Nodulation and Nitrogen Fixation in Rice

POTENTIAL AND PROSPECTS

Edited by G.S. Khush and J. Bennett

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The International Rice Research Institute (IRRI) was established in 1960 by the Ford and Rockefeller Foundations with the help and approval of the Government of the Philippines. Today IRRI is one of the 16 nonprofit international research and training centers supported by the Consultative Group on International Agricultural Research (CGIAR). The CGIAR is sponsored by the Food and Agriculture Organization of the United Nations, the International Bank for Reconstruction and Development (World Bank), and the United Nations Development Programme (UNDP). The CGIAR consists of 50 donor countries, international and regional organizations, and private foundations.

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Foreword

Cereals are the world's major source of food. Global food security depends on reaching ever higher levels of sustainable grain production. The introduction of high-yielding varieties of wheat and rice has led to large increases in cereal production in many countries. But high levels of production are not possible without additional nutrient input. If cereal plants were able to utilize atmospheric N_2 as their primary source of N nutrition, serious economic and ecological problems associated with the use of inorganic and organic fertilizers could be mitigated. Research on biological N_2 fixation, particularly the *Rhizobium*-legume symbiosis, and research on plant molecular genetics have progressed to the point where it is not unrealistic to design research strategies aimed at developing N_2-fixing capacity in cereals.

Rice is particularly well-suited to serve as a model cereal for such investigations. Most of the biotechnology tools necessary for such research have been or are being developed for rice, and are available in the public sector. IRRI has an ongoing research program on biological N_2 fixation and has significant germplasm and field research facilities. Scientists throughout the world have relevant materials and knowhow.

An International Workshop to Assess the Potential for Nodulation and Nitrogen Fixation in Rice was held at IRRI to assess knowledge and recommend research strategies for the future.

Costly workshops must have a special objective to be justified in times of financial constraints. Planning a research program requires careful ex-ante evaluation of the chances of success. We did both. IRRI, following the outcome of the workshop-type Think Tank, has decided to develop a New Frontier Project that hopefully will become the umbrella for a joint effort among research centers worldwide sharing the same commitment: to reduce dependency on mineral N resources. The reduction in production costs will benefit farmers and consumers. Nonrenewable energy—mostly gas—can be saved for the generations to come. The environmental contribution—a reduction in CO_2 emission—is notable as well. We all know that a “man on the moon” project carries the risk of failure. However, in view of the food pressure in the 21st century, we are obliged to attempt what some see as impossible—to achieve all that is possible.

Dr. G.S. Khush of IRRI and Dr. Desh Pal S. Verma of Ohio State Biotechnology Center were the convenors. Dr. Khush and Dr. John Bennett served as technical editors. The proceedings were edited by Ms. Kate Kirk, assisted by Ms. Tess Rola.

Klaus Lampe
Director General

Introduction: feasibility of nodulation and nitrogen fixation in rice

J. Bennett and J.K. Ladha

More rice must be produced from less land with a minimum of costly and environmentally adverse inputs 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 intense than in supplying N to the crop.

IRRI's objective of increasing the yield plateau of rice grown under tropical conditions from 10 to 15 t/ha for the next 30 yr demands that attention be paid to sustainable methods of N supply.

Every ton of rice harvested contains about 12 kg N. Soil N and biological N_2 fixation (BNF) together account for more than half of the N used in rice cultivation; the remainder comes from chemical fertilizers. Any increase in grain yield per hectare will depend on increased N supply.

Yields per hectare are critically dependent on the nature, amount, and timing of N supply (George et al 1992). Synchrony between N supply and demand is crucial. Whether derived from green manure or soil reserves, N supply does not naturally coincide with plant demand. Through rapid mineralization immediately after flooding, excessive N is released in the first 2-3 wk after planting, when N demand is lowest, and more than 90% may be lost through bacterial and other processes (George et al 1992). The result is a shortage of N at later stages of plant growth. Although N supply can in principle be synchronized through multiple applications of N fertilizer, fertilizer and associated labor are in reality scarce and expensive. Furthermore, more than half the applied N fertilizer is lost through denitrification, ammonia volatilization, leaching, and runoff, resulting in financial loss and local and global pollution (George et al 1992). It is for these reasons that the feasibility of nodulation and N_2 fixation in rice itself must be investigated.

