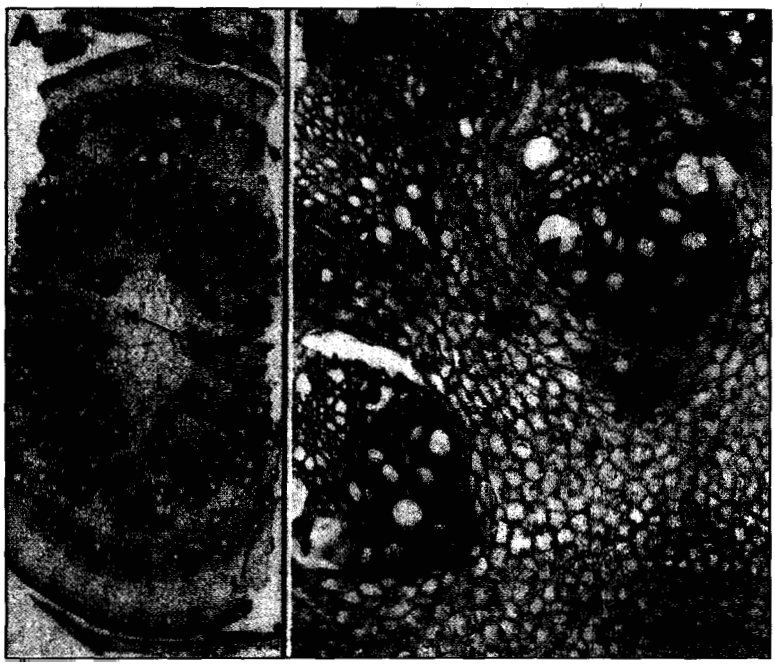

Fig. 4. Localization of *OsENOD40* mRNA in the rice stem. Stem sections from a 26 d-old rice seedling were hybridized with digoxigenin-labeled antisense RNA of *OsENOD4O.* Dark-purple color denotes hybridization signals. (A) A transverse section of the stem internode. L = lateral (large) vascular bundle, I = intermediate (small) vascular bundle, N = nodal plexus (anastomosing bundle). Note that only some lateral vascular bundles (L, arrow) exhibit positive signals. Bar = 50 μm. (B) Higher magnification of a lateral vascular bundle showing the expression of *OsENOD40 in* xylem parenchyma surrounding protoxylem. Px = protoxylem, Mx = metaxylem, St = sieve tubes. Bar = 25 μm.

tions at a higher magnification revealed that *OsENOD40* transcripts in the lateral vascular bundles are located exclusively in the xylem parenchyma cells surrounding the protoxylem (Fig. 4B). We noticed, however, that, in a single transverse section of a stem, not all lateral vascular bundles exhibited positive hybridization signals.

In rice, the network of the stem vascular system is very complex, particularly during the vegetative growth phase because of retarded elongation rates of the stem internodes. Thus, a single transverse section of a stem represents an array of vascular bundles that are at different stages of development. The differential expression of *OsENOD40* among lateral vascular bundles in a single stem section indicated that the expression of *OsENOD40* is coupled to specific developmental stages of the lateral vascular bundle. To delineate the developmental stage(s) of lateral vascular bundles that showed abundant expression of *OsENOD40,* we performed in situ hybridization studies in greater detail using contiguous transverse sections of stems obtained from rice plants at the 8th leaf stage. In the plants examined, the 8th leaf blade was in the mid-expansion stage, whereas the 9th leaf, though already emerged, had not begun expanding. In the stem sections of these plants, *OsENOD40* transcripts were detected only in the lateral vascular bundles that conjoin the newly forming 9th leaf, whereas, in contrast, the lateral vascular bundles connecting the expanding 8th leaf did not exhibit any *OsENOD40* transcripts. These findings demonstrated that *OsENOD40* is expressed only at the early stages of the development of lateral vascular bundles that conjoin the emerging leaf, and its expression is down-regulated after the onset of leaf expansion.

Regulation of *OsENOD40* gene is similar to that of legume *ENOD40*

Our study showed that, in rice, the expression of *OsENOD40* is induced exclusively in the developing lateral vascular bundles in the stem. In legumes, the expression of *ENOD40* occurs predominantly in the vascular bundles of mature nodules (Kouchi and Hata 1993, Yang et a1 1993), but substantial expression is detected in the stems as well. Because the legume root nodules show "stem-like'' anatomy (Sprent and Raven 1992), it was of interest to study the expression behavior of the *OsENOD40* promoter in legume nodules. For this purpose, a fusion construct containing the *OsENOD40* promoter and b -glucuronidase (GUS) reporter gene was made and transgenic nodules carrying this fusion gene were developed on soybean hairy roots employing the *Agrobacterium rhizogenes* -mediated transformation technique. Strong GUS activity was observed in the peripheral cells of the vascular bundles of the nodules. The expression pattern of *OsENOD40-GUS* in transgenic soybean nodules was essentially the same as that of the soybean *ENOD40-GUS* construct and was in good agreement with the results of *in situ* hybridization of soybean *ENOD40* mRNAs (Kouchi and Hata 1993, Yang et al 1993). In turn, when soybean *ENOD40-GUS* fusion was transformed into rice, GUS activity was also detected specifically in root and stem vascular traces (P.M. Reddy, J.K. Ladha, and S.K. Datta, unpublished data). These findings clearly indicate that legume and rice *ENOD40s* share similar, if not identical, regulatory mechanisms. A preliminary sequence comparison of the 5'-flanking region of

OsENOD40 and *GmENOD40* revealed the presence of several motifs common to both sequences.

Possible functions of *ENOD40* common in legumes and nonlegumes

The expression of legume *ENOD40* is not confined exclusively to the nodules because it is also induced in other parts of the plant such as the stems and roots. In soybean stems, significant levels of *ENOD40* transcripts have been localized in the cells adjacent to the secondary phloem as well as in the vascular cambium (Kouchi and Hata 1993, Yang et a1 1993). In roots of *Sesbania rostrata* and *Phaseolus vulgaris*, the expression of *ENOD40* was detected in the pericycle and parenchyma cells in the vascular bundles of primary and lateral roots, respectively (Corich et a1 1998, Papadopoulou et a1 1996). The possible functions of legume *ENOD40* have been argued mainly in favor of the induction of cortical cell divisions that lead to the initiation of nodule primordia (see Mylona et a1 1995). The expression of *ENOD40* in the root pericycle, however, is not necessarily accompanied by cortical cell divisions (Minami et a1 1996). Further, even during the late stages of nodule development, *ENOD40* continues to be expressed abundantly in nodule vascular bundles in fully mature nodules. These findings imply that *ENOD40* has a primary function other than inducing cortical cell divisions. *OsENOD40* and legume *ENOD40s* have a common feature in that their expression is most abundant in vascular bundles, indicating that they share common roles in differentiation or function of vascular bundles or both.

OsENOD40 expression is detected only in the developing vascular bundles in the stem. In rice, the formation of vascular bundles initiates just below the leaf primordium and develops both acropetally into the developing leaf and basipetally into the stem internode (Kawahara and Chonan 1968). *In situ* hybridization studies indicated that the expression of *OsENOD40* appears to be associated only with basipetal differentiation of the vascular bundles. Examination of contiguous transverse sections of rice stems demonstrated that *OsENOD40* transcripts are localized only in developing lateral vascular bundles that conjoin a newly emerging leaf. In a developing vascular bundle of rice, this early stage is accompanied by an increase in the number of protoxylem and metaxylem poles that eventually culminate in the maturation of a vascular bundle (Kawahara and Chonan 1968). Thus, the expression of *OsENOD40* specifically at the stage of differentiation of protoxylem and metaxylem poles indicates that it may have a function in the differentiation of vascular bundles. This speculation is further supported by the observation that a *Lotus japonicus* mutant, *albl,* which forms ineffective nodules without any apparent expression of *ENOD40,* shows incomplete differentiation of nodule vascular bundles (H. Imaizumi-Anraku, M. Kawaguchi, H. Kouchi, submitted).

An alternative possible function of *ENOD40* is its role in transporting photosynthates or other solutes as postulated earlier (Kouchi and Hata 1993, Yang et a1 1993). In this regard, it is noteworthy that, in rice, *OsENOD40* expression is confined to parenchyma cells in lateral vascular bundles that join the newly forming leaf. Such

a nascent leaf, which is enveloped by a preceding leaf sheath, is a strong sink for photosynthates and other solutes from mature leaves and underground parts. Moreover, the xylem parenchyma in rice stems and the pericycle in nodule vascular bundles have been suggested as performing an essential role in the transport of solutes (Chonan et a1 1985, Pate et al 1969). Thus, the expression of *OsENOD40* in parenchyma cells in developing vascular bundles may be implicated in the strong sink activity caused by a newly emerging leaf.

Extensive transgenic studies together with detailed analyses of promoter sequences of legume and nonlegume *ENOD40s* may provide more insights into *ENOD40* functions and regulation mechanisms common to legumes and nonlegumes.

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Notes

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Chitin perception in legumes and rice: What distinguishes a nodulating plant?

Gary Stacey, R. Bradley Day, Pallavolu M. Reddy, Jonathan Cohn, Serry Koh, Mitsuo Okada, Yuki Ito, Naoto Shibuya, and Jagdish K. Ladha

We are interested in using *Rhizobium*-legume symbiosis as a model for developing a comparable nitrogen-fixing symbiosis in rice. Our method is to first identify recognition steps critical to the development of the symbiosis between rhizobia and legumes. Once these steps are identified, we can then ask whether rice possesses the same ability as the legume. If we can identify traits that are unique to legume nodulation, we can develop a rational approach for creating a rice plant with the ability to form a symbiosis with rhizobia. This comparative approach has the added benefit of identifying those unique features of legumes that permit a nitrogen-fixing symbiosis.

Nod signals are substituted lipo-chitin molecules produced by rhizobial *nod* gene products and are essential for nodulation. Previous work suggested that Nod signal recognition involves at least two recognition events, perhaps mediated by two different receptors. Our work in soybean suggests that one receptor can recognize the Nod signal but also responds to simple chitin oligomers. The second receptor appears to require the specific chemical structure of the Nod signal. Binding studies with ^{125}I-labeled chitin oligomers identified a chitin-binding site on the plasma membrane of soybean and rice that shows similar specificity and binding parameters. Binding to this "receptor" correlates with the activation of plant defense responses (e.g., oxidative burst) in both soybean and rice. We have also isolated cDNA clones from a variety of legumes and rice whose sequence is similar to an apyrase recently shown to be a Nod signal-binding protein in *Dolichos biflorus.* The presence of this protein in rice could explain the ability of rice to recognize Nod signals, as evidenced by the induction of *ENOD12*-GUS fusion.

The traditional view is that chitin, a polysaccharide composed of ß-1→4-linked N-acetylglucosamine, is found in fungal cell walls, insect exoskeletons, and the hard carapace of crustaceans. In all of these locations, chitin is thought to play a structural role. Compelling evidence now exists, however, that chitin can also act as a signal molecule. For example, chitin oligomers, presumably released from fungal cell walls, act as elicitors of a plant defense response against invading fungal pathogens (reviewed in Stacey and Shibuya 1997). Similarly, substituted lipo-chitin molecules pro-

duced by rhizobia *are* the signals that induce nodulation on legumes (reviewed in Cohn et a1 1998). These latter studies have led people to question whether chitin may also be present and functioning in other organisms. For example, it is now well accepted that several vertebrates (e.g., fish and frogs) make chitin (reviewed in Wagner 1994). Indeed, Wagner (1994) has proposed that the ability to make chitin is likely an ancient trait.

An exciting possibility is that chitin oligomers are an undiscovered class of signal molecules in plants and animals. For example, DG42 protein, from the frog *Xenopus laevis,* shows sequence similarity to chitin synthase and is specifically expressed during frog embryogenesis (Semino and Robbins 1995). A similar chitin synthase ortholog in zebra fish has been shown to synthesize chitin in vitro (Semino et al 1996). In plants, De Jong et a1 (1992) demonstrated that a temperature-sensitive line, ts11, of carrot, defective in embryogenesis, could be "rescued" by chitinase. Subsequently, it was shown that the addition of rhizobial lipo-chitin molecules could rescue the embryogenesis defect of this carrot line (De Jong et a1 1993). Could chitin or lipo-chitin molecules play an important developmental role in both plant and animal embryogenesis? Such speculation has led some to suggest that rhizobial lipo-chitin molecules could be mimics of endogenous plant substances important for plant growth and development (eg, Spaink et a1 1993). We favor such a hypothesis, although definitive proof is still lacking. If this hypothesis is correct, several assumptions would follow. For example, if chitin molecules are playing an important developmental role in plants, then (1) all or most plants should produce such chitin molecules and (2) all or most plants should have the ability to recognize and respond to such chitin molecules. Of course, every new hypothesis leads to a variety of new questions. For example, if all plants make and respond to chitin, then what distinguishes legumes, which respond to rhizobial Nod signals by producing a nodule structure, from plants such as rice that are not nodulated? Could the differences lie in their ability to recognize chitin molecules and in the mode by which they react to such recognition?

Rice nodulation?

Nodulation of rice by rhizobia may still be an impossible dream. As outlined below and also described in other chapters of this volume, however, recent surprising results suggest that rice does possess many of the abilities that, only a few years ago, we would have attributed strictly to legumes. Our approach is to proceed step-by-step to compare and contrast legumes with rice to identify those steps in the nodulation process that may be absent in rice. Once identified, these steps would be obvious engineering targets for developing a nodulating rice plant.

Lipo-chitin Nod signals

Structure

Table 1 lists the known lipo-chitin Nod signals produced by rhizobia. In all cases, these molecules are substituted chitin oligomers, usually of four to five GlcNAc resi-

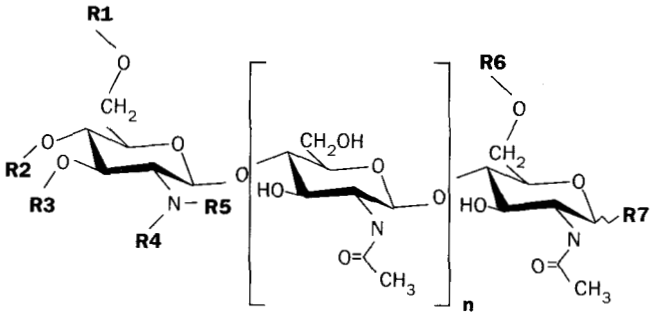

Table 1. Chemistry of some of the known Nod signals.

Species	n	R1	R2	R3	R4	R5	R6	R7	Ref.[a]
Bradyrhizobium japonicum	23	Ac	H	H	C18:1(9) C18:1(9,Me) C16:1(9) C16:0	H	2-OMeFuc	H	1,2
B. elkanii	23	Ac	Cb	H	C18:1(11)	Me,H	2-OMeFuc or Fuc	Glyceryl	2,3
Rhizobium sp. NGR234	3	Cb or H	Cb or H	Cb or H	C18:1(11) C16:0 C16:1	Me	3-*O*-SO_3H, 2-*O*-MeFuc, or 4-*O*-Ac, 2-*O*-MeFuc	H	4
Sinorhizobium fredii (USDA 257)	1,2,3	H	H	H	C18:1(11)	H	2-*O*-MeFuc or Fuc	H	5
Azorhizobium caulinodans	2,3	Cb	H	H	C18:1(11) C18:0(11)	Me	Ara or Fuc	H	6
R. loti	3	H	Cb	H	C18:1(11) C18:0	Me	4-O-A Fuc	H	7
R. etli	3	H	Cb or H	Cb or H	C18:1(11)	Me	4-O-AcFuc	H	8,9
R. tropici	3	H	H	H	C18:1(11)	Me	SO_3H	Mannosyl	10
S. meliloti	1,2,3	Ac	H	H	C16:2(2,9) C16:3(2,4.9,)	H	SO_3H	H	11,12, 13
R. leguminosarum bv. *viciae*	2,3	Ac	H	H	C18:1(11) C18:4(2,4,6, 11)	H	H or Ac	H	14,15

[a] References: 1 = Sanjuan et al (1992); 2 = Carison et al (1993); 3 = Stokkerrnans et al (1996): 4 = Price et al (1992); 5 = Bec-Ferte et al (1994); 6 = Mergaert et al (1993); 7 = Lopez-Lara et al (1995): 8 = Cardenas et al (1995): 9 = Poupot et al (1995); 10 = Poupot et al (1993); 11 = Lerouge et al (1990); 12 = Schultze et al (1992); 13 = Roche et al (1991); 14 = Spaink et al (1991); 15 = Firmin et al (1993).

dues, mono-N-acylated at the nonreducing end and carrying a variety of substitutions at both the reducing and nonreducing terminal GlcNAc residues. Each rhizobial species produces a variety of Nod signals with specific substitutions (Denarie et al 1996).

Specificity: a mirage?

Nod signal structure does not correlate with rhizobial phylogeny but with the host range of the particular *Rhizobium.* This suggests that each host plant has evolved to recognize the specific Nod signal repertoire produced by its compatible symbiont. Purified or chemically synthesized Nod signals with the appropriate structure will elicit, when applied to roots, many of the responses seen upon rhizobial inoculation (reviewed in Cohn et al 1998). Research using various rhizobial mutants blocked in the decoration of the basic lipo-chitin Nod signal structure led to the general view that Nod signal recognition was highly specific. Indeed, the model was developed in which a legume receptor recognized a tightly defined chemical structure and thus explained the host range specificity seen in many rhizobial-host interactions.

The structure of the Nod signals can be used to place rhizobia into two general classes. The first is composed of rhizobia that nodulate tropical and temperate legumes of the Genistae and Loteae tribes (e.g., *Bradyrhizobium japonicum, Rhizobium* sp. NGR234, *Sinorhizobium fredii, Rhizobium loti,* and *Azorhizobium caulinodans).* These rhizobia produce Nod signals that are N-acylated with fatty acids of general lipid metabolism (e.g., vaccenic acid, C18:1D11). The second class is composed of rhizobia that nodulate temperate, herbaceous legume genera belonging to Cicereae, Trifolieae, and Vicieae, which produce Nod signals that are N-acylated by polyunsaturated fatty acids (e.g., C16:2D2E,9Z, *Sinorhizobium meliloti;* C18:4D2E, 4E, 6E, 11Z, *R. leguminosarum* bv. *viciae;* C20:3 and C20:4, *R. leguminosarum* bv. *trifolii;* C18:2 and C18:3, *R. galegae*) (see Table 1 for references). For historical reasons, most research on Nod signal synthesis, structure, and function has been done using rhizobia that fall into this latter group (e.g., *S. meliloti* and *R. leguminosarum* bv. *viciae*). These studies have caused researchers to think about the specificity of Nod signal action.

Rhizobium sp. NGR234 has the broadest known host range of any *Rhizobium* able to nodulate legume species from 112 genera (Pueppke and Broughton 1999). Recently, Broughton and Perret (1999) pointed out that almost the only species that strain NGR234 cannot nodulate are found in the genera that belong to Cicereae, Trifolieae, and Vicieae. Thus, the apparent strict requirement of these plants for polyunsaturated fatty acids and other substituents (e.g., sulfation) may be the exception, not the rule, for *Rhizobium*-legume interactions. Even in this case, however, the data are not as clear as we might believe from reading the numerous reviews on this subject. For example, although nonsulfated *S. meliloti* Nod signals (isolated from a NodH mutant strain) fail to induce root hair deformation and cortical cell division in alfalfa roots, published reports mention that such mutants can still nodulate alfalfa (e.g., Ogawa et al 1991). Likewise, more recent reports have focused on the critical role of fatty acid unsaturation and length in determining the host range of *S. meliloti* (Demont-Caulet et al 1999). Again, however, *S. meliloti* mutants (such as NodFE) that secrete

Nod signals N-acylated with vaccenic acid still form nodules on various *Medicago* cultivars (Ardourel et al 1994).

Nodulation of Afghanistan pea by strains of *R. leguminosarum* bv. *viciae* requires the presence of the *nodX* gene product that 0-acetylates the terminal, reducing N-GlcNAc residue of the Nod signal (Firmin et a1 1993). At first glance, these data are entirely consistent with the view that Afghanistan pea possesses a Nod signal receptor that requires this 0-acetylation for recognition. Transfer of the *B. japonicum nodZ* gene into an *R. leguminosarum* bv. *viciae nodX* mutant, however, confers on this strain the ability to nodulate this pea cultivar (Ovtsyna et a1 1998). NodZ is a fucosyltransferase that transfers a fucose residue to the terminal, reducing GlcNAc residue. It is hard to imagine a "specific" receptor that cannot distinguish between an 0-acetyl and an O-fucosyl substituent if these are, indeed, critical residues for receptor recognition.

As with these studies, a critical reading of the literature reveals many reports that appear, at least superficially, to be inconsistent with the idea that Nod signal structure is the "stricto senso" determinant of nodulation specificity. For example, although the Nod signals produced by *R. etli* and *R. loti* are identical in structure, these two species have distinct host ranges (Cardenas et a1 1995). *R. tropici* and *R. etli* produce chemically different (sulfated and acetylfucosylated, respectively) Nod signals, but can both effectively nodulate common bean *Phaseolus vulgaris* (Poupot et a1 1993, 1995). Two of the Nod signals produced by *R. leguminosarum* bv. *viciae* are identical to the major Nod signals produced by *R. leguminosarum* bv. *trifolii,* but these two rhizobia have distinct host ranges (Spaink et al 1995, Orgambide et al 1995).

For the *B. japonicum* -soybean symbiosis, we have extensively studied the chemical specificity required for Nod signal action (Stokkermans et al 1995. Minami et a1 1996a,b). Our initial studies with a *B. japonicum* NodZ mutant suggested that the presence of a 2-O-methylfucose residue on the terminal nonreducing GlcNAc was critical for biological activity and, by assumption, host recognition (Stacey et al 1994). Subsequently, however, using a variety of natural and chemically synthesized lipochitooligosaccharides (LCOs), we found that fucosylation was required only when the LCO was a pentamer (Stokkermans et a1 1995). If the LC0 was a tetramer, fucosylation rendered the molecule inactive in eliciting root hair curling or cortical cell division in soybean. Hence, both the chemical substitutions and the LCO chain length were critical. Consistent with the discussion above, the fatty acid substituent did not appear to be a critical factor.