Rice yields and nitrogen supply

In well-managed irrigated lowlands, as much as 80-110 kg N is added per hectare per crop through BNF, irrigation water, and precipitation (Roger and Ladha 1992). With the exception of 10-20 kg N/ha left in roots and stubble, this N is removed as grain and straw, with about 40 kg N in each component, equivalent to a grain yield of about

3.5 t. In the most favorable situations, yields can be increased to 8-10 t/ha in tropical environments and 10-13 t/ha in temperate environments by adding a combination of green manure and chemical N fertilizers.

Many rice varieties give higher yields under temperate conditions than in the tropics (Yoshida 1981, Kropff et a1 1992). This is due principally to the longer grain-filling period under lower temperatures (40 d as against 25 d) and greater insolation under clearer skies. The greater sink size in japonica rice grains is an added factor. IRRI's objective of raising maximum yields to 15 t/ha per crop under tropical conditions could in principle be achieved by lengthening the grain-filling period and increasing sink size. With current rates of photosynthesis, one extra day of grain filling would increase yield by 0.3 t/ha (Kropff et al 1992). Extending the grain-filling period by 15-20 d would give the desired increase in yield, provided that the panicles were able to accommodate the additional biomass. IRRI is currently assessing genetic variation in grain-filling duration to determine the feasibility of increasing yield in this way.

It is recognized that the grain competes with the rest of the plant for absorbed N (Kropff et al 1992). Competition with the photosynthetic apparatus of the leaves is especially severe. As the grain fills, leaves senesce to release N from proteins, the rate of photosynthesis falls rapidly, and biomass increase becomes limited. To delay leaf senescence and simultaneously extend the grain-filling period, the plant would have to receive additional N.

Chemical N fertilizer applied at the reproductive phase delays leaf senescence and increases yield (Kropff et al 1992). In some rice varieties, an additional 30 kg N fertilizer/ha applied during the reproductive phase increases yields by 0.5-0.8 t/ha over controls, corresponding to harvesting the products of an additional 2-3 d of photosynthesis. Generalizing from this degree of yield response to added N, we can suggest that increasing yield to 15 t would require an additional 190-300 kg N/ha during grain filling. It is sobering to note that this supplement is comparable to the total amount of N_2 fixation achieved by a legume during an entire growing season (Table 1).

The yield responsiveness of rice to added N must also be increased. Work at IRRI points to the existence of significant genotypic variation in N uptake efficiency during flowering (J.K. Ladha, unpubl. data). Varieties with greater N uptake efficiency are likely to show enhanced yield response to N fertilizer. It may be equally important to identify varieties with enhanced sink capacity in the grain. Supplementary N may be most effective when provided at panicle initiation rather than at flowering because this is the time when sink size in the panicle is principally determined (Yoshida 1981).

Rice and biological nitrogen fixation

Rice is unique among cereal crops in its suitability for N supply through BNF. Its ability to grow in flooded soil aids weed control and renders it less dependent on external sources of N. The reducing environment of the flooded soil provides ideal conditions for growth of a range of diazotrophic bacterial genera including *Pseudomonas, Azospirillum, Enterobacter,* and *Klebsiella* (Ladha 1986). The surface of ricefield water provides a suitable environment for cyanobacteria, free-living and living symbiotically with the water fern azolla (Roger and Ladha 1992, Watanabe and Liu

Table 1. Estimates of dinitrogen fixed by different N_2-fixing systems in agriculture.

N_2-fixing system	Bacterium	Maximum rate of N_2 fixed (kg N/ha per crop)
Free-living/associative		
Rice-cyanobacteria	*Anabaena, Nostoc*	80
Rice-bacterial association	*Pseudomonas*	30
Sugarcane-bacterial association	*Acetobacter*	160
Symbiotic		
Rice-azolla	*Anabaena*	100
Legume symbiosis		
Soybean	*Bradyrhizobium*	237
Clover	*Rhizobium*	280
Sesbania rostrata	*Azorhizobium*	360
Actinomycete symbiosis		
Alder	*Frankia*	150

Sources: Bohlool et al (1992), Ishizuka (1992).

1992). In areas of abundant rainfall, aquatic legumes, such as *Sesbania* and *Aeschynomene,* can be grown for a month or two before the rice season to provide green manure (Ladha et al 1992). Finally, in areas of less abundant rainfall where only one rice crop can be grown, rotation of rice with a grain legume or forage legume has been practiced since ancient times (George et a1 1992, Peoples and Craswell 1992).

It must be recognized, however, that conventional BNF has only a limited capacity to render rice independent of external sources of N. The intrinsic rates of N_2 fixation by free-living and associative diazotrophs in ricefields are low compared with the rates of N_2 fixation by legumes (Table 1), while azolla shows intermediate productivity (Bohlool et a1 1992). It has been estimated that two cycles of azolla growth (30 d per cycle) in a ricefield prior to transplantation of rice would provide enough N to support the succeeding crop. However, exploitation of azolla is not always possible. The fern itself is subject to a wide range of biotic and abiotic stresses, inocula are difficult to maintain over fallow seasons, and many environments throughout Asia do not lend themselves to the use of land and water resources in this way (Watanabe and Liu 1992). In addition, azolla, like grain and forage legumes, has other uses which are often more profitable to the farmer than its exclusive use in sustaining soil N levels for rice cultivation. Extensive use of legumes for nonfertilizer purposes can actually lead to a net negative impact on soil N in some cases because only about 50% of the N in legumes is derived from BNF activities; as much as 50% comes from soil N resources (Peoples and Craswell 1992).

New approaches to biological nitrogen fixation

The inescapable conclusion from the above is that rice productivity is severely limited by available N. The highest sustainable yields with available BNF could reach 3-4 t/ha in the rainfed lowlands and 4-5 t/ha in irrigated rice. Yields beyond those levels will not be attainable without chemical N fertilizers or innovative forms of BNF. In upland

conditions, where the BNF option is difficult to exploit and where other factors such as phosphorus deficiency intervene, the highest sustainable yields are unlikely to exceed 2 t/ha. It is with this background that we turn to consider three unconventional approaches to BNF in rice that could give additional N inputs:

1) a more efficient association between rice roots and a diazotroph,
2) symbiotic N_2 fixation through nodulation of rice with bacteria such as rhizobia or *Frankia,* and
3) transfer of N_2 fixation capacity to the rice genome.

In discussing these possibilities, we acknowledge that a great deal more needs to be learned about diazotrophs, nodulation, and rice N metabolism before the full dimensions of the problem can be defined.

Forced association between rice and a diazotroph

Rice-diazotroph associations are well-known but their contribution to N supply is minor (Table 1). The association between sugarcane and diazotrophs is much more productive. The reasons for this difference are not understood. Field observations show that sugarcane productivity can be maintained despite continuous cropping, minimal N fertilizer inputs, and periodic removal of 100-200 kg N/ha with each cane harvest (Lima et al 1987). Trials using ^{15}N indicate that sugarcane obtains 20-55% of plant N from associative fixation (Urquiaga et al 1989, 1992). In Brazil, the key associative organism appears to be *Acetobacter diazotrophicus.* In Australia, a wider range of diazotrophs has been implicated, including *Pseudomonas, Enterobacter, Acetobacter, Beijerinkia,* and *Klebsiella pneumoniae* (Li and MacRae 1991).

Several genera of diazotrophs are associated with the rhizosphere of rice (Ladha 1986). They include *Azospirillum, Clostridium, Enterobacter, Alcaligenes,* and *Pseudomonas.* Fujii et al (1987) have described benefits to rice of the use of strains of *Klebsiella oxytoca* and *Enterobacter cloacae.* In the case of *K. oxytoca,* a 6% increase in soil and plant N content and a significant incorporation of $^{15}N_2$ were observed. Up to 40 kg N/ha per crop have been attributed to association with unidentified diazotrophs (App et al 1984).

An intimate association between rice and a free-living or loosely associated diazotroph might be forced by exploiting mechanisms operative in other plant-microbial interactions. Plant lectins can bind to specific chemical classes of carbohydrate, including carbohydrates on bacterial cell surfaces (Etzler 1985, Bowles 1990). Expression of an appropriate lectin gene in rice roots might promote an intimate association with a free-living diazotroph that displayed the corresponding carbohydrate moiety. The association might be enhanced further if exudates from the root could act as chemical attractants for the diazotroph or could be preferentially and specifically exploited by it as a C source. A situation resembling this proposed state is seen with *Agrobacterium,* which infects many dicotyledonous plants in response to a phenolic attractant and then transfers the T-DNA segment of its large Ti plasmid to them (Binns and Thomashow 1988). Among the genes present on T-DNA are genes for synthesis of opines, the preferred C and N sources for the bacterium. Such a system might be able to force a tighter association between rice and the diazotroph, and permit a more efficient transfer of fixed N between partners than that seen with free-living or weakly

associative bacteria. However, it is unlikely that such a system would contribute more than 50 kg N/ha per crop, unless access to plant C stimulated respiration in the diazotroph and permitted higher levels of N_2 fixation than those currently recorded (Table 1).