These results offered an explanation for a paradox that arose from earlier work on the nodulation host range of a *B. japonicum* NodZ mutant. As previously discussed, our original results pointed to the critical nature of Nod signal fucosylation for biological activity on soybean. Thus, it was surprising to find that a NodZ mutant, incapable of producing fucosylated Nod signals, was perfectly normal for nodulation of soybean (Stacey et al 1994). The results described by Stokkermans et al (1995) offered an explanation for this observation because we assumed that the NodZ mutant must be synthesizing an active, nonfucosylated tetrameric Nod signal. Indeed.

recently, we succeeded in purifying such a Nod signal from the culture supernatant of the NodZ mutant and have shown that it is active on soybean roots (Cohn et al 1999).

More recently, we have extended these studies by examining the chemical requirements for Nod signal action on an alternative host of *B. japonicum,* rice bean *(Vigna umbellata).* Again, we found that this plant responded only to the fucosylated pentameric Nod signal but could be nodulated by a *B. japonicum* NodZ mutant (Cohn et a1 1999). Therefore, we immediately tested the nonfucosylated tetrameric Nod signal, assuming that this molecule would be active. To our surprise, however, this molecule also lacked activity on rice bean roots. Thus, we were again left with a paradox: How can a *Rhizobium,* which cannot produce fucosylated Nod signals, nodulate a plant that requires a fucosylated Nod signal for recognition? To our surprise, we found that a mixture of nonfucosylated Nod signals was active in inducing cortical cell division and nodule formation on rice bean (Cohn et a1 1999). Thus, in this case, we can apparently overcome the chemical requirement for fucosylation by adding a mixture of nonfucosylated Nod signals. Such a mixture is naturally produced by the *B. japonicum* NodZ mutant and this appears to explain its ability to nodulate rice bean.

Specificity is multifactorial

We do not intend to leave the impression from the discussion above that Nod signal action lacks specificity. Numerous reports clearly show that Nod signal chemistry does affect activity. What is now becoming clear, however, is that the situation is much more complex than originally imagined. Nod signal chemical specificity is likely determined at several levels, including, but not limited to, receptor interaction.

Careful reading of the literature suggests three possible steps at which Nod signal specificity could be determined (Fig. 1). The most discussed is the interaction between the Nod signal and a specific receptor. As discussed below, however, it seems almost certain that legumes have at least two receptors (or at least two chemically distinct recognition events) with differing chemical specificity. The chemical decorations of the basic lipo-chitin Nod signal have been shown to protect the molecule from the action of plant chitinases (Staehelin et al 1994). Therefore, Nod signal specificity could also be determined by the ability of the molecule to survive in the plant in the presence of an enzymatic attack. Finally, recent work has shown that the N-acylation of the Nod signal probably plays an important role in mediating the uptake of the Nod signal into the plant cell (Philip-Hollingsworth et al 1997). Therefore, uptake may represent a third step in which Nod signal specificity may be determined. If all of these mechanisms are operating, it is the sum of their respective activities that determines the actual specificity seen in any given *Rhizobium*-host interaction.

To engineer a nodulating rice plant, it may not be necessary to create a plant with the same recognition events as a legume. What we need is information on which of these legume steps are essential for Nod signal action and then see whether rice has or can be given this ability.

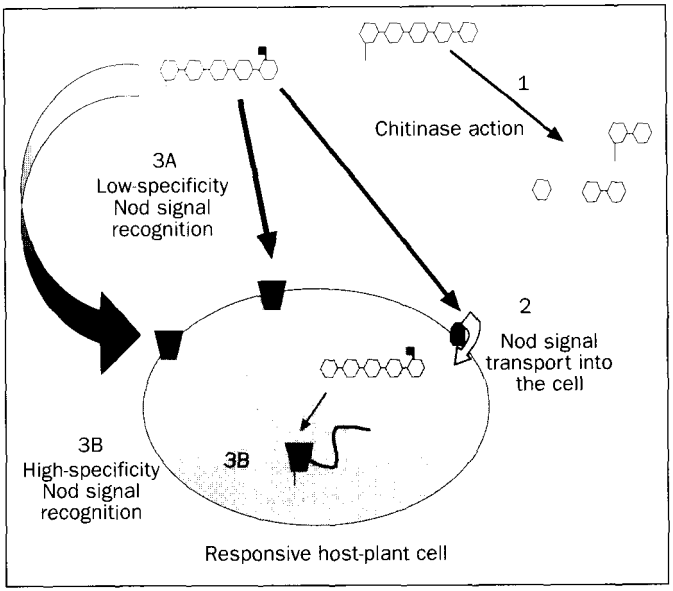

Fig. 1. Speculative model to explain the multifactorial specificity of Nod signal action. (1) Lipo-chitoohgosaccharide (LCO) lacking protective substituents (■) are rapidly degraded by plant chitinases. (2) Some published results (see text) favor the idea that the acyl group of the Nod signal plays a role in transporting the molecule into the cell. This step could affect specificity. (3) Nod signal-receptor interaction. Published results suggest that at least two recognition steps are involved (perhaps mediated by two separate receptors). (3A) Low-specificity recognition. (38) High-specificity recognition. If Nod signals are internalized before recognition, this step could occur intracellularly. (See also Fig. 2.)

Host recognition of Nod signals

Multiple receptors?

The fact that Nod signals act at very low concentrations and show some degree of chemical specificity suggests that perception is probably mediated by specific protein receptors. Such a receptor should be able to recognize the Nod signal at subnanomolar concentrations and transduce this binding signal to other cellular components. In addition, with due regard to the considerations above, the Nod signal receptor should demonstrate some chemical specificity.

Several laboratories have sought to define the nature of the Nod signal recognition event by conducting structure/function studies using either rhizobia1 mutants defective in Nod signal synthesis or a collection of natural and synthetic LCOs. The first study of this kind was published by Ardourel et al (1994). Working with *S. meliloti* mutants, they demonstrated that different plant responses (e.g., root hair deformation and preinfection thread formation) had different Nod signal structural requirements. They proposed a model in which a low-stringency signaling receptor mediated events such as root hair deformation, while a second, stringent high-affinity entry receptor

was necessary to trigger rhizobial invasion. Likewise, Felle et al (1996) examined the ability of various LCOs to trigger either cytosolic alkalinization or membrane depolarization in alfalfa root hairs. Sulfated Nod signals, which were morphogenic on alfalfa, were able to induce membrane depolarization and rapid intracellular alkalinization in root hairs. Nonsulfated Nod signals elicited an intracellular pH change. but in the absence of membrane depolarization. This study again suggests the presence in alfalfa of two Nod signal recognition events that are independently coupled to cellular events associated with nodulation.

In our laboratory, we undertook structure/function studies using a variety of natural and synthetic LCOs. For example, Stokkermans et al (1995) demonstrated that only four LCOs–LCO-V(C18: lDl1, MeFuc), LCO-V(C18:lD9, MeFuc), LCO-V(C16:0, MeFuc), and LCO-IV(C16:O) — could elicit a morphogenic response on soybean roots. These studies demonstrated that fucosylation and chitin chain length were the most important determinants for Nod signal specificity.

We then sought to extend this work by using the expression of early nodulin genes as a measure of Nod signal activity. Nodulins are plant proteins whose expression is specifically enhanced during nodulation. Early nodulins are expressed early (within 48 h) after rhizobial inoculation. Minami et al(l996a) examined the ability of chitin oligomers and LCOs to induce expression of the early nodulin *ENOD40.* This study showed that expression of *ENOD40* mRNA could be induced transiently by a simple nonacylated chitin pentamer. Sustained expression of *ENOD40,* however, required a soybean-specific Nod signal (i.e., one of the four LCOs listed above). In a subsequent paper, Minami et al (1996b) showed that the addition of any single LCO to soybean roots failed to elicit expression of the early nodulin *ENOD2.* When a mixture of LCOs was added, however, *ENOD2* expression was clearly induced. Analysis of these mixtures demonstrated that addition of a nonacylated chitin pentamer, along with one of the four morphogenic LCO molecules, was enough to induce *ENOD2* expression. Taken together, these studies by Minami et al (1996a,b) pointed to the presence of two Nod signal recognition events in soybean that vary in their chemical specificity and are independently coupled to cellular events (Fig. 2). Because expression of *ENOD40* and *ENOD2* mark different stages in nodule ontogeny, these results actually suggest that different Nod signal recognition events are required for the temporal development of the nodule. A low-stringency recognition step is coupled with the initial steps in nodule ontogeny (e.g., transient *ENOD40* expression), but is not enough to sustain nodule development. Likewise, activation of a high-affinity recognition step can result in sustained *ENOD40* expression and the formation of an incomplete nodule primordium. Only when both of these recognition events are simultaneously activated, however, does nodule development proceed to the formation of vascular tissue and the expression of *ENOD2.*

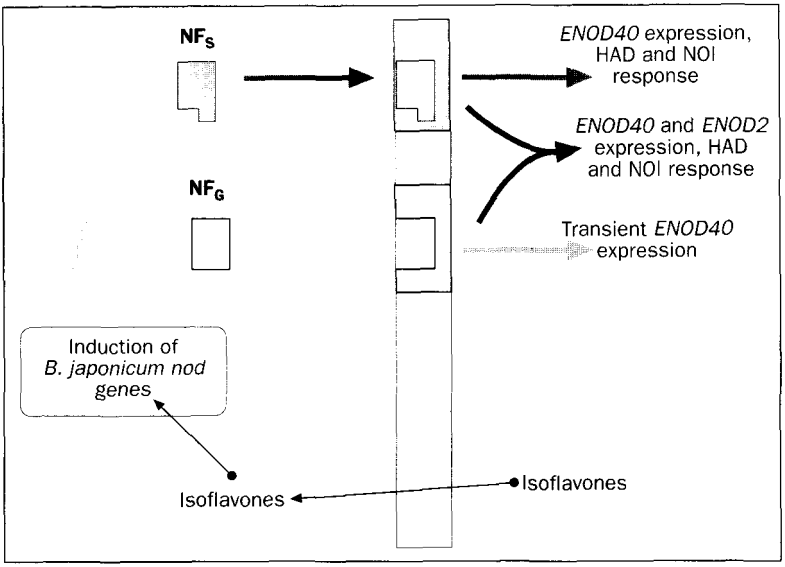

Fig. 2. Model for Nod signal recognition in soybean. A nonspecific recognition event can perceive a simple chitin pentamer (NF_G) resulting in the transient expression of *ENOD40*. A specific recognition event is required to perceive the specific lipochitooligosaccharide Nod signal (NF_S) resulting in a morphogenic response (e.g., HAD, root hair deformation, and NOI, induction of a nodule primordium) and sustained *ENOD40* expression. The induction of *ENOD2* expression is only seen when both the NF_G and NF_S recognition events are activated.

Chitin-binding proteins

Rice. Rice is probably the best characterized plant for chitin-binding proteins and chitin reception (reviewed in Stacey and Shibuya 1997). Chitin oligomers can induce defense responses in rice (reviewed in Stacey and Shibuya 1997). For example, Yamada et al (1993) showed that purified chitin oligomers (d.p. = 7–8)[1] induced phytoalexin biosynthesis in suspension-cultured rice cells at nanomolar concentrations. The same chitin fragments induced various cellular responses in rice, including transient depolarization of membrane potential (Kuchitsu et al 1993a), ion flux (K. Kuchitsu et al, unpublished), protein phosphorylation (Kuchitsu et al 1993b), transient generation of reactive oxygen species (Kuchitsu et al 1995) and jasmonic acid (Nojiri et al 1996), and transient expression of plant defense-related proteins (e.g., Minami et al 1996c). The chitin structural requirements for these responses were the same, suggesting that a single receptor was probably involved. Chitin oligosaccharides have also induced various defense-related responses in wheat (Barber et al 1989), oat (Ishihara et al 1996), melon (Roby et al 1987), and tomato (Felix et al 1993).

[1]d.p. = degree of polymerization.

In an effort to identify the rice receptor involved in chitin perception, a ^{125}I-labeled tyramine conjugate of N-acetylchitooctaose was used as a ligand to identify a chitin-binding protein in microsomal (Shibuya et al 1993) and plasma membrane (Shibuya et al 1996) preparations of suspension-cultured rice cells. These studies revealed the presence of a single binding site with an apparent dissociation constant (Kd) of 5.4 nM. This binding activity was localized in the plasma membrane. The binding specificity correlated well with the specificity for chitin elicitor activity on rice cells. For example, the binding site favored large chitin oligomers (d.p. = 7–8) and these were also the most active elicitor molecules.

Affinity labeling of the plasma membrane revealed the presence of a chitin-binding protein with a molecular weight of approximately 75,000 (Ito et a1 1996). Subsequently, this protein was successfully solubilized from the plasma membrane and purified to apparent homogeneity using an N-acetylchitoheptaosyl-lysil-agarose column (Shibuya et al 1996). The purified protein exhibited the same binding affinity as the plasma membrane preparations. These results clearly indicate the high likelihood that this 75-kDa protein represents the receptor involved in chitin-elicited defense responses in rice.

Soybean. The ability of nonacylated chitin oligomers to induce *ENOD40* expression in soybean suggested that a chitin-binding protein could be involved in Nod signal recognition. Our hypothesis was that Nod signal receptors could be a special class of chitin-binding proteins found in a wide variety of plants. Therefore, we began a study to identify and characterize chitin-binding proteins in soybean (Day et a1 2000). These studies have used the published work in rice as a guide. For example, we have used ^{125}I-labeled tyramine conjugates of N-acetylchitooctaose and N-acetylchitopentaose as ligands to identify chitin-binding proteins in microsomal membrane preparations from both soybean suspension-cultured cells and root preparations. Figure 3 demonstrates the saturable binding of ^{125}I-labeled N-acetylchitopentaose to soybean plasma membrane preparations. Using both the N-acetylchitooctaose and N-acetylpentaose, we identified a binding protein in soybean plasma membranes with an apparent Kd of approximately 50 nM. The binding site has slightly better affinity for the N-acetylchitooctaose than for the N-acetylchitopentaose. Indeed, competition

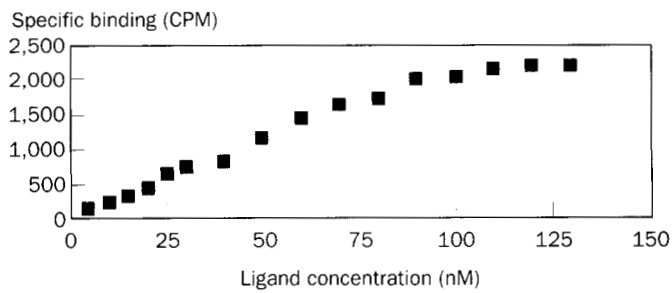

Fig. 3. Binding of ^{125}I-(GlcNAc)$_5$-APEA to a plasma-enriched membrane fraction from 5-d-old soybean roots. CPM = counts per minute, APEA = 2-(4-aminophenyl) ethylamine.

experiments using chitin oligomers (d.p. = 2–8) demonstrate that this binding site prefers the higher molecular weight oligomers. In this regard, the binding site is almost identical to that reported for rice (see above). These data also suggest that the chitooctaose and chitopentaose are interacting with the same binding protein in the soybean plasma membrane. Affinity labeling using a ^{125}I-labeled N-acetylchitooctoase ligand identified an 8.5-kDa protein in the plasma membrane. Addition of unlabeled ligand eliminated this binding, indicating that the 85-kDa protein is a specific chitin-binding protein. The binding specificity of this 85-kDa protein for various chitin oligomers correlated well with the ability of the same oligomers to induce an oxidative burst response in soybean suspension-cultured cells. The specificity demonstrated was quite distinct from that found for the induction of *ENOD40* by chitin oligomers. Therefore, we concluded from this work that the 8.5-kDa protein is not involved in Nod signal recognition but, similar to the situation in rice, may be involved in eliciting a defense response in response to fungal cell wall fragments.

Nod signal-binding proteins

LCO-binding proteins have been identified in microsomal membrane preparations from *Medicago* (Bono et al 199.5. Niebel et al 1997. Gressent et al 1999). The first site, termed N(od) f(actor) b(inding) s(ite) 1, was identified in root preparations from *M. truncatula* and *M. varia* cell suspensions using tritium-labeled NodRm-1(Ac,S,C16:2), the major Nod signal from *S. meliloti* (Bono et al 199.5). NFBS 1, however, had a relatively low affinity for the Nod signal (Kd = 86 nM) and low specificity for LCOs. Moreover, a similar binding activity could be detected in membrane preparations from tomato roots. Subsequently, a second binding activity (NFBS 2) was found in microsomal membrane preparations from *M. varia* cell suspensions (Niebel et al 1997). NFBS 2 exhibited a high affinity for the *S. meliloti* Nod signal (Kd = 2 nM). Recently, the chemical specificity of NFBS 2 was examined in more detail using a ^{35}S-labeled NodRm-1(Ac,C16:2Δ2,9) ligand (Gressent et al 1999). NFBS 2 was highly enriched in the plasma membrane fraction. Binding studies demonstrated that substitutions on the terminal nonreducing GlcNAc residue of the Nod signal, including O-acetylation, N-acylation, and C-4 hydroxylation, were critical determinants of binding specificity. For N-acylation, the binding specificity of NFBS 2 correlated well with the structure/function studies of Demont-Caulet et al (1 999) discussed previously. The surprising finding of the study by Gressent et al (1999) was that binding to NFBS 2 did not require sulfation of the Nod signal, which is thought to be a critical determinant in Nod signal action on alfalfa. Thus, although NFBS 2 demonstrates some of the characteristics expected of an alfalfa Nod signal receptor (e.g., high affinity and N-acylation specificity), it lacks other characteristics (e.g.. requirement of sulfation). Further work will be required to demonstrate the relevance of NFBS 2 to nodulation.

Lectins have long been implicated in the legume nodulation process, but a precise role for these interesting proteins has not been defined. Recently, Etzler et al (1999) reported on a unique lectin (Db46) isolated from the roots of the legume *Dolichos biflorus*. This lectin is not related to common legume seed lectins and is not

an agglutinin due to its monomeric nature. The *D. biflorus* lectin binds to Nod signals from a variety of rhizobia. Db46, however, showed the highest apparent affinity for the Nod signal produced by *B. japonicum* and *Rhizobium* sp. NGR234, both of which can nodulate *D. biflorus*. Sequence comparisons revealed that the *D. biflorus* lectin was probably an apyrase (i.e., NTPase). Indeed, Etzler et a1 (1999) demonstrated that the lectin possessed ATPase activity, which increased significantly upon the addition of the Nod signal. For this reason, the lectin was termed a lectin-nucleotide phosphohydrolase (LNP). The *D. biflorus* LNP (i.e., Db46) was found on the surface of root hairs using fluorescent antibody labeling. The properties of this protein suggest that it could play a role in Nod signal recognition and transduce this binding signal by way of its apyrase enzyme activity.

We have now extended these studies by demonstrating the presence of orthologs of the *D. biflorus* LNP in other legumes (e.g., soybean and *M. truncatula*). The soybean LNP and *M. truncatula* LNP are rapidly induced upon rhizobial inoculation and therefore classify as early nodulins (J. Cohn et al, R.B. Day et al, unpublished). Further work is required to demonstrate conclusively that LNP proteins play an essential role in nodulation. Nonetheless, they remain likely candidates for being a bona fide Nod signal receptor.

Recently, we have identified an ortholog of a legume LNP protein in rice. The presence of this protein could explain the ability of rice to respond to Nod signals.

Rice response to Nod signals

If rice can be engineered to interact with rhizobia, one requirement will be the presence in rice of many of the proteins known to be required for legume nodule development. Therefore, recent reports of the presence of legume nodulin homologues in rice provide some hope that a nodulating rice plant may be possible. For example, Reddy et a1 (1999) showed that several early nodulin genes hybridized to DNA from a variety of rice cultivars. This work was subsequently confirmed by the isolation of a rice cDNA showing considerable sequence similarity to the legume *ENOD93* gene (Reddy et al 1998a). More recently, a rice homologue of the early nodulin *ENOD40* has been isolated (Kouchi et a1 1999, and this volume).

Another requirement for rice nodulation is the ability of rice to respond to the rhizobial Nod signal and to respond to this signal by inducing genes necessary for nodulation (e.g., early nodulins). Surprisingly, this trait does exist in rice. Reddy et al (1998b) showed that the addition of Nod signals isolated from *Rhizobium* sp. NGR234 to ENODl2-b-glucuronidase (GUS) transgenic plants resulted in a significant induction of GUS expression. In this case, simple chitin oligomers were not active; only bona fide Nod signals induced *ENOD12* expression. Also, similar to what is seen in legumes, *ENOD40-GUS* expression in transgenic rice is restricted to vascular traces (P.M. Reddy et al, unpublished). The results with the *ENODIZ-GUS* plants are surprising because they indicate that rice possesses not only the mechanism to recognize rhizobial Nod signals but also the signal transduction chain that links such recognition to gene transcription. These results would not be so surprising, however, if our

original hypothesis, that chitin and LCO signaling are ancient traits, is correct. Moreover, the presence of Nod signal-binding proteins, such as homologues of legume LNPs, in rice would be expected. Only future experimentation will prove whether our speculation has merit.

Conclusions

A nodulating rice plant may still be only a remote possibility. What is now clear, however, is that rice possesses many of the traits that. until recently, would have been considered unique to nodulating legume species. The challenge to future research is to identify those specific traits that distinguish a nodulating plant from one that does not nodulate. These will then need to be engineered into rice to confer nodulating ability.