By far the most abundant organism associated with rice (80% of total microbial count) is a pseudomonad which has recently been named *Pseudomonas diazotrophicus* because of its significant, albeit low, rate of N_2 fixation (Watanabe et a1 1987). It is not known whether this bacterium associates specifically with rice or if lectins are involved. It may simply be an aggressive colonizer. It would be intriguing to identify the factors which limit its N_2-fixing rate.

Agrobacterium was mentioned above by way of analogy. However, two recent discoveries about *Agrobacterium* serve to bring it to center stage. Firstly, it was reported that *A. tumefaciens* is itself a diazotroph (Kanvinde and Sastry 1990). It can fix N_2 in the free-living state, it can grow on N-free medium, it can reduce acetylene to ethylene and incorporate $^{15}N_2$. Secondly, *Agrobacterium* can infect rice and transform it by T-DNA transfer (Raineri et al 1991). Chan et al (1992) found that transformation was enhanced by one or more factors contained in culture medium conditioned by potato suspension cells. This indicates that certain critical but as yet unidentified molecules are important in the establishment of a rice-*Agrobacterium* association.

Nodulation

Nodulation of legumes. The development of symbiotic N_2 fixation between legumes and rhizobia is a multistep process in which genes from both host plant (nodulin genes) and bacterium *(nod, nif, fix, exo, lps,* and *ndv* genes) play essential roles (Rolfe and Gresshoff 1988, Long 1989, Nap and Bisseling 1990). Small signal molecules pass between the two organisms, activating genes and eliciting developmental responses which culminate in the formation of a cluster of bacterial cells rich in nitrogenase and protected from external O_2 by a complex molecular barrier comprising infected and uninfected host cells with elevated levels of specific cell wall proteins. Nodules take sucrose from the phloem, convert it to succinate, and through bacterial respiration generate the ATP and reduced ferredoxin required for conversion of N_2 to ammonia. The plant component of the nodule takes up the ammonia and assimilates it into glutamine and asparagine in temperate legumes or into the ureides allantoic acid and allantoin in tropical legumes. The assimilate is then exported to the rest of the plant via the xylem (Schubert 1986).

The nodulation process begins with the secretion of flavonoids from roots into the rhizosphere (Long 1989). Flavonoids are host-specific and act as chemoattractants to certain rhizobia. Each flavonoid activates the *nodD* gene of specific rhizobial strains. The *nodD* gene in turn switches on other bacterial *nod* genes, enabling the cell to synthesize and secrete specific chemicals (Nod factors) which trigger nodule formation in the host. Nod factors appear to be lipo-oligosaccharides (Lerouge et al 1990, Spaink et al 1991). The basic oligosaccharide structure of Nod factors appears to be determined by a subset of *nod* genes present in all rhizobia *(nodABC).* Strain-specific structural modifications of the Nod factor framework are encoded by the variable

nodEFGHLMN genes (Vance 1990). Lectins are host proteins which recognize specific structural determinants on the bacterium and promote specific associations (Etzler 1985, Diaz et al 1989). Flavonoids, Nod factors, and lectins together contribute to the specificity of the host-symbiont interaction (Keen and Staskawicz 1988). Among the steps in nodulation triggered by Nod factors are curling of root hairs, formation of infection threads, and initiation of cortical cell division for prenodule formation (Nap and Bisseling 1990).

Plant genes affecting nodulation and N_2 fixation have been detected genetically and biochemically (Vance 1990, Caetano-Anollés and Gresshoff 1991). Genetic analysis has revealed 15 such genes in pea alone. Some of these genes contribute to the specificity of nodulation, others to its regulation (supernodulation, repressibility by nitrate). The host genes expressed during nodulation and detected biochemically are known collectively as nodulins (Vance 1990). Early nodulins are involved with the host responses so far described and are frequently cell wall proteins (Nap and Bisseling 1990). Late nodulins are concerned with the structure and function of the mature nodule (Vance 1990). About half of the known late nodulins participate in C and N metabolism within the nodule and include sucrose synthase, glutamine synthetase, NADH-dependent glutamate synthase, aspartate transaminase, and, in the case of nodules producing ureides, uricase and xanthine dehydrogenase. The other half include cell wall proteins contributing to the formation of the O_2 barrier protecting nitrogenase. The most abundant late nodulin is leghemoglobin, which ensures rapid O_2 transport from host to microsymbiont at prevailing low O_2 partial pressures (Appleby 1984).