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Rhizobium nodulation and interaction with legumes and nonlegumes

Barry G. Rolfe, Ulrike Mathesius, Joko Prayitno, Francine Perrine, Jeremy J. Weinman, John Stefaniak, Michael Djordjevic, Nelson Guerreiro, and Frank B. Dazzo

Our research program has been investigating the signal exchanges that occur during the early stages of *Rhizobium* infection of clovers and the nonlegumes *Parasponia* and rice. We propose that, early during clover infection, the *Rhizobium* lipochitin-oligosaccharides cause the induction of chalcone synthase, the first enzyme of the flavonoid pathway in the inner cortical cells of the root. This is then followed by an alteration of auxin flow. Subsequently, the inner cortical cells accumulate specific flavonoids, followed by auxin accumulation in those cells that then divide to form a nodule primordium. In *Bradyrhizobium* infection of *Parasponia,* fluorescent compounds, whose properties are consistent with those of flavonoids, accumulate in dividing cells during nodule formation. Thus, flavonoids may not be the unique feature of legumes that enables legumes to form symbioses with *Rhizobium.*

The rice-*Rhizobium leguminosarum* bv. *trifolii* interaction was studied by several approaches: first, by analyzing rice plants and showing that they produce various chemical signals, including flavonoids, that might interact with rhizobia; second, by investigating the effects of bacterial inoculation on rice seedling growth and finding that some *Rhizobium* strains inhibit while others can stimulate rice growth; and, third, by using proteome analysis to investigate the molecular genetic basis of *Rhizobium* strain ANU843 inhibition of rice plants. The possibility of establishing a more effective *Rhizobium*-nonlegume interaction is potentially available in rice because some of the phenylpropanoid pathway compounds that could interact with *Rhizobium* are also present in nonlegume roots.

A marked increase in environmental awareness and a concern for sustainable agriculture have occurred over the past decade. This concern extends to agriculture continuing to be the foundation for human civilization throughout the 21st century and beyond. We therefore need technological advances to obtain more yield per resource for sound economic reasons (Rolfe et al 1998). This awareness has highlighted the need to both further promote plant growth and find alternatives to nitrogen fertilizer, possibly by harnessing or manipulating *Rhizobium*-legume-nonlegume systems more effectively.

Different legumes release various sets of compounds, which will be perceived as either inducers or anti-inducers of the nodulation genes by soil rhizobia. These signaling compounds are mostly flavonoids, which are derived from the phenylpropanoid pathway. When the extracts from white clover seedlings were characterized, three particularly active compounds were found. One was the inducer molecule 7,4'-dihydroxyflavone (DHF), which could induce *Rhizobium nod* genes (Redmond et a1 1986, Djordjevic et al 1987). The other two substances, present at about 3–4 times the concentration of the active flavones, were the isoflavone formononetin and the 7-OH coumarin umbelliferone (Djordjevic et a1 1987). Both of these compounds inhibited the reporter gene fusions from being induced by the stimulatory molecules. In clovers, the release of the inhibitory substances varies for different regions of the developing clover root, as does the production of the stimulatory compounds (Rolfe 1988). While the inducer is chiefly released from plant cells behind the growing root tip in the zone of emerging root hairs, the release of the anti-inducer occurs in the region between this zone and the root tip and elsewhere on the root (Rolfe 1988). A chief determinant of the sites of nodule initiation could be the ratio of inducer:anti-inducer. Fluctuations in the concentrations of these compounds in the plant will affect the expression of *Rhizobium nod* genes, but perhaps also the behavior of the dividing plant cells and the phytohormone balance in the root (Jacobs and Rubery 1988).

We have investigated the cellular, genetic, and molecular processes occurring during the establishment of symbioses between *Rhizobium leguminosarum* bv. *trifolii* and clovers and compared these processes with those in the nonlegume *Parasponia* (McIver et a1 1989, Djordjevic and Weinman 199 1). We describe here the characteristics of some of the signal transduction events associated with clover nodulation and an investigation of these events in the nonlegumes *Parasponia* and rice. One question investigated in this study was whether flavonoid or other signaling compounds from rice would act in a similar fashion (as inducers and anti-inducers of *Rhizobium nod* genes) as the compounds found in legumes. In rice, it was possible to show a variation in the levels of chemical signals that might influence *Rhizobium* gene expression, which could be extracted from different regions of the plant.

Recently, *Rhizobium leguminosarum* bv. *trifolii* strains were shown to naturally colonize roots of rice plants in Egypt, where rice and berseem clover have been rotated annually for more than 700 years (Yanni et al 1997, Dazzo et al 1998). In laboratory experiments, several of these strains can promote rice growth. The extent of these growth responses was influenced by the rice cultivar and inoculant strain. Furthermore, two field experiments have shown that inoculation of rice seedlings with two of these endophytic *Rhizobium* strains can significantly increase grain yield and agronomic N-use efficiency of rice plants (Yanni et al 1997, Dazzo et a1 1998). The molecular and genetic characterization of these growth-enhancing endophytic strains has only just begun (Yanni et al 1997, Rolfe et al 1998).

In contrast to the above, *R. leguminosarum* bv. *trifolii* strain ANU843 was found to inhibit rice seedling growth when plants were grown in liquid medium. The elimination of specific plasmids of ANU843, however, abolished this growth-inhibition

phenotype. To describe the molecular genetic basis of the rice growth-inhibition phenotype of ANU843, we used 2-dimensional gel electrophoresis (2-DE) to examine gene expression within *R. leguminosarum* bv. *trifolii* strain ANU843 and its plasmid-cured derivative strains.

Materials and methods

Bacterial strains

Rice-associating *Rhizobium leguminosarum* bv. *trifolii* strains were isolated from either the surface-sterilized root tissues (E strains, E4, E11) or the root rhizosphere (R strains, R4) of rice plants grown at Sakha Kafr El Sheikh in the middle Nile Delta region of Egypt (Yanni et a1 1997). The archetypal *R. leguminosarum* bv. *trifolii* strain ANU843 (Rolfe et a1 1980) is a nonrice-associated bacterium. Strain ANU843 has five plasmids, ranging from 180 kbp to ~700 kbp in size, and these, together with the chromosome, constitute a fully integrated genomic complex (Guerreiro et al 1998). Two derivative strains, CFNS 152 (PC cured) and CFNS309 (pe cured), were used. Broad host-range strain NGR234 can nodulate a large group of legumes and the nonlegume *Parasponia* (Bender et al 1988). *Agrobacterium tumefuciens* strain C58 and its plasmid pTi-negative derivative strain A136 were used to represent A. tumefuciens. The reporter strain ANU845 (pMD1) was used to detect and analyze rhizobia1 *nod* gene-inducing signals in rice plant extracts as described elsewhere (Le Strange et al 1990). Strain CP279 is a *Bradyrhizobium* strain able to nodulate *Parasponia* (Le Strange 1997).

Specific transgenic plants

Specific transgenic clovers have been developed and used to study root development and *Rhizobium* infection (Larkin et a1 1996, Djordjevic et a1 1997, Mathesius et al 1998a,b).

Single-leaf plantlet bioassay

A bioassay technique to speed up the analysis of transgenic plants was developed for the rapid analysis of plant signals during nodulation and root morphogenesis (Rolfe and McIver 1996). The rooted leaf assay has enabled the routine examination of large numbers of independently transformed plants and the analysis of the expression of specific plant genes (Mathesius et al 1998a,b).

Bacterial growth media

Bacterial strains were grown on Bergersen's modified medium (BMM) at 29 °C for 3 d before inoculation of plants (Rolfe et al 1980). Bacteria for inoculation were suspended in sterile water.

Plant growth media

Nitrogen-free modified Fahraeus medium (NFM) has been described previously (Rolfe et al 1980, Rolfe and McIver 1996). Where specified, 10 mM KNO_3 was added to this

medium. Hoagland #2 (Sigma Chemical Co.) medium contains 15 mM nitrogen as 6 mM KNO_3, 4 mM $Ca(NO_3)_2$, and 1 mM $NH_4H_2PO_4$. Colonization studies used liquid media of NFM plus 10 mM KNO_3 in Magenta jars (containing 250 mL liquid medium).

Plant growth studies

Rice cultivars Calrose and Pelde were used (seeds were obtained from Dr. L. Lewin, NSW Department of Agriculture) and the procedures were as described elsewhere (Prayitno et a1 1999).

Extraction and analysis of signal molecules from rice plants

Rice seeds were obtained from Dr. L. Lewin, NSW Department of Agriculture. Fully developed, not discolored rice seeds of cultivars Pelde and Calrose were selected and surface-sterilized by first dehusking and then soaking in 99% EtOH for 3 min, followed by two rinses in Milli-Q water. The seeds were then soaked for 35 min in a quarter dilution, with Milli-Q water, of sodium hypochlorite 12.5% w/v, containing 10–13%available chloride, obtained from Ajax Chemicals, Auburn, NSW. The seeds were then washed four times in Milli-Q water. They were then transferred to petri dishes (12 per petri dish) containing BMM (Bergersen 1961) in 1.2% Difco Bacto Agar. The seeds were overlayed with 0.8% soft-water agar. The petri dishes were then incubated in the dark at 29 °C for 2–3 d until the seeds germinated.

The germinated seeds were then transplanted onto large plates containing Fahraeus medium with 15 mM KNO_3 solidified with 1.2% Difco Bacto Agar (Rolfe et a1 1980), 12 per plate. These were placed into a growth cabinet having 12 h daylight, light intensity of 460 $\mu Es^{-1}m^{-2}$ at shelf surface, 30 °C day/20 °C night temperature, and relative humidity at 70%.

Seven days after sterilization, the first batch of rice seedlings was harvested. Three replicates of each cultivar were harvested at each sampling date. Fifteen plants per replicate were randomly selected from the plates and cut into five sections as follows: zone 1 (R2), the lower root region; zone 2 (RI), a 1-cm section of the upper root from the seed down; zone 3, seed; zone 4 (Shl), a 1-cm section of the shoot (including the culm) from the seed up; zone 5 (Sh2), the upper shoot region (see Fig. 3A). The corresponding sections were placed into 4 mL of a 50% ethanol/Milli-Q water solution in glass scintillation vials and left in the dark in a 29 °C incubator overnight (20 h). The following day, the EtOH/H_2O "extracts" mixture was pipetted off from each vial and passed through a 0.45-ym Acrodisc syringe filter into a new 1-dram glass vial. These vials were then stored at −80°C to be assayed for ß-galactosidase activity. Further batches of rice seedlings were harvested on days 10, 14, and 21. At these sampling dates, the plants were cut into seven sections to ascertain whether signal-producing cells moved with the elongating zones of the plants. Thus, as shown in Figure 3A, zone 1 (R2) was the lower root region; zone 2 (Rl'), a 1-cm "mid" root section; zone 3 (R1), 1 cm of upper root from the seed down; zone 4, seed; zone 5 (Shl), a 1-cm section of the shoot (including the culm) from the seed up; zone 6

(Shl'), the next 1-cm section of the shoot; zone 7 (Sh2), the upper shoot region (Fig. 3A).

ß-galactosidase assay

Samples of signal extracts in 50% ethanol/H_20 were taken from −80 °C storage, thawed, vortexed, and 200 µL pipetted into new 1-dram glass vials. These were then evaporated to half volume (to remove the ethanol) in a vacuum oven at 40 °C and −90 to −100 kPa pressure for 1 h. These "signals" were used to induce transcriptional activation of *nod4::lacZ* in ANU845 (pMDl), which had been grown in BMM broth to an OD_{600} of 0.204. Induction was carried out for 2 h at 29 °C. Naringenin at a concentration of 1×10^{-6} µM was used as the positive control.

In vitro assays with 2-nitrophenyl-P-D-galactopyranoside as a substrate were carried out to measure 13-galactosidase activity resulting from the induction of the *nodA::lacZ* fusion in the reporter bacteria, as per Miller (1972). The 42 °C incubation step was omitted, however. Activity was calculated as described in Miller (1972), but then the values obtained were divided by the dry weights of the tissue extracted and also by the volume of extract obtained. Hence, the level of *nodA* induction is reported as activity g^{-1} dry wt mL^{-1} of extract $\times 10^{-3}$.

Analysis of global changes in protein expression by proteome analysis

Proteome analysis was used to resolve the complex mixtures of *Rhizobium* strain ANU843 proteins on a 2-dimensional polyacrylamide electrophoresis (2-DE) gel. The growth of strains and the procedures used were as previously published (Guerreiro et al 1997, 1998, 1999).

Results

Rhizobium-induced responses in the roots of transgenic white clovers

When legume roots are exposed to rhizobia or their lipochitin-oligosaccharides (LCOs), a signal transduction chain is initiated and a disturbance to the root phytohormone balance takes place (Mathesius et al 1998a). In clovers, this response leads to the initiation of inner cortical cell division and the formation of a nodule primordium. The clover root responses are specifically induced by clover-nodulating rhizobia and are not induced by nonnodulating mutants or by foreign rhizobia (Mathesius et al 1998a).

Specific transgenic clovers containing the auxin responsive promoter, *GH3-gusA* fusion (Larkin et al 1996), were developed to study how auxins might be involved in nodule formation. To analyze the steps in signal transduction, spot inoculation of the roots of rooted leaves was used to apply auxin, certain flavonoids, NPA (N-(1-naphthyl) phthalamic acid, an auxin transport inhibitor), *Rhizobium,* and *Rhizobium* LCOs to precise sites on the roots of these transgenic white clovers (Mathesius et al 1998a). The results, summarized in Figure 1, show the changes taking place over 96 h following *Rhizobium* inoculation, which result in alterations of auxin levels, presumably via blockage of polar auxin transport in the roots within 24 h of their

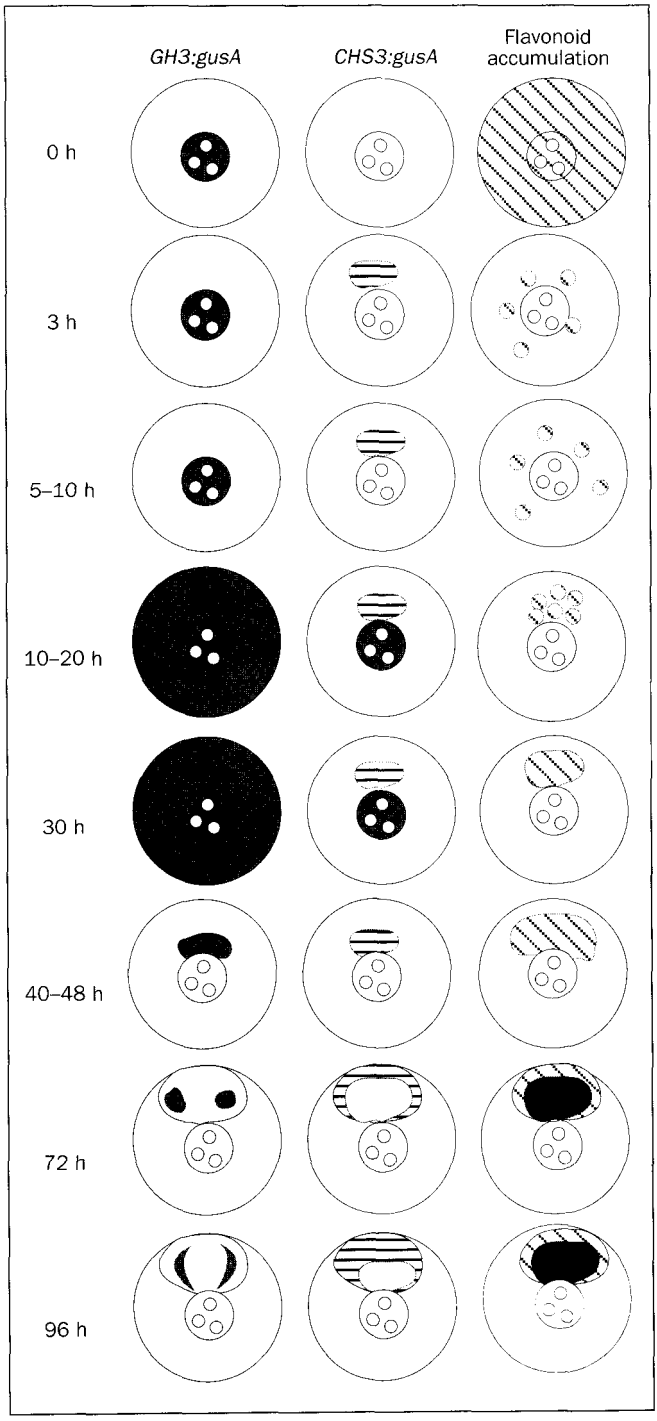

Fig. 1. The expression of the auxin responsive promoter construct *(GH3:gusA)* and the chalcone synthase *CHS3:gusA* construct and the accumulation of specific flavonoid compounds in cells and tissues during nodule initiation in white clover roots. The diagram shows the typical expression pattern of the three markers in root sections through the site of spot inoculation with the nodulating *Rhizobium leguminosarum* bv. *trifolii* strain ANU843 at different times after inoculation.

application. These changes can be mimicked by the application of *Rhizobium,* NPA, certain flavonoids, or LCOs.

The site-specific induction of the flavonoid pathway was also examined before and during nodule initiation in white clovers to test whether flavonoids induced by *Rhizobium* could mediate the effects on the auxin balance. The complex regulation of flavonoids was studied by (1) examining the fluctuation of the chalcone synthase *(CHS3)* gene (Djordjevic et al 1997) during *Rhizobium* infection. (2) fluorescence microscopy, and (3) microspectrofluorometry. A chalcone synthase-regulated β-glucuronidase (GUS) transgene *(CHS3:gusA)* was up-regulated from 3 h postinoculation (p.i.) until cell division (around 40 h p.i.) in inner cortex cells underlying the inoculation site (Fig. 1). Intracellular fluorescence was found to occur in the vacuoles of inner cortex cells underlying the inoculation site from 13 h p.i. until the fluorescent cells divided. Fluorescence emission spectra of contents of individual fluorescing cortex cells were measured in situ and compared with emission spectra of compounds purified from root extracts. The fluorescing compound located in cells of the inner cortex after *R. leguminosarum* bv. *trifolii* inoculation was identified as a water-soluble derivative of 7,4'-dihydroxyflavone. As the nodule progenitor cells form a distinct nodule primordium, a change in the type of fluorescent compounds present in these cells was observed. Nodule primordium cells contained a different fluorescent compound that was identified as the isoflavonoid formononetin. As with *GH3:gusA* expression, CHS3:gusA expression and flavonoid accumulation were only induced in inner cortex cells by a nodulating *Rhizobium* strain and by clover-specific LCOs, but not by nonnodulating rhizobia.

Is flavonoid accumulation in nodule progenitor cells a widespread feature of legume nodule formation?

We examined other legumes to determine whether the responses observed in clovers also occurred in them. Although we had not specifically identified the precise chemical nature of any fluorescent compound observed, we did observe fluorescent compounds in alfalfa, pea, and siratro. These fluorescent compounds occurred in the cells that participate in nodule formation, but the compounds were different in each case. Only nodule-forming rhizobia induced the changes in fluorescent properties of these cells. It is possible that these fluorescent compounds are also flavonoids. The results indicate that fluorescent flavonoids are useful markers in nodule organogenesis in clover and may have direct roles in nodule formation.

Nodulation in the mature root region

The changes described above in *GH3:gusA* expression, induction of CHS promoters. and accumulation of flavonoid compounds occur when rhizobia are inoculated in the most developmentally susceptible zone just behind the root tip, but do not occur when the mature root zone is inoculated with rhizobia (Mathesius et al 2000). The results suggest that rhizobia cannot induce the same nodulation-specific signals in the mature root, similar to what occurs near the root tip. Similar patterns of *GH3:gusA* expression, *CHS3:gusA* expression, and flavonoid accumulation, however, occur in cor-

tical cells next to sites of lateral root emergence. The reason why nodules can be initiated at lateral root emergence sites may be that, during plant development, the cortex cells next to the lateral root primordium have already been activated by the plant to a "nodulation competent state." Consistent with this hypothesis is the result that shows that increasing the numbers of lateral roots also leads to a significant increase in nodule numbers at lateral root emergence sites (Mathesius et a1 2000). These results suggest that rhizobia can nodulate sites along the root where the plant has previously provided signals during lateral root formation that activate cortical cells similar to what rhizobia do.

Temporal expression of a basic-chitinase during nodulation

As was observed in the transgenic *GH3:gusA* and *CHS3:gusA* clovers, another temporal expression pattern of a root gene during *Rhizobium* infection was observed in transgenic white clovers containing the tobacco basic-chitinase promoter-*gusA* gene (Pittock et a1 1997). In cultured tobacco tissues, this chitinase gene was shown to be strongly induced by ethylene and its transcription is negatively regulated by the presence of certain levels of both auxin and cytokinin (Shinshi et a1 1987). *Rhizobium* infection did induce a temporal expression of the promoter in the inner cortical cells in the susceptible region, which subsequently divides to form a nodule primordium between 1 and 4 h p.i., but not during the next 14 d of nodule development. This finding was specific for clover-nodulating rhizobia (Pittock et a1 1997). The expression of this promoter was seen in lateral roots only after their emergence from the main root.

In summary, several transgenes show the temporal nature of the changes taking place in *Rhizobium*-inoculated roots. A comparison between lateral root and nodule formation shows that flavonoids are generally present in dividing cells and that the same flavonoid, previously identified as formononetin (Mathesius et a1 1998b), is produced in the root tip and in lateral and nodule primordia. Fluorescent compounds with properties consistent with those of flavonoids also occurred in dividing cells in other legumes and nonlegumes. The unexpected finding was the temporal pattern of changes observed with the basic-chitinase promoter-*gusA* gene, which could be indicative of the changing pattern of the auxin:cytokinin ratios taking place in the *Rhizobium*-inoculated root.