The bacterial *nif* and *fix* genes are expressed after the nodulin genes and in response to the successful formation of the O_2 barrier between host and symbiont (Rolfe and Gresshoff 1988, Vance 1990). They include genes for 1) the two subunits of nitrogenase (the Fe protein and the FeMo protein), 2) proteins involved in the assembly of active nitrogenase, and 3) the ferredoxins and flavodoxins which provide reducing power to nitrogenase for the conversion of N_2 to ammonia.

Does nodulation occur naturally in rice? In investigating the feasibility of forcing nodulation in rice, it would be prudent to ascertain if nodulation occurs naturally in rice but at such a low frequency or under such unusual conditions that it has so far escaped detection in the field. If this is correct, then our efforts should be directed toward designing a rational search for the phenomenon and then attempting to increase its frequency through genetics and management. This is not an entirely absurd notion, given the comparatively recent discoveries of rhizobial nodulation of the nonlegume *Parasponia* and nonrhizobial nodulation by *Frankia* in a range of nonlegumes.

How should we search for nodulation in rice? One approach would be to examine the roots of land races and wild species growing under N-limited conditions—where nodulation would be an advantage and where the amount of mineralized N would be insufficient to repress nodulation. Another possibility is to examine the capacity of rice to support nodulation by rhizobia and other microsymbionts derived from other plant species. Studies of this type have been reviewed by Kennedy and Tchan (1992). In such studies, nodulation either occurred at very low frequencies (Bender et al 1990) or required an enzymatic pretreatment of the roots (Al-Mallah et al 1989). Nitrogen

fixation was reported by only one group (Jing et al 1990) but their work awaits confirmation.

A third approach would be to examine the rice genome itself for the presence of genes required for N_2-fixing symbiosis. Given the intrinsic sensitivity of bacterial nitrogenase to O_2, it would be appropriate to look for an analog or homolog of leghemoglobin. Hemoglobins have been found not only in legume nodules but also in rhizobia1 nodules on *Parasponia* and in *Frankia* nodules on *Casuarina* (Appleby et al 1983). However, *Trema,* a non-nodulated relative of *Parasponia,* also contains a hemoglobin gene; the amino acid sequence homology between the hemoglobins of *Parasponia* and *Trema* is 93% (Landsmann et al 1986). Furthermore, hemoglobin has been detected in sterile, un-nodulated roots of plants capable of nodulation (Appleby et al 1988). Thus, hemoglobin may be present in roots of all plants as a normal component but may serve an as yet unknown function. If this is the case, discovery of a hemoglobin gene in rice would not necessarily indicate that rice has the potential for nodulation.

If nodulation of rice occurs in nature, there may be problems in reproducing the phenomenon under conditions of intensive cultivation. In spite of many decades of research on soybean nodulation, the preparation of efficient inoculants capable of establishing themselves in the face of competition from indigenous microsymbionts remains difficult (Dowling and Broughton 1986, Keyser and Li 1992).

Transfer of nodulin genes to rice. If rice contains none of the genetic apparatus required for nodulation, an unknown number of nodulin genes and an unknown number of regulatory genes would have to be transferred from legumes or from genera nodulated by *Frankia* (such as *Casuarina, Alnus,* and *Myrica*). It may also be necessary to modify these genes considerably to render them functional in their new milieu. Since nodules will demand photosynthate from the plant in return for reduced N, it may be necessary to modify the source-sink relations of the rice plant to accommodate nodules. Our knowledge of N metabolism in rice roots would also have to be greatly expanded.

A common feature of all nodulation processes is a meristematic response in the host (Nap and Bisseling 1990). In the case of rhizobium nodules, the root cortical cells proliferate in response to Nod factors. Empty nodulelike structures are produced in alfalfa simply by treatment with these bacterial lipo-oligosaccharides (Lerouge et al 1990). Thus, some of the host activities of nodule formation, once triggered by a Nod factor, can proceed without further participation by the microsymbiont. Other activities such as the formation of glutamine synthetase may or may not be induced in empty nodules (Vance 1990). Thus, in soybean, induction of glutamine synthetase is dependent on ammonia produced by the symbiont, whereas in alfalfa induction of this key enzyme occurs even in empty nodules.

In nodulation by *Frankia,* division of host cortical cells is followed by lateral root growth, but it is not known whether lipo-oligosaccharides are involved here as well. In view of the importance of having general principles to guide us in the present task, it would be valuable to determine whether *Frankia* also secretes lipo-oligosaccharides. If it does, and given the similarity between Nod factors and the oligosaccharin class of plant development regulators (Tran Thanh Van et al 1985), the strong implication

would be that different forms of nodulation have been superimposed on the same oligosaccharin-based plant developmental control system. It would then be important to determine whether rice is responsive to rhizobial lipo-oligosaccharides and whether related molecules can be isolated from rice itself. It seems essential to retain the Nod factor/cortical cell interaction in any rice nodulation process so that the time and place of host differentiation are determined by the presence of the appropriate microsymbiont.

One way of assessing whether regulatory genes would have to be transferred to rice is to determine whether the nodulin gene promoters that respond to Nod factors in legumes will respond similarly in rice. One experimental system would be to fuse the promoter of an early nodulin gene such as *enod2* (Dehio and de Bruijn 1992) to the *gus* reporter gene (Jefferson et al 1987), transform rice with the construct and examine the expression of the GUS enzyme in rice before and after treatment of the plant with Nod factors. This experiment is reminiscent of a study in which the wound-inducible *pin*II promoter from potato was fused to the *gus* gene, transformed into rice and shown to be activated by wounding (R. Wu, pers. commun.). As in potato, the rice wound response was systemic: both wounded and unwounded tissue synthesized the GUS protein. This result shows that rice has a wound response, that it is systemic, and that the potato promoter can be integrated into the rice signal transduction pathway. If *enod* promoters are operative in rice, transfer of regulatory genes to rice to ensure that the nodulin genes function correctly may not be necessary. If activation of the *enod/gus* chimeric gene occurred in the above experiment with Nod factors and transgenic rice, it would seem unnecessary to transform rice with legume genes encoding the receptor for the Nod factor and other proteins required for transduction of the Nod factor signal.

Rhizobia enter their hosts without eliciting a defense response. Apparently, 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). Mutations in these genes produce modified cell walls and result in defective or aborted nodulation; in some cases they may also trigger a host defense reaction. It would be important to understand more about the factors which trigger defense responses in rice and to ensure that this response is not elicited by putative microsymbionts.

It is possible to imagine a minimal nodulation system, where the microsymbiont enters the host through epidermal cracks (like *Rhizobium* on peanut, *Azorhizobium* on *Sesbania* or *Photorhizobium* on *Aeschynomene*) or through the root cuticle followed by intercellular penetration (as observed with *Frankia* on some tree species). This might avoid the complexities of entry through root hairs and triggering of host defense responses. We could use a microsymbiont that does not require a flavonoid from the plant for activation of the *nod* genes. Mutants of this type are known in *Rhizobium* (flavonoid independent transcription activation mutants) and they remain highly effective in nodulation (Spaink et al 1989). Perhaps we could employ a microsymbiont which was able to form its own O_2 barrier, like *Frankia* strains on *Alnus* (Tjepkema and Schwintzer 1986).

One concern aroused by the idea of nodulation in rice relates to competition between nodules and the rest of the plant for photosynthate. Competition of this nature

exists in legumes (Pate and Layzell 1990). The direct cost of N_2 fixation in legumes is estimated to be 1-2 t glucose/ha per crop under the best conditions (Penning de Vries et al 1989). However, the impact on yield is probably relatively small because the energetic load of N_2 fixation is spread over the entire growing period of the crop and not concentrated in the pod-filling period. Nevertheless, some attention should be given to this question in rice. It is therefore encouraging to learn (M.J. Kropff, IRRI, 1992, pers. commun.) that the energy load to be expected in rice from operating N_2 reduction in nodules can probably be quantified using existing simulation models developed to quantify production and yield of leguminous species (Penning de Vries et al 1989, Kropff 1990).

Genetic engineering strategies which depend on major alterations of the bacterium to reduce the engineering of the host will have to take into account the effect of such modifications on the competitiveness of the modified bacterium.

It would be desirable to address the central question of the effect of Nod factors on rice not only with extracts of rhizobia that nodulate soybean, pea, etc., but also with extracts from microsymbionts nodulating *Sesbania, Aeschynomene,* and *Myrica,* especially those known to compete well in the rice-growing environment.