Fluorescence detection in roots of *Parasponia andersonii* during nodule initiation

Fluorescent intracellular compounds that accumulated in cortex cells of legumes that participate in nodule initiation and development were identified as flavonoid compounds (Mathesius et a1 1998b). We investigated whether the formation of fluorescent compounds during nodule formation is unique in legumes or is also associated with nodule formation in the nonlegume Parasponia. After 3, 6, 10, and 14 d p.i., fluorescence accumulation was detected at the spot inoculation site (Fig. 2). In control roots, fluorescence was detected in the root tip (Fig. 2A) but not in the mature cortex cells (Fig. 2B). After 3 and 6 d p.i., no intracellular fluorescence was present in

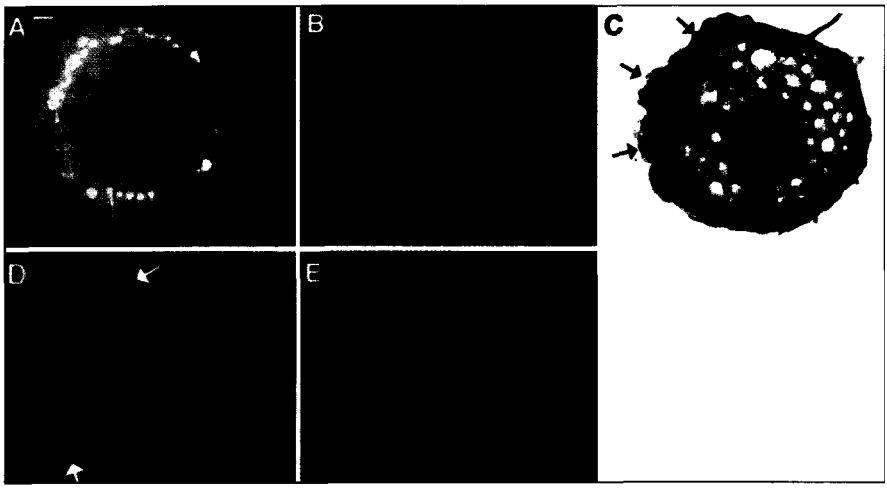

Fig. 2. Formation of fluorescent compounds during nodule formation in *Parasponia. Parasponia* seedlings grown as described in Bender and Rolfe (1985) were spot-inoculated at the zone of emerging root hairs with strain CP279 (Le Strange 1997). Flavonoids can be detected in situ using fluorescence microscopy after staining of live sections with the flavonoid-specific dye, diphenylboric acid-2-aminoethylester (DPBA). After 3, 6, 10, and 14 d postinoculation (p.i.), flue rescence accumulation was detected at the spot inoculation site. (A) Control root fluorescence in the root tip; (B) no intracellular fluorescence in the mature cortex cells, after 10 d p.i.; (C) the first epidermal cells had divided and infection threads appeared (arrows); (D) the divided epidermal cells and some outer cortex cells contained blue fluorescent intracellular compounds after DPBA treatment under ultraviolet light (arrows). Pericycle cells (P) underlying the divided epidermal cells had divided and also showed fluorescence accumulation, in this case of green color. (E) Nodules sectioned 2 wk p.i. showed blue fluorescence throughout the nodule primordium (n).

the inoculated roots. After 10 d p.i., the first epidermal cells had divided and infection threads were detected (Fig. 2C). The divided epidermal cells and some outer cortex cells contained blue fluorescent intracellular compounds. Pericycle cells underlying the divided epidermal cells had divided and also showed fluorescence accumulation, in this case of green color. The area of dividing blue fluorescent cells subsequently grew larger toward the middle and inner cortex. The group of divided green fluorescent pericycle cells also grew. Nodules sectioned 2 wk p.i. showed blue fluorescence throughout the nodule primordium (Fig. 2E).

Chemical signals produced by rice cultivars Pelde and Calrose that stimulate *nod*-gene expression

Prayitno et a1 (1999) studied the effect of inoculation with either the rice-associating rhizobia or with strain ANU843 on the growth of rice cultivars Pelde and Calrose. Some strains stimulated the growth of these cultivars, whereas others inhibited their growth. These growth experiments with the two cultivars indicate that an interaction was taking place between the rhizobia and the rice seedlings. Thus, rice seedlings were analyzed for possible signal molecules that might interact with *Rhizobium* cells. Extracts made from seedlings of both Pelde and Calrose were tested with the reporter

strain ANU845 (pMDI), which contains the NGR234 *nodD* gene and an inducible promoter. Although signal production followed a similar pattern in both cultivars, the major difference was the level of activity obtained (Fig. 3B). Both cultivars produced higher levels of signals extracted from the different sections of rice seedlings early in development, peaking at day 10 (D10) and then declining to basal levels by D 14. The results depicted in Figure 3B clearly show that signal production varies with the age of the seedlings.

At D7, the greatest activity was observed in the Sh1 and Sh2 regions in both cultivars (Fig. 3B). The Sh2 activity was elevated, however, due to A420 absorbance of 0.5 unit by the chlorophyll present in this extract. D7 Pelde Shl signal extracts

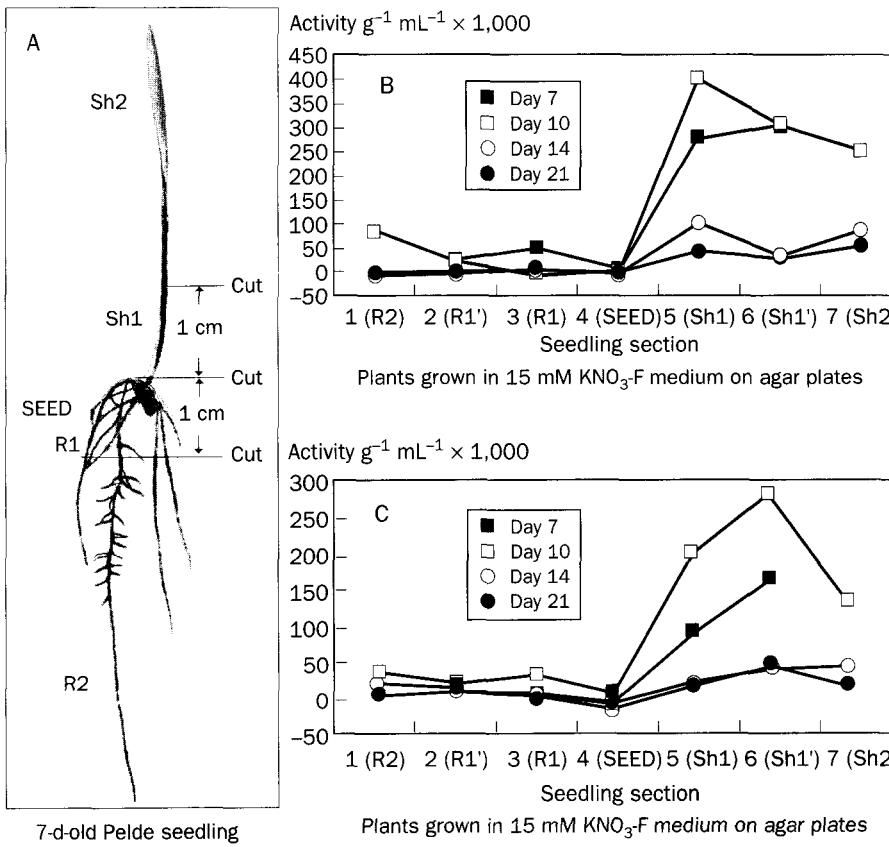

Fig. 3. Signal production in rice seedlings. (A) Rice plants were cut into 5 sections: zone 1 (R2), the lower root region: zone 2 (R1), a 1-cm section of the upper root from the seed down: zone 3 (seed); zone 4 (Shl), a 1-cm section of the shoot (including the culm) from the seed up; zone 5 (Sh2), the upper shoot region. (B) The signals detected in sections of cv. Pelde seedlings at day 7, 10, 14, and 21. D7 Shl signal extracts produced an activity of 270 x 10^3 units, D14 was 100 x 10^3 units, and D21 was ~30 x 10^3 units. These were compared with signals detected in sections of cv. Calrose seedlings (C) at day 7, 10, 14, and 21.

produced an activity of 270 × 10^3 units, which is 3.5 times that of Calrose Shl at D7. At D10, the Sh1 level of activity was 1.5 to 2 times that of D7 for both cultivars, and the activity of Pelde was twice that of Calrose. The activity detected dropped significantly by Dl4 in both cultivars, but the Pelde Shl activity (100 × 10^3 units) was still 6 times the activity of Calrose. By D21, the activity had dropped to −30× 10^3 units for Pelde and 15 × 10^3 units for Calrose.

In the R1 region on D7, Pelde activity of 47 × 10^3 units was 8 times the activity of Calrose. On D10, Pelde signals from the R1 region recorded an activity of −11× 10^3 units, whereas Calrose had an activity of 30 × 10^3 units. Pelde signals from the R1 region on D10 were inhibiting the induction of ß-galactosidase, whereas Calrose signals from this region were able to induce the NodD1 construct from strain NGR234. By D14, the inhibitory signals from Pelde were gone and both cultivars produced activity readings only slightly above that of water (< 10 × 10^3 units). Similar levels of activity were recorded on D21.

Interaction of *R. leguminosarum* bv. *trifolii* strain ANU843 with rice seedlings

To mimic the responses of rice grown under flooded conditions, a procedure was developed using Magenta jars with liquid medium. Cultivar Pelde seedlings were inoculated with the clover strain ANU843 and the plants grown for 3 wk. In comparison with the uninoculated plants, the strain ANU843-inoculated seedlings were very inhibited in their growth and were light yellow in color (Fig. 4).

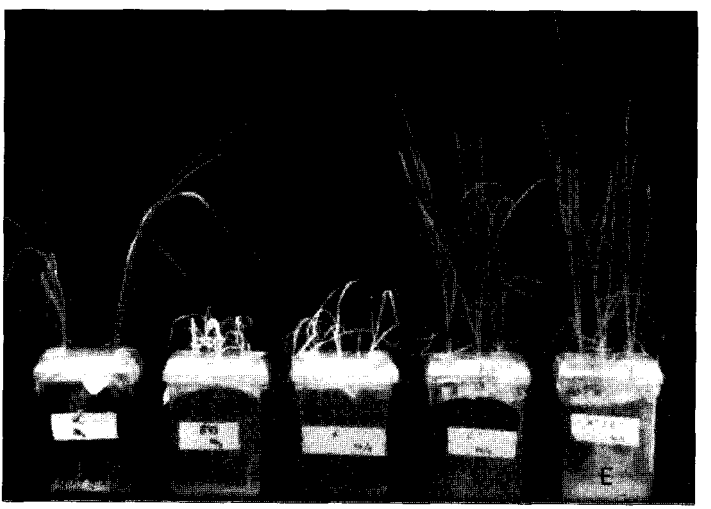

Fig. 4. Rice cultivar Pelde seedlings grown for 3 wk in Magenta jars with liquid NFM10 medium and inoculated with strain ANU843 and its plasmid-cured isolates: (A) uninoculated seedlings, (B) plants inoculated with strain ANU843, (C) inoculated with strain ANU845 pSym (pa)-cured, (D) inoculated seedlings with strain CFNS152, (E) plants inoculated with strain CFNS601 that has been cured of both pa and pc plasmids.

Table 1. Effect of bacterial inoculation on the growth of cultivar Pelde seedlings.

Inoculant strain	Plant response[a]
Uninoculated control	Plants grow, leaves green
ANU843	Growth inhibited, leaves yellow
ANU845pSym$^-$	Growth inhibited, leaves yellow
CFNS152 pc$^-$	Plants grow, leaves green
CFNS309pe$^-$	Plants grow, leaves green
CFNS601pa $^-$pc$^-$	Plants grow, leaves green
R4	Plants grow, leaves green
E11	Growth inhibited, leaves yellow
E4	Growth inhibited, leaves yellow
NGR234	Growth inhibited, leaves yellow
C58	Growth inhibited, leaves yellow
A136pTi$^-$	Growth inhibited, leaves yellow

[a] Seedlings grown for 21 d in F10 medium.

Although many of the individual plasmids of strain ANU843 can be cured, their presence does influence the phenotypic properties of the strain (Guerreiro et a1 1998). To test whether information encoded on these plasmids could influence the interaction between strain ANU843 and rice, rice seedlings were inoculated with various derivative strains of ANU843, which had lost one of their plasmids (Fig. 4). Strain ANU845, which had lost its Sym-plasmid, pa, behaved like the parental strain ANU843 inhibiting rice growth. Derivative strains, however, lacking either the pc plasmid or the pe plasmid or both plasmids pa and pc, did not inhibit rice seedling growth, and the plants were green at 3 wk (Fig. 4).

These experiments were then extended to examine the effects of other strains on rice plant growth (Table 1). Rice seedlings inoculated with *Agrobacterium tumefuciens* strain C58, its Ti-plasmid-cured derivative strain A136, or with *Rhizobium* strains E4, E11, or NGR234 all inhibited plant growth and produced yellow plants by 3 wk. In marked contrast was the finding that rice-associating strain R4 did not inhibit rice growth and the plants were green after 3 wk of incubation (Table 1).

Proteome analysis of strain ANU843 and its plasmid-cured derivatives

The finding that the two derivative strains—CFNS 152 (pc cured) and CFNS309 (pe cured—of *R. leguminosarum* bv. *trifolii* ANU843 did not inhibit rice seedling growth prompted the reexamination of gene expression in these strains in an attempt to describe the genetic and chemical basis of strain ANU843 inhibition of rice growth. Previously, proteome analysis was used to monitor the global changes in gene expression in strain ANU843 and the detection of plasmid-encoded functions (Guerreiro et al 1997, 1998). We began 2-DE protein maps for strain ANU843 grown in the presence and absence of the flavone DHF (Fig. 5A). Image analysis of 2-DE protein gels of strain ANU843 revealed nearly 2,000 gene products, which represents about 36% of the estimated coding capacity of the strain, assuming that one gene gives one pro-

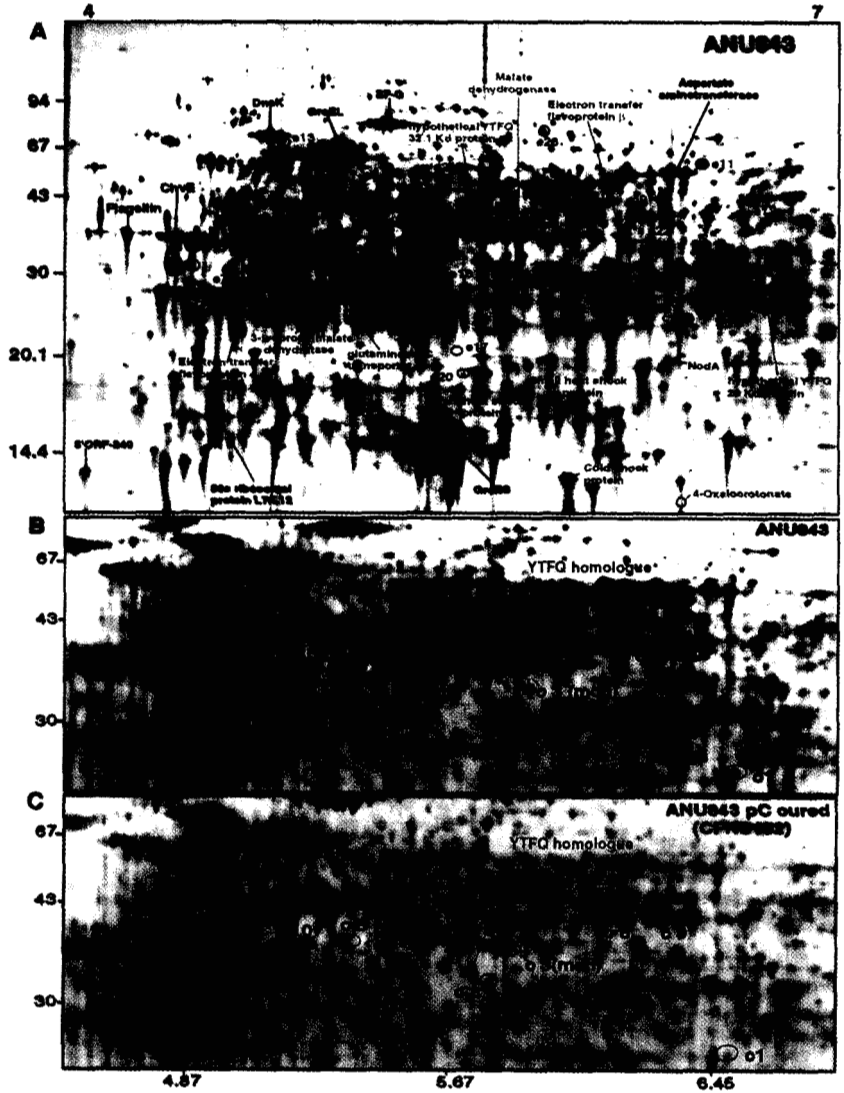

Fig. 5. Proteome pattern of strains ANU843 and CFNS152 showing proteins affected by plasmid curing: (A) proteome pattern of strain ANU843 showing the 39 proteins affected by curing plasmid pe (proteins circled or named in blue), curing plasmid pc (proteins circled or named in green), or curing plasmid pa, the pSym (proteins circled or named in red). The identity of several constitutively expressed proteins are labeled in black. (B and C) Proteins that are consistently altered in expression levels by curing pc are identified in ANU843 (panel B) and strain CFNS152 (cured of plasmid pc) in panel C. Protein c2 is homologous to an unidentified open reading frame (YTFQ) in *E. coli.* Strain CNFS152 was kindly provided by Tomas Stepkowski.

tein product (Guerreiro et a1 1997). A 2-DE *Rhizobium* protein database has been constructed for strain ANU843 based on both sequencing and amino acid composition analysis of more than 100 constitutively expressed proteins. Furthermore, we have used proteome analysis of a series of plasmid-cured derivatives of strain ANU843 to add to this database (Guerreiro et a1 1998). Figures 5B and 5C show that, under the conditions used, the plasmid-encoded gene products contributed only a small proportion of the 2,000 proteins visualized on the gels.

Strain CFNS 152 is a derivative of ANU843, which has had its plasmid pc eliminated. Plasmid pc is about 300 kbp in size and, with its removal from strain ANU843, 10 protein differences can be detected: four proteins were lost, four were up-regulated, and two were down-regulated (Fig. 5C) (Guerreiro et a1 1998). At present, we have been able to characterize only four of these gene products, with the other six still under investigation to identify their possible function. The change in the regulation of six of the proteins, particularly the malate dehydrogenase, suggests a complex interaction between the different replicons that affect gene expression in strain ANU843.

In strain CFN309, the largest plasmid, the pe plasmid, which is approximately 700 kbp in size, has been eliminated and, as a consequence, 26 protein differences can be detected (data not shown). This represents a loss of 15 proteins, two newly synthesised proteins, the up-regulation of five, and the down-regulation of four. Because of the number of changes associated with the elimination of the pe plasmid, we are concentrating on strain CNFS 152 in an attempt to describe the genetic and chemical basis of strain ANU843 inhibition of rice growth.

Discussion

Our aim in this research was to investigate the complex series of signal exchanges that occur during the early stages of Rhizobium infection of clovers and the nonlegume *Parasponia.* With this information, we studied the rice-*Rhizobium leguminosarum* bv. *trifolii* interaction by several approaches: first, by analyzing whether rice plants produced chemical signals that might interact with rhizobia; second, by studying the effects of bacterial inoculation on rice seedling growth; and third, by using proteome analysis to investigate the molecular genetic basis of *Rhizobium* strain ANU843 inhibition of rice plants.

Summary of the signal transduction experiments

The early steps in signal transduction involve transient changes in the apparent levels of auxin and flavonoids in the root. Our experiments have supported the proposal that the following series of events give rise to nodulation. Early during infection, the *Rhizobium* LCOs cause the induction of the flavonoid pathway in the inner cortical cells of the root. These events are followed by an alteration of auxin flow. Subsequently, the inner cortical cells accumulate fluorescent flavonoids, followed by auxin accumulation in those cells, which then divide to form a nodule primordium (Mathesius et al 1998a,b).

Our results suggest that fluorescent compounds, whose properties are consistent with those of flavonoids, accumulate in dividing cells during nodule formation in the nonlegume *Parasponia.* Therefore, flavonoids are probably not the unique feature of legumes that enable legumes to form symbioses with *Rhizobium.* The results further suggest that flavonoids may play a role in organ formation. The fact that *nod*-gene-inducing substances as potential nodulation signals are also present in nonlegume roots suggests that the phenylpropanoid pathway could be further exploited in nonlegumes in the quest to establish *Rhizobium*-nonlegume interactions.

Plant signal production

By sectioning the seedlings of cultivars Calrose and Pelde, it was possible to show that there was a variation in the levels of *nod*-gene-inducing substances extracted from the different sections of the plant. In addition, this variation in levels changed with the development of the seedlings. Therefore, either different amounts of signal molecules are present or different types of signal molecules are produced in the sections studied in both cultivars. The Shl section, which is the first section of the growing shoot that includes the culm region, is the major zone containing signals that can activate the NGR234 NodDl reporter system. These signals in the Shl region are produced early during seedling development, reaching a peak at day 10.

The different signal extracts from the various sections were investigated by reverse-phase thin-layer chromatography and the chromatograms treated with diphenylboric acid 2-amino ethyl ester. This treatment produced shifts in the colors seen under 365-nm ultraviolet light, which suggested that flavonoids were present in some of the fractions (J. Stefaniak, unpublished data). At this stage, however, there is no evidence that the *nod* genes of rice-associating rhizobia can be activated by these molecules or that LCOs are necessary for association. It might be advantageous for rice-associating bacteria to be attracted to this Shl region early in development while there are many possible open intercellular spaces in the culm region (F.B. Dazzo, unpublished, Yanni et a1 1997). There are fluid-filled spaces between the developing leaf sheaths at the base of the stem, which the bacteria could enter and colonize and grow on the potentially nutrient-rich environment present in this region.

In nearly all situations, the level of *nod*-gene-inducing activity detected in root extracts was greater for cv. Pelde extracts than for those of cv. Calrose. This correlates with the observation that Pelde shows greater growth promotion than Calrose when inoculated with *Rhizobium* strain R4 (Prayitno et a1 1999). In our experiments, however, some *Rhizobium* strains inhibited the growth of both Pelde and Calrose. So are the same plant-signal molecules promoting different effects in strain R4 and in these "rice-inhibiting strains," leading to growth enhancement with strain R4 and growth reduction with the rice-inhibiting strains? Or are there different signal molecules that are active from a cocktail of signals extracted from each section?