Nodulation site. Lambers and Visser (1984) and Layzell et al (1990) have found that utilization of photosynthate in nodules is limited at all times by the availability of O_2, especially under adverse conditions. Ladha et al (1992) hypothesized that the higher biomass and N_2 fixation potential of *S. rostrata* grown in submerged soils might be due to stem nodulation. Nitrogen fixation in stem nodules may not be limited by O_2. This brings up the important issue of the nodulation site in rice and the impact of ecosystem.

In upland conditions, root nodulation of rice would resemble root nodulation in soybean, but in rainfed lowland and irrigated rices, root nodules may suffer O_2 deficiency, especially late in crop development when the aerenchyma is degenerating. If so, the stem might be a more appropriate site for nodulation. Stem nodulation has the further advantages that 1) the inoculum could be supplied as a spray of the most effective rhizobia1 strain, and 2) the inoculum would not have to compete with native rhizobia. It might in fact be wise to consider developing stem nodules in rice with *Photorhizobium* from *Aeschynomene.* This diazotroph contains bacteriochlorophyll and appears to use light as a source of energy and reducing power for N_2 fixation (Eaglesham et al 1990, Evans et al 1990, Ladha et al 1990). It may not require ATP derived from the respiration of carbohydrates of plant origin.

Transfer to the field. If nodulation of rice is eventually achieved in the laboratory, would it work in the field? This is not an unreasonable question given the problems of achieving high levels of nodulation and N_2 fixation in legumes themselves (Keyser and Li 1992). One of the major problems is the inoculum. It seems often to be the case that rhizobia modified for superior performance in the laboratory or greenhouse fail to establish themselves in nature. Given the peculiarities of the rice-growing environment, it will probably be necessary to choose microsymbionts that are already adapted to aquatic plants (e.g., rhizobia from *Sesbania, Aeschynomene* and *Neptunia,* or *Frankia* growing on *Myrica* species). In this connection, it is interesting to note that *Azorhizobium caulinodans* isolated from *S. rostrata* is able to associate with the

rhizosphere of rice and show nitrogenase activity (Ladha et al 1989). By contrast, rhizobia from several nonaquatic legumes fail to show nitrogenase activity in rice.

The diversity and variability of rice ecosystems must also be recognized. These range from the irrigated with controllable levels of flooding, through rainfed with uncontrollable levels of flooding and drying, to upland with meager water supply and little scope for utilization of BNF through either green manure or co-cultivation of rice with azolla or cyanobacteria. Deepwater and tidal wetlands offer still more diversity. It is likely that these ecosystems will have to be treated quite differently with respect to nodulation/N_2 fixation in rice, but it cannot be doubted that rice and rice farmers in each ecosystem stand to gain enormously from developments of this nature.

Nitrogen fixation without a microsymbiont

Given the complexity of the interactions between host and microsymbiont, it might be simpler to transfer the biochemistry of N_2 fixation to the host and avoid nodulation entirely. In that case, we can ignore nod genes and concentrate on *nif* and *fix* genes and associated metabolic reactions (Vance 1990).

Nitrogenase is a multisubunit protein which requires Mo and Fe as cofactors. Its substrates are N_2, ATP, and reduced ferredoxins/flavodoxins, and ammonia is the key product. Variable amounts of H_2 are a wasteful byproduct of the nitrogenase reaction (Pate and Layzell 1990). ATP and reducing power are generated by a bacterial electron transport chain which oxidizes succinate derived from C compounds and employs O_2 as the terminal electron acceptor. The terminal oxidase has a very low K_m for O_2 and operates efficiently at the low intracellular O_2 tensions that are required for maintenance of nitrogenase activity (Bergensen and Turner 1980). However, the oxidase requires a high flux of O_2. As mentioned above, the kinetics of O_2 binding and release displayed by leghemoglobin in the cytosol of host cells provide the means of achieving high fluxes at low O_2 concentrations.

Where should nitrogenase be expressed in plants? From the point of view of energetics, the chloroplast of leaf cells would be the ideal location because the chloroplast could supply ATP and ferredoxin directly to the enzyme without the need to produce carbohydrate as an intermediate. The chloroplast is also the site of nitrite reduction and therefore possesses the enzymes required for ammonia assimilation. However, the fact that O_2 is a byproduct of chloroplast photosystem II raises the problem of protecting nitrogenase. If we assume that the problem of O_2 partial pressure could be solved, then N_2 fixation could in principle be transferred to chloroplasts by transferring fewer genes than would be required for the transfer of nodulation. The essential genes would include those encoding the two subunits of nitrogenase, together with the genes required for assembly of the Mo and Fe cofactors of the enzyme. The genes for these proteins would be transferred to the nucleus of the rice plant but would require modification to provide each polypeptide with a N terminal transit sequence specifying entry into the chloroplast (Keegstra et al 1989). Of course, it is not obvious that as complex an enzyme as nitrogenase could be assembled in one cellular compartment from component polypeptides synthesized in another. As an alternative to transferring nitrogenase genes to rice from rhizobia, they could be transferred from *Klebsiella* (Vance 1990) or several other bacteria (Bishop and Joerger 1990).