The exudation of flavonoids from certain sites along the root may be a mechanism that enabled signal exchange between legumes and soil bacteria. The exudation of such signals, however, seems to be confined to the R1 region on the roots of day-7 Pelde seedlings (Stefaniak, unpublished data).

The signal extracts on D10 from the Pelde R1 region are an exception as they can inhibit ß-galactosidase activity in reporter strains and presumably the induction of the NGR234 NodDl reporter system. Interestingly, on D10, there is a pronounced increase in activity in signals from the R2 region (the root section starting 2 cm down from the seed) in Pelde. This correlates with the zone of secondary root emergence and hence possible sites of bacterial entry into the root tissue. The inhibitory signals in R1 may also produce negative chemotaxis, that is, repel the bacteria and redirect them to the newly active chemo-attracting zone now in R2.

Interaction between bacterial strains and rice cultivars

We found previously that different rhizobia can either promote or inhibit the growth of rice seedlings and that environmental growth conditions can greatly influence these growth effects (Prayitno et al 1999). We then took two approaches to investigate this plant-microbe interaction system (Prayitno et al 1999). First, we used a specific genetic marker (the green fluorescent protein) to investigate root colonization patterns. These studies showed that *Rhizobium* preferentially colonizes in clumps, along grooves on the rice root surface, or at the emerging lateral root zones and root tips. But *Rhizobium* bacteria could also colonize intercellularly in lateral roots formed on the main roots near the culm region of the seedling, forming long lines of individual bacterial cells inside the growing lateral root (Prayitno et al 1999).

Second, when cultivar Pelde seedlings were inoculated with the clover strain ANU843, they became very inhibited in their growth and leaves were yellow in color by 3 wk. Rice seedlings inoculated with derivative strains of ANU843, strains CFNS 152 and CFNS309, which have been cured of specific plasmids, however, were not inhibited and were green after 3 wk. Proteome analysis was then used to investigate the molecular genetic basis of this rice interaction (Fig. 5). Significant advances in bioinformatics and techniques for proteidpeptide identification have made proteome analysis a primary tool for characterizing gene expression and regulation in complex biological systems (Wilkins et al 1995, Guerreiro et al 1997, 1998).

We have concentrated on strain CNFS 152 in an attempt to describe the genetic and chemical basis of strain ANU843 inhibition of rice growth because of the lower number of protein changes associated with the elimination of the pc plasmid. We have identified only two of the 10 proteins that were altered during pc loss. To understand the rice-interaction phenotypes of strains CNFS 152 and ANU843, the other eight proteins need to be investigated in more detail. To do this more effectively, however, we need to identify those conditions in which plasmid-borne genes are expressed and affect the complex replicon interaction of strain ANU843. Future experiments will involve plasmid mobilization and cosmid cloning studies.

Finally, the possibility of establishing a more effective type of *Rhizobium*-nonlegume interaction is potentially available in rice because many of the phenylpropanoid pathway compounds that could interact with *Rhizobium* are also present in nonlegume roots. The finding that some *Rhizobium* strains can markedly inhibit rice seedling growth while others can enhance plant performance, however, has important implications for the future development of inoculum strains, as well as

for the use of rice-legume crop rotations. What is not known are the effects of various natural ratios of stimulatory and inhibitory bacteria in the soil on the early stages of rice seedling development. Analysis of the resident bacterial populations of rice-growing areas will be very informative and provide important information on their effect on initial seedling growth, final yield, and grain nitrogen content.

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Notes

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Exploring genetic programs for root endosymbioses

Clare Gough and Jean Denarie

The study of the interaction of plants with diazotrophic bacteria has clearly shown that it is only in intercellular, endosymbiotic interactions that significant amounts of fixed nitrogen are provided to the host plant. While only a minority of plants can establish such root endosymbioses with diazotrophic bacteria, most plants are able to establish endosymbiotic associations with arbuscular endomycorrhizal fungi. Therefore, a large majority of plants, including rice, wheat, and maize, possess the genetic information required to pave the way for successful symbiotic root infection, involving the reactivation of cortical cells in the presence of a microsymbiont, the accepted penetration of the symbiont in host cells, and the synthesis of a membranous interface with the symbiotic partner to facilitate metabolic exchange. We summarize the genetic and genomic approaches used on the model legume *Medicago truncatula* to identify the legume genetic program controlling root endosymbioses, that is, the genes involved in nodulation, in endomycorrhizae formation, and in both types of symbioses. We propose to generate and study expressed sequence tag libraries of mycorrhized roots in rice to identify rice genes involved in endomycorrhizae formation, that is, the rice genetic program for endosymbiotic interactions. A comparison of the *M. truncatula* and rice programs for root endosymbioses should allow a critical assessment of the feasibility of exploiting and improving the existing symbiotic genetic program of rice to make endosymbiotic associations possible between nitrogen-fixing bacteria and this major crop.

Among the approaches that have been proposed to extend biological nitrogen fixation (BNF) to nonlegume plants such as rice (Ladha et a1 1997, Rolfe et al 1998). three main strategies can be distinguished: (1) selection of efficient endophytic or rhizospheric associations between diazotrophic bacteria and plants, (2) transfer of bacterial *nif* genes to plants, and (3) transfer of nodulation ability to nonlegumes. The first strategy, which has already been the subject of several studies (see Ladha et al 1997, Reinhold-Hurek and Hurek 1998), can be considered to be a short- to medium-term objective, while the second and third strategies represent two very long-term objectives.

The study of the interaction of plants with diazotrophic bacteria has clearly shown that only endophytic associations provide significant amounts of fixed nitrogen to the host plant. Two types of such symbioses can be envisaged: one with bacteria in the intercellular spaces or vascular bundles and one involving intracellular associations. One of the most efficient BNF systems known is the *Rhizobium*-legume symbiosis (see Mylona et al 1995). Nitrogen fixation by the *Rhizobium*-legume symbiosis provides such significant benefits to host plants because of an intracellular infection that allows intimate and efficient metabolic and signal exchange at the cellular level between the nitrogen-fixing bacteria and host cells. This infection is also accompanied by the formation of a novel and specific nitrogen-fixing organ, the nodule, which allows optimal oxygen availability and facilitates metabolite exchange between the nitrogen-fixing tissues and the rest of the plant.

While relatively few plants (only legumes and actinorhizal plants) are able to form nodules in association with nitrogen-fixing bacteria, the ability of plants to form endosymbiotic associations with microorganisms is much less restricted. In fact, the majority of plants, including rice, possess the genetic information required to pave the way for successful symbiotic root infection, involving reactivation of cortical cells in the presence of a symbiont, accepted penetration of the symbiont in host cells, and synthesis of a membranous interface with the symbiotic partner to facilitate metabolic exchange. Such a genetic program is activated by arbuscular endomycorrhizal fungi, which are capable of establishing highly efficient symbioses with more than 80% of all land plants examined (Gianinazzi-Pearson 1996, Harrison 1997, 1999). The scientific community working on nitrogen fixation has largely ignored this feature of nonlegume plants and yet recent genetic research on legume nodulation has allowed the identification of plant genes that are involved in both endomycorrhizae formation and nodulation (reviewed in Gianinazzi-Pearson 1996, Albrecht et a1 1999, Harrison 1999), making more apparent the existence of this genetic program for endomycorrhizal endosymbiosis in most plants. Multiple research efforts are now concentrating on model legume plants (see Cook et al 1997) to facilitate rapid and coordinated studies aimed at unraveling the genetic programs of these two important endosymbiotic associations between plant roots and microorganisms.

The first part of this chapter briefly summarizes the arbuscular mycorrhizal and *Rhizobium*-legume symbioses. Subsequently, we describe how plant genes that control common steps in these interactions have been identified, and how the legume *Medicago truncatula* can be exploited as a model system to characterize the function of these genes and to identify and characterize genes specifically involved in nodulation, mycorrhization, or both. To have, even in the long term, a chance of transferring to rice the ability to establish intracellular symbiosis with nitrogen-fixing bacteria, it is necessary to identify both the endosymbiotic (endomycorrhizal and nodulation) genetic programs of legumes and the endosymbiotic (endomycorrhizal) genetic program of rice. These objectives are considerable, but in the final part of this chapter we will describe how progress in rice and legume genomics should provide, on a global scale, a wealth of information concerning genes involved in these programs, in the coming five years.

Two major types of root endosymbioses

The arbuscular mycorrhizal symbiosis

Arbuscular mycorrhizae, formed between the roots of most higher plants and obligate biotrophic fungi of the order *Glomales,* are by far the most common root endosymbiotic association (Smith and Read 1997, Harrison 1997. Gianinazzi-Pearson 1996). The endomycorrhizal symbiosis is extremely ancient and structures identified as arbuscules have been found in fossil tissues of an early Devonian land plant, providing evidence for at least a 400 million-year-old association between terrestrial plants and arbuscular mycorrhizal fungi (Remy et al 1994). Moreover. the ability of plants to associate with fungi may have been a major turning point in land colonization. Mycorrhizal fungi procure and transport phosphate and other nutrients from the soil to plant roots. In turn, the host plant provides fixed carbon to its fungal partner (see Harrison 1999). In contrast to the *Rhizobium*-legume association. there is very little host specificity in arbuscular endomycorrhizal symbioses. A fungus can interact with diverse host-plant genera and a certain host plant can interact with several fungal species and genera.

Fungal hyphae enter host plants through the root epidermis via specific structures, called appressoria, which are formed on the surface of roots. Once inside the root, hyphae are either intercellular or intracellular, depending on the host plant. In the case of intercellular infection, the process that has been most commonly studied, hyphae enter the root between two epidermal cells and become surrounded by de novo synthesized cell-wall material. In the inner cortex, the fungus invades root cells. generating arbuscules, which are highly ramified structures thought to play an important role in metabolic exchange between the two partners. Although the fungus is intracellular at this stage, it is not in direct contact with the host cytoplasm because a perifungal membrane, originating from the plant plasma membrane, invaginates and surrounds the arbuscules.

Little is known about the exchange of signals between the two partners, although exudates from host roots, including flavonoids. enhance spore germination and elongation and branching of hyphae (Nair et al 1991, Tsai and Phillips 1991, see Harrison 1999). In addition, rhizobial Nod factors have been reported to enhance arbuscular mycorrhizal colonization of soybean roots and this phenomenon has been suggested to be linked to increased flavonoid secretion by roots (Xie et al 1995). The plant genetic control of endomycorrhization has not yet been studied in much detail mainly because of the difficulty in screening for mutants, but also because of the recalcitrance of the fungal partner to growth in pure culture. Moreover, the model plant *Arabidopsis thaliana,* like the majority of the members of the Brassicaceae, cannot form arbuscular mycorrhizae, precluding its use as a genetic system to study plant-mycorrhizal fungal interactions.

The *Rhizobium*-legume symbiosis

Symbiotic bacteria of the genera *Rhizobium, Bradyrhizobium, Azorhizobium,* and *Sinorhizobium,* collectively referred to as rhizobia, elicit on their leguminous hosts

the formation of specialized organs, called nodules, in which atmospheric nitrogen fixation occurs. At least as much nitrogen is fixed annually by these symbiotic associations as by the chemical fertilizer industry worldwide. Genetic analyses of the bacterial partner have led to the identification of *nod* genes, which are involved in the control of host specificity, infection, and nodulation. The regulation of *nod* gene expression is under the control of plant signals, essentially flavonoids, excreted in the rhizosphere. The *nod* genes specify the synthesis of symbiotic signals, Nod factors, that are lipo-chitooligosaccharides (Dénarié et al 1996). While the role of Nod factors in the control of the specificity of infection and nodulation has been studied quite extensively, little is known about the mechanisms of Nod factor perception and transduction in the host plant. Purified Nod factors are capable of eliciting, in roots of legume hosts, many of the plant responses characteristic of the bacteria themselves (Dénarié and Cullimore 1993). These responses include the initiation of cortical cell division, the activation of plant genes that are specifically induced during early stages of nodulation (nodulin genes), cytoskeleton reorganizations, and the triggering of a plant organogenic program leading to nodule formation.

The induction of these responses by Nod factors and the identification of lines of alfalfa that can spontaneously form nodules in the absence of rhizobia (Truchet et al 1989) indicate that genetic control of the nodulation program is governed by the plant. Furthermore, the complexity of the organogenic process suggests that numerous plant genes are involved. Molecular studies have identified nodulin genes, which have been subdivided into early and late nodulin genes, according to the timing of their expression (reviewed in Mylona et al 1995). Early nodulin genes are associated with infection, organogenesis, and bacterial invasion of the root nodule. Late nodulin gene products are thought to be involved in nodule function and include the oxygen transporter leghemoglobin, enzymes of carbon and nitrogen metabolism, and proteins located in the peribacteroid interface. Genetic studies of pea nodulation mutants have identified at least 40 plant genes involved in different stages of the symbiotic interaction (see Borisov et al 1998).

Legume genes involved in both nodulation and mycorrhization

Many parallels can be drawn between root nodule formation induced by rhizobia and root colonization by arbuscular mycorrhizal fungi (Fig. 1; Albrecht et al 1999, Harrison 1999). In both cases, during the penetration of root tissues, the microsymbionts are separated from host cells by plant cell wall-derived material and the plant plasma membrane. In both cases, the target tissue for the microsymbiont is the root cortex, and extensive defense reactions are not triggered. The intracellular invasion and colonization of cortical cells are linked to reactivation of the host cytoplasmic compartment and nucleus, as well as proliferation of the microsymbiont and its development into specific structures, the bacteroids in nodules and the arbuscules in mycorrhizae. Both bacteroids and arbuscules are separated from the plant cytoplasm by a host-derived perisymbiotic membrane. The peribacteroid membrane (in nodules) and the

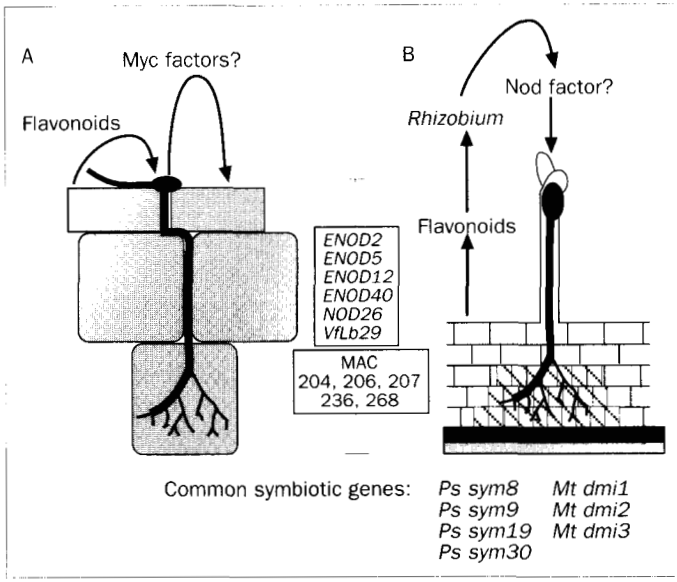

Fig. 1. Diagrammatic illustration of mycorrhiza (A) and nodule (B) development. showing legume nodulin genes expressed during both interactions (*ENOD2, ENOD5, ENOD12, ENOD40, NOD26, VfLb29*), infection thread and peribacteroid compartment glycoconjugates that are immunodetected in plant-derived components of the arbuscule interface (MACs), and common symbiotic genes of pea (*Ps sym8, Ps sym9, Ps sym19, Ps sym30*) and of *Medicago truncatula (Mt dmi1, Mt dmi2, Mt dmi3)*. (A) Root-exuded flavonoids stimulate fungal growth prior to infection and fungal Myc factors may be involved in signaling to the plant before and/or during infection. (B) Root-exuded flavonoids induce rhizobial *nod* genes, resulting in the production of rhizobial symbiotic signals, the Nod factors, which are involved in host specificity, infection, and nodulation. For details, see text.

periarbuscular membrane (in mycorrhizae) are thought to be extended surfaces between the symbiotic partners that facilitate bidirectional exchange of metabolites.

Legumes are able to establish root endosymbioses with both endomycorrhizal fungi, to form phosphate-acquiring arbuscular mycorrhizae. and rhizobia, to form nitrogen-fixing nodules. Direct screening for plant mutants affected in their ability to form arbuscular mycorrhizae is a very arduous task. Legume mutants that are altered in their ability to form nodules (Nod-) are easy to screen for, however, and pioneering genetic studies have led to the finding that certain such Nod- mutants of pea *(Pisum sativum)* and of faba bean *(Vicia faba)* are simultaneously altered in their ability to form arbuscular mycorrhizae (Myc-), while their ability to interact with soil pathogens is unaffected (Duc et a1 1989, Gollotte et al 1993).

This shows that legume genes, which are essential for both rhizobial and endomycorrhizal symbioses, exist. Four such loci have now been identified in pea *(Ps sym8, Ps sym9, Ps syml9, Ps sym30)* (Albrecht et a1 1999) and Nod-/Myc-mu-

tants have been characterized in other legume species, including *Medicago trancatula* (Sagan et a1 1995, 1998), bean *(Phaseolus vulgaris)* (Shirtliffe and Vessey 1996). and *Lotus japonicus* (Szczyglowski et al 1998, Wegel et al 1998). Also, in pea, mycorrhiza mutants have been identified that are both affected at the stage of arbuscule development and show a delayed nodulation phenotype (Gianinazzi-Pearson 1996).

Interestingly, the *sym8* gene of pea, which is one of the four genes controlling both the *Rhizobium*-legume and endomycorrhizal symbioses, controls a step of the signal transduction cascade, induced by Nod factors as well as mycorrhizal fungi, and which leads to *PsENOD5* and *PsENOD12A* expression (Albrecht et al 1998). Other early nodulin genes have been identified that are also induced in both symbioses—*MsENOD2* and *MsENOD40* in alfalfa (van Rhijn et al 1997), a leghemoglobin gene *(VfLbb29)* in broad bean (Frühling et al 1997), and the aquaporin-encoding gene *NOD26* in soybean (Wyss et al 1990). Certain infection thread and peribacteroid compartment glycoconjugates (MACs) are immunodetected in plant-derived components of the arbuscule interface (Gianinazzi-Pearson 1996).

Expression of nodulin genes in nonlegumes

Furthermore, certain nodulin genes are expressed in nonlegumes and are subject to regulatory mechanisms similar to those described in legumes. For example, Nod factors are able to induce expression of the *MtENOD12* promoter when introduced into rice (Reddy et al 1998). The chitooligosaccharidic backbone of Nod factors has been shown to be unable to elicit this response, indicating that rice roots specifically perceive N-acylated Nod factors, which can act as signal molecules to activate *MtENOD12* expression. As in legumes, the presence of an excess of combined nitrogen in the growth medium inhibited the expression of *MtENOD12* in transgenic rice plants, suggesting that at least part of the nitrogen-mediated regulatory mechanism responsible for symbiotic responses in legumes is present in rice.

The expression of the early nodulin gene *ENOD40* has also been studied in rice (Kouchi et al 1999). Two *ENOD40* homologues, *ObENOD40* and *OsENOD40,* have been isolated from rice and shown to potentially encode small oligopeptides with considerable amino acid sequence homology to the corresponding oligopeptides of *ENOD40s* of legumes. Subsequent in situ hybridization studies revealed that the transcription of the *OsENOD40* gene is confined to parenchyma cells surrounding the protoxylem during the early stages of development of lateral vascular bundles conjoining an emerging leaf. When the *OsENOD40* promoter, fused to a reporter gene, was introduced into soybean, expression was also found to be restricted to peripheral cells of nodule vascular bundles. This indicates that the promoter activity of the rice *ENOD40* gene is essentially the same as that of the soybean *ENOD40* gene and that *OsENOD40* and legume *ENOD40s* probably share common functions in the differentiation and/or functioning of vascular bundles (Kouchi et al 1999).

The observation that nodulin gene expression in legumes and nonlegumes shares certain characteristics, including the ability to be induced by lipo-chitooligosaccharides, suggests that legumes may have recruited. for Nod factor recognition and transduc-

tion, mechanisms that were already existing in nonlegumes and possibly used in mycorrhizal interactions. If this is true, then the ability to respond to chitin oligomer derivatives may be an inherent property of all plants having the genetic program to establish an endosymbiotic interaction with endomycorrhizal fungi. This, in conjunction with the observed relatedness of the differentiation responses in both types of symbioses, suggests that the symbiotic partners trigger the activation of a welcoming "red carpet," consisting of genetic programs that pave the way for infection through the root tissue (Gianinazzi-Pearson and Dénarié 1997).

Medicago truncatula, a model legume to study plant-microbe interactions

Most genetic studies of nodulation have been performed in pea, soybean, and alfalfa. The large or complex genomes of these plants, however, render map-based cloning of the symbiotic genes identified by mutagenesis very difficult. Furthermore, *Arabidopsis thaliana* is not able to establish either nodulation with rhizobia or endomycorrhization with arbuscular mycorrhizal fungi. It has therefore been important to identify (an)other model plant(s) to study these root endosymbioses and the natural choice was among legume plants because they can establish both types of root endosymbiosis. Our laboratory proposed *Medicago trancatula* a few years ago (Barker et a1 1990) and several laboratories worldwide have now decided to concentrate their research efforts on this model legume (Cook et a1 1997. Penmetsa and Cook 1997. Liu et al 1998. Cook 1999). A second model legume, *Lotus japonicus,* which, in contrast to *M. truncatula,* forms determinate nodules, was suggested by Handberg and Stougaard (1992) and is also studied by several laboratories (see Cook et al 1997, Szczyglowski et al 1998).

M. truncatula was chosen because of its amenability to molecular genetic analysis; it has a diploid genome, approximately one-sixth of the size of pea (–500 Mb), has a short life cycle, is autogamous, has a high level of natural polymorphism, and can be efficiently transformed and regenerated. In addition, the microsymbiont of *M. truncatula, Sinorhizobium meliloti,* is the most extensively studied rhizobial species. Extensive mutageneses have been performed on *M. truncatula:* gamma-ray mutageneses in Dijon, France (Sagan et a1 1995, 1998). and EMS mutageneses in Texas, USA (Penmetsa and Cook 1997). Nodulation mutants have been identified in both laboratories, as well as by our group in Toulouse, and a large collection of nodulation mutants is now available.

Identification of *Medicago truncatula* genes involved in rhizobial entry

We are currently exploiting the collection of *M. truncatula* Nod– mutants to genetically dissect the mechanisms of rhizobial initiation of infection in root hairs and the Nod factor perception and signal transduction pathway(s). By cytological and histological studies, we have first determined at what stage of the symbiotic interaction the various mutants are blocked. Approximately 30 mutants were found to be blocked at a very early stage of infection; they show none of the early, typical responses to bacterial inoculation such as the formation of infection threads and of nodule meristems.

Genetic characterization of these mutants has shown that we are dealing with recessive, monogenic mutations and allelism tests have enabled us to define at least six complementation groups (R. Catoira, C. Galera, C. Gough, E de Billy, F. Maillet. V. Penmetsa, D. Cook, C. Rosenberg, and J. Denarie, manuscript in preparation).

The characterization of genes specifically involved in nodulation should reveal the type of functions that are needed for rhizobial infection and nodule ontogeny, in addition to those coded for by genes common to nodulation and mycorrhization. Three main groups of nodulation-specific genes can be envisaged: (1) rhizobial host-specificity genes controlling bacterial entry into root hairs, (2) genes involved in the Nod factor signal transduction cascade, and (3) genes controlling specific steps of nodule organogenesis and functioning. Whereas in the endomycorrhizal symbiosis very little host specificity has been observed, the *Rhizobium*-legume interaction is generally highly specific. Host specificity is controlled at several levels, and the synthesis and structure of Nod factors often play a prominent role in the control of bacterial infection. The symbiotic characterization of bacterial mutants producing modified Nod factors has suggested that there are at least two pathways for Nod factor perception: one pathway not very stringent for Nod factor structure, leading to the induction of symbiotic responses (root hair deformation, nodulin gene expression, and cortical cell activation), and another pathway highly stringent for Nod factor structure, leading to rhizobial entry into the root hairs (Ardourel et al 1994).

To identify *M. truncatula* genes specifically involved in the control of rhizobial entry, we have adopted two approaches. First, by screening *M. truncatula* Nod- mutants for their ability to form arbuscular mycorrhizae, two classes of mutants have been identified: mutants affected in both symbioses (Nod^-/Myc^-) and mutants affected specifically in nodulation (Nod^-/Myc^+) (M. Harrison, V. Gianinazzi-Pearson, and D. Morandi, personal communications). By analyzing *M. truncatula* Nod^-/Myc^+ mutants that elicit normal responses to Nod factors in epidermal cells, but are unable to initiate infection thread formation, we have identified a gene that controls infection initiation (R. Catoira, C. Gough, T. Timmers, G. Truchet. V. Penmetsa, D. Cook, and J. Denarie, manuscript in preparation). Second, we have studied natural ecotypes of *M. truncatula* that show alterations in their symbiotic interactions with rhizobial mutants, producing modified Nod factors. One hundred twenty natural populations of *M. truncatula* have been screened for their nodulation properties with *Sinorhizohium meliloti nodL* mutants producing Nod factors lacking the O-acetyl group, with a *nodFL* mutant producing Nod factors with a modified N-acyl chain and lacking the O-acetyl group at the nonreducing end, and with *nodH* mutants producing Nod factors lacking the O-sulfate group at the reducing end. Ecotypes showing different Nod factor structural requirements for the elicitation of symbiotic responses, such as the formation of infection threads and nodule induction, could be grouped into at least three classes having different requirements for the O-acetyl, O-sulfate, or N-acyl Nod factor substitutions. We are currently performing complementation tests among these ecotypes to determine the number of plant genes involved in specific Nod factor recognition.

In the case of one of these ecotypes, DZA315, a *nodL* mutant of *S. meliloti* has been found to be unable to initiate the formation of infection threads, indicating that

the presence of the O-acetyl group is required for entry into root hairs. In contrast, the presence of the O-acetyl group is not required to elicit root hair curling or transcription of the early nodulin gene *MtENOD11* or for the activation and mitosis of cortical cells. Moreover, genetic analysis. by crosses with cultivar Jemalong. has revealed that the control of stringency for the O-acetyl group is monogenic and dominant (T. Huguet, P. Toquet, E Maillet, G. Truchet, T. Timmers, C. Rosenberg. and J. Dénarié. manuscript in preparation).

Identification of *Medicago truncatula* genes involved in the Nod factor signal transduction cascade

In addition to the Nod^-/Myc^+ mutants described above, 12 Nod^-/Myc^- mutants have so far been characterized and found to be blocked early for infection by both symbionts. These mutants are no longer able to form curled root hairs or infection threads in the presence of rhizobia and, although arbuscular mycorrhizal fungi can still form appressoria on roots of these mutants, they fail to develop intercellular hyphae. In collaboration with E.P. Journet (CNRS-INRA, Toulouse) and M. Sagan (INRA, Dijon; Sagan et al 1998). we have established that at least three *M. truncatula* genes *(dmil, dmi2,* and *dmi3*) are involved in early stages common to both symbioses.

The responses of Nod-/Myc- mutants to Nod factors are now being studied to dissect the Nod factor signal transduction cascade in *M. truncatula.* Certain mutants have been found to be pleiotropically affected in early nodulin gene expression and in root hair deformation, while retaining their ability to specifically recognize Nod factors, strongly suggesting that the mutated genes encode components of the Nod factor signal transduction cascade. Three such genes have already been identified, all of which control early steps common to both rhizobia1 and endomycorrhizal symbioses. Another gene that may encode a component of the Nod factor signal transduction cascade and that controls a step specific for nodulation has also been identified (R. Catoira, C. Galera, C. Gough, F. de Billy, F. Maillet. V. Penmetsa, D. Cook, C. Rosenberg, and J. Dénarié, manuscript in preparation). *dmi1* and *dmi2* have been mapped on the genetic map of M. truncatula (T. Huguet. INRA-CNRS, Toulouse, personal communication) and are now being isolated. by map-based cloning, in collaboration with T. Huguet (INRA-CNRS, Toulouse) and D. Cook (Texas A&M University).

The discovery that "common" legume symbiotic genes are involved in the Nod factor signal transduction cascade suggests that the characterization of homologous genes in rice might help to elucidate how putative mycorrhizal signals, Myc factors, are perceived by rice. In *M. truncatula,* Nod factor perception leads to the induction of symbiotic responses such as root hair deformation, nodulin gene expression, and cortical cell activation. Such responses are thought to prepare the way for bacterial infection. The early nodulin genes, which are expressed in the endomycorrhizal symbiosis and in rice, are genes whose activation is known to be nonstringent for Nod factor structure. This suggests that the nonstringent Nod factor perception pathway in legumes may have evolved from a perception mechanism present in nonlegumes. More-

over, lipo-chitooligosaccharide perception in rice has probably not been maintained to recognize Nod factors, and, although the natural ligands for this perception mechanism are unknown, the elucidation of its function in rice should help us understand at least some of the early steps in the establishment of arbuscular mycorrhizae.

Toward a genomic approach to elucidate molecular mechanisms underlying root endosymbioses

Medicago truncatula genomics

We have postulated the presence of three categories of symbiotic genes in legumes, namely, genes common to nodulation and mycorrhization. nodulation-specific genes, and mycorrhization-specific genes. The analysis of *M. truncatula* mutants has so far allowed the identification of genes common to nodulation and mycorrhization, as well as nodulation-specific genes. Since it has been shown that part of the genetic program is shared between the *Rhizobium*-legume symbiosis and the endomycorrhizal symbiosis and because both legumes and nonlegumes are able to establish arbuscular mycorrhizae with the same fungal species, it is reasonable to assume that most vascular plants possess such common symbiotic genes. This finding has important implications, including the hypothesis that at least part of the genetic program for endosymbiosis should be readily identifiable in important crops such as rice, wheat, and maize. Therefore, as soon as genes controlling the steps common to both nodulation and arbuscular mycorrhizae formation have been cloned from *M. truncatula,* the corresponding genes can be searched for and studied in rice.

In parallel to genetic approaches, molecular approaches have been successfully used to identify plant genes expressed during nodulation in *M. truncatula.* For example, 29 new families of nodulin genes of *M. truncatula* have been identified by subtractive hybridization (Gamas et al 1996) and a new Nod factor-induced gene of *M. truncatula* has been identified by mRNA differential display experiments (de Carvalho Niebel et a1 1998). Such genes will provide useful molecular markers, notably to define and characterize cell- and tissue-specific responses to rhizobia and to study Nod factor-induced plant responses.

Genes expressed during mycorrhization have also been identified by molecular approaches and initial studies concentrated on previously identified plant defense genes, which, in fact, show only minor transient increases in expression following fungal colonization (reviewed in Harrison 1997, 1999). More recently, differential hybridization approaches have been used in *M. truncatula* to isolate both a gene whose expression is suppressed in response to colonization by arbuscular mycorrhizal fungi (Burleigh and Harrison 1997) and three genes whose expression is induced during the establishment of the arbuscular mycorrhizal symbiosis (van Buuren et al 1999). The isolation and genetic analysis of *M. truncatula* Nod^+/Myc^- mutants with a range of mycorrhiza-defective phenotypes would lead to the identification of genes involved in the establishment and functioning of mycorrhizae and hence a fuller understanding of the genetic complexity of mycorrhizal symbiosis. However, such mutants are dif-

ficult to screen for. In addition, the identification of plant genes by mutant analysis has limitations (gene redundancy, hot spots for mutagenesis, lethal genes. etc.).

Functional genomics is a complementary approach to identify many symbiotic genes of all three categories, and considerable progress is being made in the study of *M. truncatula* genome expression. First, several groups are developing structural genomic approaches that will also serve as tools to clone symbiotic genes identified by the characterization of nodulation mutants. In Toulouse, T. Huguet's group has generated a genetic map of *M. trancatula* using molecular markers; at Texas A&M University. a BAC (bacterial artificial chromosome) library of *M. truncatula* has been generated by D. Cook's group; in Wageningen, the group of T. Bisseling has developed FISH (tluorescence *in situ* hybridization) technology to physically map *M. truncatula* symbiotic genes on pachytene chromosomes.

In the framework of an NSF plant genome program, an important project has recently begun in the United States, with *M. truncatula* serving as a "nodal species" for legume genomics (for more information, readers are referred to the following Web site: http://chrysie.tamu.edu/medicago). EST (expressed sequence tag) libraries generated under several physiological conditions, such as nodulation, mycorrhization, and responses to pathogens, are being made and analyzed within this project. Physical maps of chromosome regions containing symbiotic genes will be constructed to facilitate positional cloning of genes of interest. A root-hair EST library has been generated and sequenced in Stanford University (Covitz et al 1998). Also in the United States, a large-scale T-DNA activation tagging project is under way at the Noble Foundation (Oklahoma) in M. Harrison's group. This project aims to generate 300,000 tagged lines that will be screened for symbiotic mutants. In Europe, a network of various laboratories is launching an integrated structural, functional, and comparative genomics program on *M. truncatula.* Finally, in France, a DNA-sequencing project of 15,000 ESTs representing genes expressed during nodulation and mycorrhization has started and should be completed by October 1999. The comparison, using both high-density filters and DNA microarray technology. of *M. truncatula* EST libraries made from uninoculated roots, *S. meliloti*-inoculated roots, and mycorrhizal fungi-inoculated roots should lead to the characterization of genes expressed specifically during each symbiosis and genes expressed in both symbioses. Such EST-sequencing programs have already been extremely successful in the discovery of new genes in *A. thaliana* (see Bouchez and Hofte 1998). The global nature of this approach, together with the existence of several such EST libraries made in different laboratories at various times after inoculation, should enable us to study large numbers of genes, that is, to identify a large proportion of the legume genetic program for root endosymbioses.

Rice genomics

Both upland and wetland rice varieties are able to form endosymbiotic associations with arbuscular mycorrhizal fungi. Studies on mycorrhizae in upland rice have included the effect of inoculation on phosphorus absorption and growth promotion (Isobe and Tsuboki 1998) and on grain yield (Ammani and Rao 1996). More studies have

been performed on paddy-field rice. however, because mycorrhizal associations are less effective in these flooded conditions that represent a much greater proportion of rice production worldwide. For wetland rice. much work has concentrated on studying the influence of inoculating plants, at the nursery stage, on fungal colonization levels as well as on grain yield (Khan and Belik 1995, Secilia and Bagyaraj 1992, Solaiman and Hirata 1996, 1997, 1998).

The global study of gene function by functional genomics is also under way in rice, which has been chosen as a model monocotyledonous plant (Izawa and Shimamoto 1996, Bennetzen 1999). Rice is an excellent model plant, having a small (~440 Mb) diploid genome, being transformable, and having a detailed genetic map. Comparative molecular genetic mapping has indicated a similar gene content and extensive map colinearity between rice and other grasses, suggesting that much information acquired studying rice can be applied to other major food crops such as wheat, maize, and barley.

A large proportion of rice genomics is being achieved by the Rice Genome Research Programme in Japan (RGRP, National Institute of Agrobiological Resources, Tsukuba, Japan). Since 1991, the RGRP has carried out large-scale EST sequencing, construction of a fine-scale restriction fragment length polymorphism map, and physical mapping of the rice genome with yeast artificial chromosome clones (Yamamoto and Sasaki 1997). Starting in 1998, the RGR program entered into a new stage of rice genomics with the sequencing of the complete genome of Nipponbare (a japonica variety), which should eventually reveal all of the genomic sequence information in the rice plant (Sasaki 1998). Although cDNA sequencing has been performed on a very large scale in rice, and has catalogued organ-specific (around 2,000 cDNA clones from roots, etc.), developmental-stage-specific (flowering and ripening panicles), and stress-specific genes, to our knowledge no EST libraries have been made from mycorrhizal rice roots. We therefore propose the generation and sequencing of such mycorrhizae-specific ESTs, the study of which would be necessary to identify rice genes whose regulation is modified during endomycorrhizal symbiosis, in other words, the rice genetic program for root endosymbiosis.

Perspectives

In the near future, genetic and genomic approaches will provide a wealth of information on the plant genetic programs for root endosymbioses, that is, the identification of hundreds of genes whose expression is modified in the course of nodulation in legumes and of mycorrhization in both legumes and rice. In the short to medium term, this information should be useful in breeding legumes for a better exploitation of symbiotic nitrogen fixation and mycorrhization, and in breeding rice for a better use of mycorrhization. Comparison of the genetic programs for root endosymbioses in legumes and rice should allow the definition of sets of genes involved in endomycorrhization, nodulation, and both types of symbioses. This information is required for a critical assessment of the possibility of exploiting and improving the

existing symbiotic genetic program of rice in such a way as to make endosymbiotic associations with nitrogen-fixing microorganisms a realistic goal for this major crop.

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Prospects for constructing nitrogen-fixing cereals

Ray Dixon, Qi Cheng, and Anil Day

The engineering of autonomous nitrogen-fixing plants is undoubtedly a long-term goal because it requires the assembly of a complex enzyme and provision of appropriate physiological conditions in the absence of the environment normally provided by the prokaryotic cell. Here we briefly review the genetic, biochemical, and physiological requirements for nitrogen fixation in the context of engineering nitrogen-fixing plants and report some of our recent findings on the expression of one of the nitrogenase structural subunits within plastids of the model photosynthetic eukaryote *Chlamydomonas reinhardtii.*

Several approaches toward developing cereals capable of nitrogen fixation are now being considered, such as the establishment of effective endophytic symbioses, the development of legume-like nodulation in cereals, and the introduction and expression of nitrogen fixation *(nif)* genes in cereal plants. In this review, we will consider the current prospects for the latter approach, that is, the engineering of autonomous nitrogen-fixing plants. This is undoubtedly a long-term goal because it requires the assembly of a complex enzyme and provision of appropriate physiological conditions in the absence of the environment normally provided by the bacterial cell. It is therefore difficult to predict whether this goal is feasible and, if it is, how long it will take to achieve. Recent advances in our knowledge of natural diversity among diazotrophs, however, may help to achieve this goal. For example, the recent discovery of the novel superoxide-tolerant nitrogenase isolated from *Streptomyces thermoautotrophicus* (Ribbe et al 1997) may allow the expression of active nitrogenase under oxic conditions in photosynthetic tissues. Second, developments in plant transformation technology may help to accelerate the introduction of a cluster of *nif* genes into plants because the simultaneous transformation of multiple genes in cereals is now possible (Kohli et al 1998). We will briefly consider here the genetic, biochemical, and physiological requirements for nitrogen fixation in the context of engineering nitrogen-fixing plants and report some of our recent findings on the expression of one of the nitrogenase structural subunits within plastids of the model organism *Chlamydomonas reinhardtii.*

Physiological considerations

Finding an appropriate location to support the synthesis and activity of nitrogenase in higher plants is difficult because most nitrogenases are irreversibly inactivated by oxygen and require high levels of adenosine triphosphate (ATP) and a suitable low-potential electron donor to support enzyme activity. The choice of location is also influenced by genetic engineering considerations because at least 16 *nif* genes are required for nitrogenase biosynthesis. The expression of these genes in the eukaryotic nucleus requires the individual "tailoring" of each gene to provide an appropriate promoter, optimize the gene for translation, and possibly modify the codon usage. Further engineering would be needed to target proteins to organelles or to the endoplasmic reticulum. Thus, although mitochondria might provide a suitable energy-rich reducing environment to support nitrogenase, this location has been ruled out, at least in the short term, because of the complexity of targeting each of the *nif*-encoded proteins into this organelle. In contrast, the plastid may provide a more suitable location for introducing the *nif* genes because chloroplast genes are expressed in a prokaryotic-like fashion and translation of polycistronic messages occurs. We have argued previously that the *nif* gene cluster could be introduced into the plastid and its transcription directed by prokaryotic regulatory proteins in association with a specific bacterial sigma factor (Dixon et a1 1997).

Photosynthesis and nitrogen fixation

Nonheterocystous cyanobacteria are able to reconcile the incompatibility between the oxygen-sensitive process of nitrogen fixation and oxygen evolution during photosynthesis by separating both processes on a temporal basis. Nitrogenase synthesis and activity occur in the dark period, supported by ATP and reductant provided by a breakdown of endogenous glucan. Several hours into the dark period, when the ADP[1]/ATP ratio increases and respiration is inhibited due to limiting substrate, nitrogenase synthesis declines and enzyme activity is lost. Photosynthesis during the light period supports the replenishment of glucan and the nitrogen fixed during the dark period is used to support protein synthesis (Gallon 1992). Although this could provide a model for the temporal separation of nitrogen fixation and photosynthesis in plant chloroplasts, the levels of ATP and reductant available during the dark period are probably insufficient to support nitrogen fixation. Moreover, there is no efficient respiratory system in the plastids under these conditions that could remove oxygen. Under conditions of illumination, reducing power is generated by Photosystem I and the chloroplast stroma provides a reducing environment, as evidenced by the reduced

[1] ADP = adenosine diphosphate.

state of thioredoxin, which acts as a signal for redox status (Danon and Mayfield 1994). Under these reducing conditions, it is possible that nitrogenase itself could help to remove oxygen. When the concentration of nitrogenase Fe protein is at least in 4-fold molar excess over the concentration of oxygen. the Fe protein is oxidized without a loss of activity (Thorneley and Ashby 1989). However, a major product of this reaction is hydrogen peroxide, and, to prevent oxidative stress. it would probably be necessary to increase the level of peroxide scavenging enzymes in the chloroplast such as ascorbate peroxidase, monodehydroascorbate reductase, and dehydroascorbate reductase.

Photorespiration might also help to protect nitrogenase from oxygen damage, but at the expense of photosynthetically generated reductant. It may also be possible to use the unusual oxygen-tolerant nitrogenase from *S. thermoautotrophicus* for nitrogen fixation in chloroplasts (Ribbe et al 1997). In this case, however, the superoxide radical is the electron donor, so, unless alternative electron carriers can be sought, this enzyme will function in the plastid only under conditions of photooxidative stress. Introducing this enzyme into chloroplasts might make plants more stress-tolerant, but nitrogenase activity would be conditional upon stress conditions. Potentially, the activity of the superoxide-dependent enzyme could be adapted to "nonstress" conditions by disabling superoxide dismutase. This may lead to increased levels of hydrogen peroxide, however, necessitating the engineering of enhanced levels of peroxide scavenging systems as mentioned above. Another point when considering thermotolerant *S. thermoautotrophicus* nitrogenase is the activity of the enzyme under normal plant growth temperatures. Because the enzyme has about 10% of the activity of conventional nitrogenase at 25°C (Ortwin Meyer, personal communication), it will also be necessary to ensure that adequate levels of the enzyme are expressed to support plant nitrogen demand.

Nonphotosynthetic plastids

In nonphotosynthetic tissues, the energy required to support nitrogen fixation would have to be imported as either ATP or adenylates from which ATP could be generated within the plastid by substrate-level phosphorylation. Reducing power could be provided by carbohydrate oxidation within the organelle although the introduction of nitrogenase might impose strong competition with other biosynthetic pathways that depend on reductant, such as the reaction catalyzed by the enzyme GOGAT during ammonia assimilation, which requires reduced ferredoxin (Bowsher et al 1996). The roots of wetland rice survive in an anaerobic environment, so nitrogenase may not be inactivated in rice root tissues, though it is still questionable whether there is sufficient ATP and reductant to support nitrogen fixation in heterotrophic plastids. Our preliminary studies below on the expression of the *nifH*-encoded protein in plastids of dark-grown *Chlamydomonas reinhardtii* may provide a model system to study whether nitrogenase activity can be established under heterotrophic conditions.

Lower plants as a model system

A commonly accepted evolutionary hypothesis is that plastids evolved from photosynthetic prokaryotes, probably cyanobacteria. These ancestors may have also been diazotrophs. DNA sequencing of lower plant genomes has revealed three genes. designated *chlL (frxC/gidB), chlN (gidA),* and *chlB,* that may have evolved from an ancestral nitrogenase (Choquet et al 1992, Lidholm and Gustafsson 1991, Ohyama et al 1986, Wakasugi et al 1994). These sequences have been identified in all Gymnosperms examined so far, ranging from algae to diverse land plants including black pine. The amino acid sequences of their putative products show significant similarities to those of the three subunits of nitrogenase and, in particular, the structural subunit of nitrogenase Fe protein (NIFH) encoded by *nifH* exhibits 32-35% identity with the putative "chlorophyll iron protein" (CHLL) encoded by *chlL* (Burke et al 1993, Fujita et al 1992, Suzuki and Bauer 1992). Mutational analysis of the *chl* genes demonstrates their involvement in the light-independent pathway for chlorophyll biosynthesis in which chlorophyll a and b can be synthesized in complete darkness. *chl* mutations prevent the conversion of protochlorophyllide to chlorophyllide in the dark and it has been suggested that the three *chl* gene products make up the structural subunits of a dark-dependent protochlorophyllide reductase (DPOR) (Fujita 1996).

Although no biochemical studies have yet been performed, the similarities with nitrogenase suggest that protochlorophyllide reductase may be composed of two protein components analogous to nitrogenase Mo-Fe protein encoded by the structural genes *nifD* and *nifK* and the Fe protein encoded by *nifH.* According to this model, CHLL may be a reductase component that transfers electrons to the other subunits encoded by *chlB* and *chlN* to permit substrate reduction. The sequence identity between CHLL and NIFH is sufficient to model the structure of this plastid-encoded component based on the crystal structure of nitrogenase Fe protein (Burke et al 1993) and suggests that, like the Fe protein, CHLL contains an ATP-binding site and conserved cysteine residues for binding a [4Fe-4S] cluster (Fig. 1). The conservation of cysteine residues that are putative ligands to an Fe-S cluster suggests that DPOR, like nitrogenase, may be an oxygen-sensitive enzyme. It has been argued that DPOR evolved from an ancestral nitrogenase to perform chlorophyll biosynthesis under anaerobic conditions. To prevent photooxidative damage during porphyrin biosynthesis in the presence of oxygen and light, a second enzyme, light-dependent protochlorophyllide reductase (LPOR), has evolved to permit chlorophyll biosynthesis under aerobic conditions (Reinbothe et al 1996). One obvious corollary to this argument is that, if DPOR is indeed an oxygen-sensitive enzyme, then the chloroplast environment during the dark period may be sufficiently anoxic to prevent oxygen damage to nitrogenase. Furthermore, if CHLL, by analogy with nitrogenase Fe protein, is a reductase component, which consumes ATP and reducing power to donate electrons for substrate reduction by DPOR, how are enough energy and reductant provided in the dark period to support this?

We have used *Chlamydomonas reinhardtii* as a model system to explore the potential functional relationships between nitrogenase Fe protein and CHLL. This

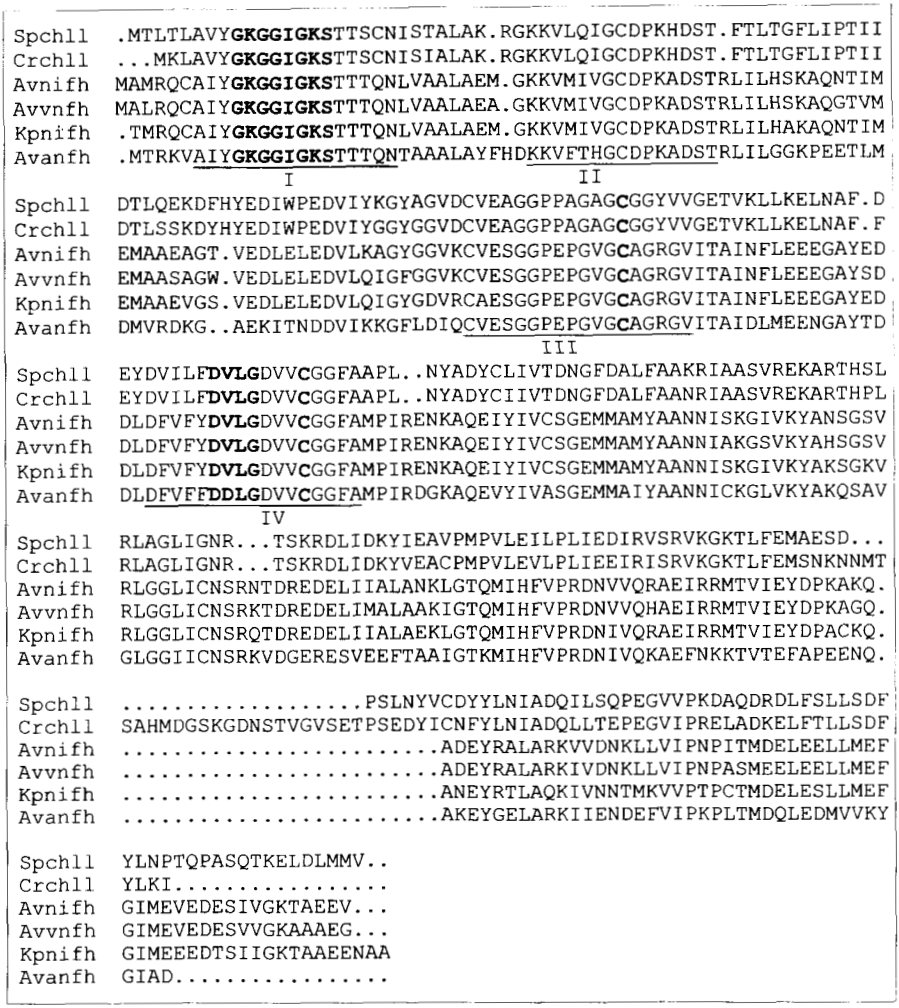

Fig. 1. Amino acid sequence alignment of CHLL and NIFH from various species. The conserved nucleotide-binding Walker A motif **(GXXXXGKS)** and Walker B motif **(DXXG)** are shown in green and conserved cysteines for binding the [4Fe-4S] cluster are shown in red. Other similar amino acid residues are underlined. Spchll = *Synechococus* sp. CHLL; Crchll = *C. reinhardtii* CHLL; Avnifh = *A. vinelandii* Mo-nitrogenase Fe protein; Avvnfh = *A. vinelandii* V-nitrogenase Fe protein; Kpnifh = *K. pneumoniae* Mo-nitrogenase Fe protein; Avnifh = *A. vinelandii* alternative nitrogenase Fe protein.

organism grows heterotrophically on an acetate-containing medium in the dark and produces green colonies under these conditions because of the action of DPOR. Mutations in the plastid *chl* genes give rise to a "yellow-in-the-dark phenotype" and fail to synthesize chlorophyll a or b under these conditions, thus providing a marker for DPOR activity. Because chloroplast transformation is well established in this organism, we have exploited this to make targeted insertions in the plastid genome in which the coding sequence of *chlL* is precisely replaced either by coding sequences of the *Klebsiella pneumoniae nifl* gene or by the marker gene *uidA.* This has been achieved using two transformation steps. In the initial transformation, we inactivated *petB,* a photosynthetic gene closely linked to *chlL,* by inserting a spectinomycin resistance gene *(aadA). PetB*$^{-}$ mutants are unable to grow photoautotrophically and require acetate for growth. Homoplasmic spectinomycin-resistant transformants were then used as recipients for a second transformation in which we introduced a DNA fragment carrying a wild-type copy of the *petB* gene, together with closely linked *nifH* or *uidA* coding sequences flanked by the regulatory regions of *chlL.* We sought transformants in which homologous recombination had replaced the *aadA* insertion in *petB* with the wild-type *petB* gene and the *nifH* or *uidA* sequences had replaced the coding sequence of *chlL.* All of the transformants selected for further study grew on a minimal medium, but many exhibited a yellow or partially green phenotype when cultured on acetate medium and incubated in the dark, indicative of replacement of the wild-type *chlL* sequence by the foreign DNA sequences. Southern hybridization was used to identify those transformants that carried the expected insertion of foreign DNA and that were homoplasmic for the transgenes.

As mentioned above, disruption of *chlL* inactivates the dark-dependent pathway of chlorophyll biosynthesis and, accordingly, homoplasmic *uidA* transformants were yellow when cultured in the dark on acetate medium, in contrast to the wild-type and *petB:aadA* insertion mutants, which were green in the dark. The homoplasmic *nifH* transformant, however, was partially green in the dark, suggesting that it may be able to partially substitute for the function of *chlL* (Fig. 2). Pigments synthesized by the transplastomic lines in the dark were analyzed by fluorescence spectroscopy of extracts. As expected, the *uidA* transformants accumulated only protochlorophyllide in contrast to the wild-type strain, which showed the characteristic emission spectrum for chlorophyll a and b. The *nifH* transformant also accumulated protochlorophyllide, but the spectrum indicated that a high proportion of the substrate had been converted to chlorophyll. These results show that the *uidA* transformant is deficient in light-independent chlorophyll biosynthesis and the *nifH* transformant could at least partially restore the capacity for chlorophyll biosynthesis in the dark.

The ability of NIFH to partially function in the dark-dependent chlorophyll biosynthesis pathway supports the notion that CHLL and nitrogenase Fe protein have similar structural and functional properties. These similarities suggest that CHLL might function in ATP-coupled electron transfer to the other components of the protochlorophyllide reductase encoded by CHLN and CHLB, which have sequence similarities with NIFD and NIFK (Fujita 1996). It is remarkable that *K. pneumoniae* NIFH is apparently able to function with the other components of protochlorophyllide

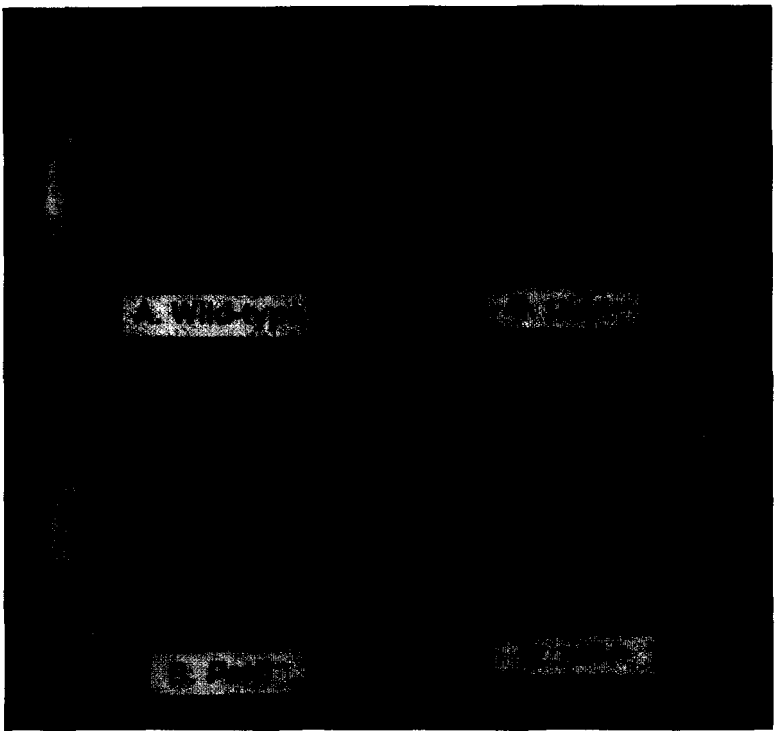

Fig. 2. Pigmentation phenotypes of wild-type and transplastomic *C. reinhardtii* strains grown on acetate medium in the dark: (A) wild-type strain, (B) *petB* mutant strain, (C) *uidA* chloroplast transformant, (D) *nifH* chloroplast transformant.

reductase because, although combinations of Fe protein and MoFe protein from diverse bacteria can result in substantial substrate reduction (Emerich and Burris 1976, Emerich et a1 1981), some combinations are not effective (Eady 1995). Fe protein is the most oxygen-sensitive component of nitrogenase and its ability to partially replace CHLL suggests that oxygen-sensitive enzymes may function in chloroplasts when grown in the dark. This is perhaps not surprising because hydrogenase, another oxygen-sensitive enzyme that is located in the chloroplast of *C. reinhardtii,* is induced following anaerobic adaptation in the dark (Happe et a1 1994). *Chlamydomonas* carries out fermentative metabolism under dark-grown conditions by degrading starch to provide ATP and reducing equivalents. It is suggested that this fermentative pathway is located entirely in plastids and that hydrogenase in the plastid can act as a sink for excess reductant (Gfeller and Gibbs 1984). This suggests that there is sufficient reductant and energy to support DPOR activity in *C. reinhardti* in the dark, but it does not explain how DPOR activity is maintained in lower plants, which have a different physiology and apparently lack hydrogenase.

Assuming that a [4Fe-4S] cluster is required for the NIFH and CHLL function in the protochlorophyllide reductase complex, the biosynthesis of this cluster is pre-

sumably achieved by ancillary proteins. In diazotrophs, the products of two genes, *nifS* and *nifU,* are necessary for Fe protein activity, which is required for the mobilization of sulfur and iron for Fe-S cluster formation (Zheng and Dean 1994, Zheng et al 1993). Homologues of these genes, however, have been found in nondiazotrophic eubacteria as well as in yeast and humans, suggesting that they may provide a ubiquitous pathway for Fe-S cluster assembly, which we presume is present in *C. reinhardtii.* The product of a third gene, *nifM,* is required for activation and stability of the Fe protein in nitrogen-fixing organisms. When *nifH* is expressed in either *Escherichia coli* or yeast in the absence of *nifM* a very low level of dimeric Fe protein is synthesized (Berman et a1 1985, Paul and Merrick 1989). The precise role of NIFM is unknown, although it has been suggested that it could have a chaperone-like role in maintaining the apoFe protein in the correct conformation to accept the [4Fe-4S] cluster. The structural similarities between Fe protein and the "chlorophyll iron proteins" suggest that an equivalent of *nifM* is present in organisms that contain *chlL.* Because homologues of *nifU, nifS,* and *nifM* are not present in the liverwort chloroplast genome (the only lower plant plastome that has been completely sequenced), it is tempting to speculate that their equivalents are encoded in the nuclear genome. Indeed, they may be represented among the nuclear mutant "y" alleles that produce yellow-in-the-dark phenotypes in *C. reinhardtii.*

Future prospects

The ability of *nifH* to at least partially substitute for *chlL* in algal chloroplasts suggests that it may be possible to engineer a nitrogen-fixing derivative of C. reinhardtii through the systematic introduction into the plastid genome of the remaining genes required for nitrogenase synthesis and activity. The enzyme would need to be expressed at high levels because nitrogenase exhibits relatively slow-reaction kinetics. This could be achieved by tailoring each of the introduced genes to optimize expression using plastid 5' and 3' regulatory signals or by exploiting similarities in transcriptional machinery between plastids and prokaryotes to use bacterial sigma factors and transcription factors to drive expression of a *nif* gene cassette in the chloroplast. Even if conditions are appropriate for nitrogenase activity in *Chlamydomonas* plastids under dark-grown conditions, however, this does not necessarily mean that the system can function in higher plants. Angiosperms do not possess DPOR, so it is possible that higher plant plastids cannot support the synthesis and activity of oxygen-sensitive proteins. Whereas assembly of CHLL protein in *Chlamydomonas* may be facilitated by nuclear equivalents of *nifU, nifS,* and *nifM,* the assembly of Fe protein in higher plant plastids will most likely require the introduction of these genes into the plastid genome. A further problem is that it is difficult to predict at present whether nitrogenase will function in a given location in a higher plant. For example, we have major gaps in our knowledge of oxygen concentrations in plant tissues. Physiological approaches such as the use of microelectrodes to measure oxygen concentrations in specific tissues, together with expression experiments using appropriate marker genes linked to anaerobically responsive promoters such as ADH, would be extremely use-

ful for identifying appropriate locations. In attempting to engineer nitrogen-fixing plants, we are taking a huge leap into the unknown. Each step along the way may lead to new difficulties or bring remarkable surprises. Whatever the outcome, at the very least we are likely to uncover more about the biosynthesis of nitrogenase, develop tools to introduce and express multiple transgenes in plants, and, in addition, expand our knowledge of plant physiology and biochemistry. These spin-offs alone justify further research in this pioneering field.

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Notes

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Appendix

During the working group meeting of the Frontier Project on Nitrogen Fixation in Rice, held at IRRI 9- I2 August 1999, two group discussions were held on (1) endophytic diazotroph associations with rice and (2) genetic manipulation of rice for nitrogen fixation and symbiosis, to take stock of current achievements and prioritize areas for future research. In addition, a brain-storming session was organized to examine the implications of energy costs involved in nitrogen fixation and its likely effect on rice yield. The following sections summarize the group discussions and the brain-storming session.

Endophytic diazotroph associations with rice

Endophytic diazotrophs were found to contribute to the nitrogen economy of nonlegumes such as sugarcane and Kallar grass growing in nitrogen-poor soils. These findings gave impetus to look for endophytic diazotrophs in rice as it was envisaged that, if such a biological nitrogen fixation (BNF) system could be identified for rice, its dependency on chemical N fertilizer could be reduced. For the past few years, several scientists worldwide have begun studies on rice-endophyte interactions with an aim to exploit the potential of endophytic nitrogen fixation for the benefit of the rice plant. The group discussion focused on three topics: (1) identification of model endophytes, including rhizobia1 endophytes, (2) BNF versus growth promotion by endophytes, and (3) biology of the rice plant.

1. Identification of model endophytes

Participants raised two issues for discussion: (1) whether to pursue investigations on understanding the underlying principles involved in rice-endophyte interactions using a well-characterized strain such as *Azoarcus* or (2) to continue efforts to identify bona fide and predominant rice endophytes for undertaking studies on plant-microbe interactions. After a thorough discussion, participants decided that there was a need to identify the "compatible" endophytes first. They suggested that if a few important predominant diazotrophic endophytes that show benefit to the plant in greenhouse/field conditions could be identified, further research could then focus on these few isolates to study rice-endophyte interactions by using molecular techniques together with marker and reporter gene technologies. They opined that an efficient rice-endophyte system may exist in nature and therefore more efforts are needed to identify such a system by using screening procedures such as the ^{15}N dilution technique. In fact, using the ^{15}N dilution technique, researchers in Pakistan found that rice variety Basmati derives 30% of its nitrogen requirement through BNF. Incidentally, these

plants also contained a high frequency of diazotrophic endophytes, including *Herbaspirillum.* Hence, to narrow down efforts to a few important endophytic strains, it is worthwhile to concentrate on screening the rice varieties that show a positive N balance as this may help in correlating BNF with the most predominant diazotrophic endophyte(s) that a particular rice variety may harbor. Questions were raised, however, about whether a positive N balance as determined by ^{15}N isotope dilution says anything about *in planta* nitrogen fixation by endophytes.

2. BNF versus growth promotion

Some rhizobial endophytes such as *Rhizobium leguminosarum* bv. *trifolii* E11 and El 2 and *Rhizobium* sp. IRBG74 have been found to enhance (rice) plant growth. This observation was considered to be quite exciting and worthy of further investigation. It is not yet known, however, whether the observed plant growth promotion effects were, in fact, due to BNF per se. The group suggested that the scope of the Frontier Project should be broadened to also include plant growth-promoting rhizobacteria (PGPR). It was considered that rhizobial strains could be important components of PGPR management strategies in the future.

As various PGPR are now being tested to assess their beneficial effects on plant growth, the group recommended that guidelines be formulated to conduct standard experiments so that the efficiency of different strains could be compared and the best strains selected for future studies.

3. Biology of the rice plant

The role of the rice plant in determining interactions with microorganisms, both endophytes as well as rhizobia, was also discussed. Questions were raised about whether the knowledge of rice genetics can be used to identify genes that play an important role in defining the endophytic associations, and whether rice varieties can be developed that would form effective associations with endophytes/rhizobia. It became obvious that diverse rice genotypes need to be screened in different environments to identify the genes that enable the formation of endosymbiotic associations. It was pointed out that rice varieties that form efficient mycorrhizal associations could be good candidates for the identification and isolation of the plant genes involved in symbiosis.

Conclusions

The group came to a consensus that the following suggestions should be examined:

1. IRRI varieties (e.g., IR42) should be tested in Pakistan and Pakistan varieties (e.g., Basmati) tested at IRRI to correlate BNF with the number and variety of bacteria found inside and outside rice plants.
2. Similarly, researchers should test these varieties in upland conditions to see whether they form associations with mycorrhizal fungi.
3. Researchers should study the efficiency of growth promotion by PGPR (e.g., *Rhizobium leguminosarum* bv. *trifolii* E11 and E12) in rice varieties that are able to form associations with mycorrhizal fungi.

4. A newsletter should be published for the effective sharing of information on standard protocols and scientific developments among the research groups.

The group also discussed ways to attract funding for research on endophyte-rice associations. The group suggested that, to attract funds, it might be a better strategy to include other cereals in the studies, instead of just rice. The group stressed, however, that it is more advantageous to continue research on rice because rice is a model monocot, and the complete genome sequence that will soon become available will enormously benefit the project in achieving its goal.

Genetic manipulation of rice for nitrogen fixation and symbiosis

Research under this heading has focused on two general areas: (1) the prospects for developing an intimate symbiotic association between rhizobia and rice, and (2) the prospects for directly introducing a functional nitrogen-fixing enzyme system into plants. Both of these projects have to be considered speculative and very long term. Work in the first area has been driven by the hypothesis that rice possesses an incomplete genetic program for nodulation. Indeed, recent results support this hypothesis. For example, selected rice cultivars have the ability to induce rhizobial nodulation (*nod*) genes. Also, using *ENOD12*-GUS transgenic plants, it was demonstrated that rice can respond to the addition of rhizobial lipo-chitin nodulation signals (Nod factors). This surprising result indicates that rice has the ability to recognize not only the Nod signals but also the signal transduction pathway to couple this recognition to gene transcription. Work in the second area has focused on the hypothesis that a functional nitrogenase system can be constructed in plants provided the appropriate physiological environment can be provided.

1. Establishment of a rhizobial-rice interaction

A. Do we need a nodule or a modified lateral root?

Discussions within the group focused on the idea that primitive rhizobial-plant interactions could provide a model for developing a rice-rhizobia1 interaction. For example, rhizobial infection of *Parasponia,* the only nonlegume nodulated by rhizobia, involves colonization of a modified lateral root. Therefore, the question was posed whether we should focus on constructing a nodule in rice or a modified lateral root that could be colonized by rhizobia (c.f., *Parasponia*).

The consensus was that a nodule structure may be necessary to provide the appropriate environmental conditions (e.g., low oxygen) for significant nitrogen fixation. Construction of a modified lateral root, however, may be a more achievable goal in the short term.

One recommendation for moving forward was to organize a European Molecular Biology Organization (EMBO) workshop where experts on plant root development could meet with researchers interested in root symbioses. This interaction could lead to practical strategies for developing a modified lateral root on rice.

Participants also pointed out that a wealth of plant genomic information will be available within 12–24 months. For example, recent estimates indicate that the

Arabidopsis genome will be completely sequenced by the end of 2000. Using this information, it should be possible to define the set of genes involved in the program for lateral root formation.

B. Is the vasicular-arbuscular (VA) mycorrhizal symbiosis a useful model for the development of a rice endosymbiosis?

Participants pointed out that rice, similar to most angiosperms but unlike *Arabidopsis,* is capable of developing a symbiotic relationship with VA mycorrhizal fungi. The nature of this symbiosis demonstrates that rice has the ability to respond to the mycorrhizal signals that trigger plant cortical cell activation and the formation of symbiotic intracellular membrane structures. All of this occurs in the absence of a plant-defense response. Moreover, this symbiosis is clearly supported by the plant. Recent work in legumes demonstrates that many of the early nodulin genes (e.g., *ENOD12, ENOD40,* etc.) are induced upon mycorrhizal infection. A subset of legume plant mutants that are defective in nodulation are also defective in the mycorrhizal symbiosis. These findings indicate that nodulation and mycorrhizal invasion require some of the same plant functions. Thus, the rice mycorrhizal endosymbiosis may be a good starting point for thinking about a nitrogen-fixing endosymbiosis in rice.

There was a consensus within the group that this concept should be pursued. In particular, functional genomics could be exploited to identify the rice genetic program for root endosymbiosis. Furthermore, transgenic *ENOD12*-GUS rice plants are available and could be used to rapidly test whether mycorrhizal infection, as in legumes, can induce transcription of this gene in rice.

Research groups are now working to identify signals produced by VA mycorrhizae that induce plant symbiotic functions. Once these signals are identified, it should be possible to engineer a nitrogen-fixing bacterium to produce these signals to help establish a novel endosymbiosis.

2. What are the prospects for engineering a nitrogen-fixing plant?

A significant accomplishment announced at this meeting was the first demonstration of the engineering of a eukaryotic alga (i.e., *Chlamydomonas*) in which the *Klebsiella pneumoniae* NifH protein functions to complement a defect in dark-dependent chlorophyll biosynthesis. There was a consensus that efforts should be made immediately to build upon this initial discovery. There is hope that the announcement of this advance will remove barriers to funding in this area.

Conclusions

Research on these topics must still be considered speculative and long term in nature. The group was encouraged, however, by the surprising, positive results that have been obtained within a relatively short time. Advancements in methodology, genomics, and knowledge of other nitrogen-fixing systems should aid future work for developing a functional nitrogen-fixing system in rice.

Energy costs of *in planta* nitrogen fixation

Biological nitrogen fixation (BNF) is a high-energy-requiring process. Consequently, it is envisaged that nitrogen fixation in rice may be such a strong sink for photosynthate that yields would be affected. Hence, a brain-storming session was organized to examine the implications of costs involved in nitrogen fixation and its likely effect on rice yield.

1. Carbon costs for endosymbiotic nitrogen fixation in legumes

A. Rice is not a nitrogen-fixing plant like nodulated legumes, so obviously no experimental data are available to assess the effects of nitrogen fixation on rice yields. Nevertheless, an assessment of energy cost incurred by nitrogen fixation in legumes such as soybean may provide a rough idea of likely energy costs imposed by nitrogen fixation in rice. With this assumption in mind, group discussion on this topic began with the presentation of background data by Dr. David Layzell, who indicated that analysis had shown that an N_2-fixing soybean plant has the following C costs, with nodules consuming about 19% of the photosynthate:

Shoot growth and maintenance	34%
Shoot respiration	17%
Root growth and maintenance	12%
Root respiration	18%
Nodule growth and maintenance	4%
Nodule respiration	15%

The energy costs for nodules could be partitioned as follows:

N_2 fixation	41%
H_2 evolution	20%
NH_3 assimilation	14%
Export of fixed N	5%
Growth and maintenance	20%

Overall, 5 CO_2 are released per N fixed.

These data are comparable with the costs incurred by nitrate assimilation by a nonnodulated soybean: approximately 5.7 CO_2 per N assimilated in nonphotosynthetic tissue, and 0–2.9 CO_2 per N assimilated in photosynthetic tissue.

B. Against these energy costs. which were significant for both dinitrogen fixation and nitrate assimilation but apparently somewhat greater for nitrogen fixation, indirect benefits of N_2 fixation could be identified. These were:
   - Environmental
   - Maintenance of C sink, which can enhance photosynthesis
   - H_2 released into the rhizosphere. which can enhance plant growth

2. Theoretical energy requirements

Dr. Ivan Kennedy drew attention to a discussion on energy costs conducted at the Rockefeller Bellagio meeting in 1997 (Kennedy and Cocking 1997). A copy of this discussion is included later with kind permission from Dr. Ivan Kennedy. The following points presented in that discussion paper indicate that the question of relative energy costs of BNF versus nitrate assimilation, or even ammonia assimilation as in paddy rice. remains unresolved:

- The extra alkalinity of nitrate assimilation over that of ammonia assimilation and dinitrogen reduction demands the synthesis of extra bicarbonate and then carboxylate, with reduced carbon dioxide evolution. This has led to underestimates of energy costs for nitrate assimilation.
- It has been suggested that the evolution of C_4 plants was a result of the alkalinity of nitrite reduction, allowing the spatial separation of inorganic nitrogen reduction and carboxylation by Rubisco. This allows the elimination of photorespiration in C_4 plants, which can be seen as a consequence of the need for oxygenase activity to produce phosphoglycolic acid in C_3 chloroplasts. Thus, a large part of photorespiration would be attributed to the pH stat role of Rubisco, largely needed for the neutralization of hydroxyl ions produced in nitrite reduction.
- The ability of plants to compensate for extra energy consumption needs to be considered, as photosynthetic systems saturate at relatively low light intensity. A case can even be made that extra energy consumption by roots would stimulate the production of biomass in tops.

3. Estimate of bacterial population needed for significant endophytic or endosymbiotic BNF in rice

The energy cost incurred by a potential nitrogen-fixing rice plant also depends on the numbers of endophytic or endosymbiotic bacterial cells that a plant has to sustain for significant nitrogen fixation. Considering this, approximate estimates of numbers of endophytic bacteria needed for effective nitrogen fixation in rice were made.

A. Dr. Barbara Reinhold-Hurek provided information on the likely numbers of endophytic bacteria needed for effective N_2 fixation in rice. An estimate, based on bacterial numbers in soybean nodules, was performed as follows:
   - A soybean nodule contains 1.5×10^9 bacteroids. This is equal to 3×10^{10} bacteroids g^{-1} dry wt.

- The N content of rice is 3 times less than that of soybean. so rice needs 1×10^{10} bacteroids g^{-1} dry wt.
- *Azoarcus* nitrogenase activity in its hyper-induced state is 10 times greater than that of soybean, so rice may need only 1 x 10^9 *Azoarcus* bacteroids g^{-1} dry wt.
- The dry wt of 1 cm of root is approximately 0.3 mg, so we should expect to see approximately 30 bacterial cells in each 1-µm section.

This may be achievable.

B. Dr. Ivan Kennedy contributed an alternative means of estimating the likely numbers of endophytic or endosymbiotic bacteria that would be required to provide a significant input of nitrogen to rice (Table 1).

Table 1. Approximate estimates of numbers of endophytic or endosymbiotic bacteria needed to provide significant fixed nitrogen to rice through nitrogen fixation.

Property	Numbers per fresh volume		
	Soybean	Wheat	Rice
Nodules			
Bacteroids as a proportion of infected cells (v/v)	0.2	–	–
Infected cells as a proportion of legume nodule (v/v)	0.5	–	–
Nodules as a proportion of whole plant (fw)	0.05	–	–
Bacteroids[a] as a proportion of total plant	P = 0.2 x 0.5 x 0.05 = 0.005 = 0.5%	–	–
100%	0.5 x 10^{12} cells cm^{-3}	–	–
Overall (0.5%)	2.5 x 10^9 cells cm^{-3}	–	–
Associative roots[b]			
CFU +2,4-D Sp7-S[c]	–	5 x 10^8 cells cm^{-3}	
CFU –2,4-D Sp7-S[c]	–	5 x 10^7 cells cm^{-3}	
CFU *H. seropedicae*[d]	–	Similar	
CFU *H. seropedicae*	–		5 x 10^8 cm^{-3}
Discrepancy (cells cm^{-3})	x 1	x 0.1	x 0.1
Nitrogen requirement	x 1	x 0.3	x 0.2
Nitrogen sufficiency		33%	50%

[a] Bergersen and Goodchild (1973). [b]CFU = colony-forming units; 2,4-dichlorophenoxy acetic acid. [c] Katupitiya et al (1995). [d]Kennedy et al (1997).

Thus, even though there are so many unknown factors, such as

- transport rates of C and N between diazotrophs and plant tissues in a nodule versus a tubular distribution in the cortex or in xylem tubes and
- relative rates of BNF between bacteroids and other endosymbionts,

it may well be possible to achieve sufficient numbers of endophytes.

Considering the low protein content of rice. it can be speculated that the rice plant requires lower rates of nitrogen fixation than legumes (Reddy and Ladha 1995). Therefore, a relatively low population of endophytic or endosymbiotic diazotrophs is enough to support the needed nitrogen fixation rates in rice, thus causing lesser energy drain than in legumes.

4. Will endophytic or other means of BNF reduce rice yield (because of extra energy demand)?

In legumes, no significant yield differences were observed when the plants were grown on dinitrogen versus nitrate as a nitrogen source (Gibson 1966). This indicates that legumes using BNF rather than nitrate nitrogen suffer no obvious yield penalties. This may be because the carbon (energy) costs for fixing dinitrogen ($DG = -687\,kJ\,mol^{-1}$) are theoretically similar to those for nitrate reduction ($DG = -605\,kJ\,mol^{-1}$) in the plants (Pate et al 1979). While there is no doubt that supplying ammoniacal fertilizer as a source of nitrogen for rice plants reduces the energy requirement for nitrogen assimilation, in upland conditions rice yields may not be compromised because of the energy demand of BNF, since the energy expended to sustain nitrogen fixation will not be excessively greater than that required to assimilate nitrate, the most common alternative nitrogen source available for the plants in aerated soils.

Even under lowland flooded conditions, in some rice varieties nitrogen absorption and plant growth and yield are generally improved when the plants are fertilized with nitrate simultaneously with ammonia (Ancheng et al 1993 and references therein; Glass and Siddiqi 1995, Kronzucker et al 1999a,b, Xiaoe and Xi 1991). This implies that the rice plant is capable of providing sufficient extra energy resources required for the assimilation of nitrate without compromising yield. It is not clear whether the capacity of the rice plant to compensate for increased energy costs is met by increasing photosynthesis rates. The fact that no yield penalty exists for crops grown on nitrate and ammonia rather than on ammonia alone suggests that energy may not be limiting.

It is normally envisaged that nitrogen-fixing symbioses in rice may be such a strong sink for photosynthate that yields would be affected. Because rice is low in protein, a much lower rate of nitrogen fixation than in legumes will be required, with less drain on the plant's photosynthates (Reddy and Ladha 1995). In addition, actual rice yields are much lower than their maximum genetic potential; therefore, the nitrogen-fixing trait may have little effect at current yield levels.

5. Comments on the problem of obtaining efficient BNF in rice

Variability in specific activities of nitrogenase in various endophytes may have a bearing on the efficiency of nitrogen fixation in rice. Hence, a question was raised:

What are the specific nitrogenase activities of various endophytic bacteria compared with bacteroids? Dr. Ray Dixon indicated that some data exist in the literature to make these estimates, allowing an assessment of the possibilities.

It was pointed out that, even if numbers were reasonable enough for significant nitrogen fixation. endophytic bacteria residing in rice would still have an O_2 problem that nodules in legumes have solved through the evolution of diffusion barriers such as leghemoglobin, which has an affinity for O_2 high enough to keep it from inactivating the O_2-sensitive nitrogenase but still providing adequate O_2 to support an ATP-generating respiratory system. Dr. Barry Rolfe mentioned the observation of nitrogen fixation in *Parasponia* nodules with their bacteroids enclosed in persistent infection threads. The composition of these threads might be of interest. providing an example of a possible permeability barrier. So, the composition of infection threads (currently unknown) demands examination. Dr. Euan James pointed out that *Parasponia* nodules still have an O_2 diffusion barrier as with legume-rhizobia1 nodules. In rice, however, most of the endophytic diazotrophic bacteria on the roots, in intercellular spaces. in xylem, etc., could not fix nitrogen and would actually be involved in respiratory protection to allow a few bacteria in the interior of the colony to express nitrogenase. The ease with which *nifH-lacZ* and *nifH-gus* expression can be obtained, however, suggests the existence of conditions suitable for nitrogenase expression for endophytes (e.g., *Azoarcus* and *Azospirillum* transconjugants in roots). Nevertheless, protective barriers with low permeability for oxygen may be required in rice for efficient nitrogen fixation.

Dr. David Layzell pointed out a need to model oxygen conditions with different distributions of diazotrophs *in planta.* This could be achieved using current models (Layzell et al 1988).

Conclusions

No definitive conclusions can be drawn about what the exact energy costs would be and the extent to which the rice plant would compensate for any extra demand by increased photosynthesis. Based on current knowledge, it was not possible to conclude exactly what the energy cost would be and whether this would reduce yield. Any yield penalty because of extra energy needs, however, would need to be small for the project to be viable. The best way forward appeared to be to assess energy costs on an ongoing basis as research is carried forward.

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1.2.3 BNF: An energy-costly process?

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The energy cost of BNF is often referred to as exceptionally high, but this conclusion bears examination. The fully balanced equation for nitrogen fixation as expressed in the nitrogenase reaction of *Clostridium pasteurianum.* the organism in which cell-free preparations of the enzyme were first isolated. is as follows:

$$N_2 + 8\text{ferredoxin}^- + 8H^+ + 16\text{MgATP}^{2-} + 18H_2O$$
$$\Rightarrow 2NH_4^+ + 20H^- + 8\text{ferredoxin} + 16\text{MpADP}^- + 16H_2PO_4^- + H_2$$

The large requirement for ATP, acting in an overall way as a dehydrating agent to increase the reactivity of reduced sites on the enzyme complex, is certainly exceptional. In microaerobic systems, as in the bacteroids of legume nodules and most associative bacteria, ferredoxin is replaced in this reaction by flavodoxin, a similar 1-electron carrier donating electrons to the nitrogenase system (see Section 2.1). This reaction shows an obvious need for reducing potential and energy as ATP. No other single biochemical reaction requires so much ATP. There is reason to believe that even more ATP may be used *in vivo* by *Cl. pasteurianum* where the increased sucrose utilisation for growth on dinitrogen compared to ammonia indicated nitrogenase activity required about 30 ATP molecules.

Clearly, symbiotic nitrogen fixation in plants involves significant energy costs—as does the Haber-Bosch fixation process. However. these facts regarding energy costs as reductant and ATP may have led to an exaggerated concept of the amount of energy required for BNF and the capacity of nitrogen-fixing symbioses to sustain a high yield of grain. Some relevant issues are:

- while there is no doubt that supplying ammonia (NH_3) as a nitrogen source for plants reduces the energy requirement for nitrogen assimilation, the proper comparison that should most often be made (except for paddy rice) is between dinitrogen (N_2) and nitrate as nitrogen sources, since nitrate is the most common alternative nitrogen source available for legumes and other plants in aerated soils;

- balanced equations (Kennedy 1988) that can be written to indicate the respective carbon substrate requirements for conversion of nitrate and dinitrogen to ammonia (NH_4^+) are:

$$NO_3^- + {}^1/_3C_6H_{12}O_6 + H_2O \Rightarrow NH_4^+ + 2HCO_3^- \qquad (DG_o = -599 kJ\ mol^{-1})$$

$${}^1/_2N_2 + {}^3/_8C_6H_{12}O_6 + {}^5/_4O_2 + {}^3/_4H_2O \Rightarrow NH_4^+ + {}^1/_2H_2 + {}^5/_4CO_2 + HCO_3^- \qquad (DG_o = -627 kJ\ mol^{-1})$$

  For oxidative phosphorylation coupled to oxygen uptake by cytochrome oxidase (38 ATP/glucose oxidised). sufficient ATP could be synthesised if only part of the hydrogen was re-utilised (Hup^+) (9.5 total ATP for complete oxidation of reductants). Thus, there is little or no theoretical difference in the minimum metabolic carbon cost for these two sources of nitrogen, excluding all considerations related to nitrate uptake or tissue maintenance for nitrate or dinitrogen utilisation;
- note that nitrate assimilation is less acidifying than dinitrogen assimilation (Kennedy 1992), with no carbon dioxide evolved. Unfortunately, none of the estimates of the carbon cost for nitrogen fixation versus that for nitrate assimilation relying on carbon dioxide efflux to measure glucose consumption (e.g., Silsbury 1977) considered this metabolic difference. As a result, calculations indicating a greater carbon requirement for dinitrogen fixation underestimated the energy cost of nitrate assimilation;
- the above equations indicate minimum carbon costs of 1.7 g and 1.9 g carbon per g nitrogen assimilated as nitrate or dinitrogen, respectively, and 4.3 g and 4.5 g carbon when the extra photosynthate required to synthesise the dicarboxylic acid (oxaloacetate) for amino acid synthesis is included; all forms of inorganic nitrogen, nitrate, dinitrogen, or ammonia require this amount of dicarboxylate. Experimental observations suggest costs for legume nodules in the range of 5–8 g carbon per g dinitrogen assimilated, including costs for growth and maintenance of nodules, nitrogenase activity, and ammonia assimilation and export (Layzell et a1 1988);
- Pate et a1 (1979) have also pointed out that the carbon costs for legumes such as lupins fixing dinitrogen are similar to those for nitrate reduced in the roots. The possibility of reducing nitrate in the leaf cells and nitrite in chloroplasts has been suggested to allow reduced carbon costs for nitrate assimilation in leaves since photosystem II can directly provide electrons for reduction of nitrite derived from the photolysis of water. However, this is too simplistic a view and may be spurious since there is a variable additional carbon cost in disposing of the second OH^- or HCO_3^- molecule produced in nitrate assimilation. One HCO_3^- will be needed to make oxaloacetate by PEP carboxylase for amino acid biosynthesis but the second will be at least partly used to synthesize organic acid (malate, see equation be-

low) to be stored in leaf cell vacuoles with potassium ions absorbed in the roots by symport with nitrate to provide cell turgor by osmosis, adding a maximum cost of 2.6 g extra carbon per nitrate assimilated in those species retaining 100% of the bicarbonate produced in nitrate reduction as organic acid anion (e.g., tomatoes, spinach):

$$HCO_3^- + 1/2C_6H_{12}O_6 \Rightarrow C_4H_4O_5^{2-} + H^+ + H_2O$$

- in cereals. at least part of this bicarbonate is ultimately excreted as bicarbonate (or as carboxylate) to the soil solution as antiport for nitrate, reducing the need for photosynthate (Kennedy 1992). Although data on this point are meagre, one measurement indicated that only 41 % of the second bicarbonate formed in nitrate assimilation is retained in the tops of wheat as carboxylate, indicating an additional minimum cost of 1.1 g carbon per nitrate assimilated other than that used for reduction (equivalent to 1.7 g carbon but lessened by nitrite reduction in the chloroplast) and amino acid formation (2.6 g). If carboxylate is excreted instead of bicarbonate (wheat is said to excrete 20% of its photosynthate from roots), the carbon cost would be proportionately increased up to a maximum of 2.6 g per nitrate reduced;
- a hypothesis (Kennedy 1990) that photorespiration in C_3 plants results at least partly from a need to neutralize the excess alkalinity from nitrite reduction in the stroma of chloroplasts (with Rubisco's oxygenase activity thus acting as a pH stat) could further increase the energy cost of nitrate assimilation in leaves of C_3 plants such as wheat.

For these physiological reasons, theoretical assumptions that nitrogen fixation in legumes or in a potential nitrogen-fixing cereal automatically involves significantly greater energy costs as photosynthate than utilisation of nitrate (Addiscott et al 1991) should be re-assessed. Furthermore, there is no reliable field evidence supporting differences in dry matter yield by legumes with different sources of nitrogen; on the contrary, a greenhouse comparison of clover grown on dinitrogen versus nitrate as nitrogen source measuring biomass production indicated no significant difference in yield (Gibson 1966). The capacity of plants to compensate for increased energy costs by increasing photosynthesis rates above saturating light intensities (a fraction of full sunlight) is also unclear. Experimental trials conducted at radiation levels limiting photosynthesis could be more instructive regarding relative energy costs of different nitrogen sources but conclusions about relative energy costs and yields in such complex physiological systems will remain risky in the absence of such trials.

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