The problem of protecting nitrogenase from oxygenic photosynthesis has been solved by photosynthetic diazotrophs in at least two ways. Filamentous cyanobacteria such as *Anabaena* and *Nostoc* employ a physical separation of oxygenic photosynthesis from nitrogenase, the latter being located in specialized cells known as heterocysts, which lack photosystem II activity. Heterocysts are also the site of nitrogenase activity in *Anabaena azollae* (Peters and Meeks 1989). The unicellular cyanobacterium *Gleocapsa gallia,* by contrast, effects a temporal separation of photosynthesis from N_2 fixation which occurs in the dark (Gallon 1980). Thus, we have four models for protecting nitrogenase from O_2: 1) the rhizobia-leghemoglobin model, 2) the 0, barrier of *Frankia* on *Alnus,* 3) the heterocyst model, and 4) the *Gleocapsa* model. A possible fifth model is that of the highly unusual bacterium *Photorhizobium* (mentioned above and also known as *Rhizobium BTAil*). Since photosynthesis in *Photorhizobium* is sensitive to inhibitors of photosystem II (Eaglesham et al 1990) but does not appear to be oxygenic, the bacterium may use an electron donor other than water. Further insights into the detailed operation of these five models may suggest a mechanism for the task of protecting nitrogenase from O_2 in rice.

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Rhizobium-legume interactions

Genesis of root nodules and the role of host genes

D.P.S. Verma

Development of root nodules involves a series of interactions between the plant and bacteria that begins with chemotaxis of rhizobia and continues through the symbiotic state to senescence of nodules. Leguminous plants have evolved a set of genes encoding nodule-specific proteins (nodulins; Legocki and Verma 1980) to facilitate nodule development. Expression of these genes permits progression of infection, nodule organogenesis, release of rhizobia from the infection thread, and establishment of the symbiotic state. Although root nodules can develop without bacterial infection, infection and organogenesis proceed simultaneously. Nodule organogenesis and endocytosis of rhizobia are not dependent on the ability of the bacteria to fix N_2.

Nodule development has recently been shown to occur under the control of specific nodulation (Nod) signals produced by rhizobia in response to the host, although hypertrophic growth can occur autonomously on roots of certain legumes (see Caetano-Anollés and Gresshoff 1991). Signal molecules have recently been purified from several rhizobia and their structures have been determined, but the molecular basis for host specificity is not yet known.

Meristematic tissue normally resists bacterial infection, but rhizobia invade newly divided cells through a highly orchestrated infection process that does not provoke host defense reactions (Verma and Nadler, 1984, Djordjevic et al, 1987). Nodule meristem initiation occurs before the infection thread is formed, and can occur prior to root hair curling (see Dudley et al 1987). This suggests that rhizobia produce diffusible Nod factors to initiate nodule organogenesis. By uncoupling the events of nodule organogenesis from endocytosis of bacteria, it may be possible to identify the genes responsible for nodule development.

Phenolic compounds, flavones, and isoflavones are exuded by the portion of the root where the root hair emerges (Peters and Verma 1990). They activate the expression of nod genes in rhizobia and stimulate production of bacterial Nod factors (de Bruijn and Downie 1991, Kondorosi 1992). The Nod signal compound from *Rhizobium meliloti* (NodRm-1) has been identified as a sulfated and acylated oligo-N-acetylglucosamine (Lerouge et al 1990). This signal is effective at very low concentrations and initiates cortical cell division leading to the formation of nodulelike structures. The signal molecule from *B. japonicum* has a similar pentaglucosamine backbone, but is

not sulfated (Verma 1992). *R. leguminosarum* biovar *viciae* also produces nonsulfated Nod signals, but they contain a unique highly unsaturated fatty acid and differ in the number of N-acetylglucosamine units (Spaink et a1 1991).

Several factors secreted by *Rhizobium trifolii* bear no resemblance to the nod compounds, but enhance nodulelike primordia on clover (Philip-Hollingsworth et a1 1991) or cause uniform cell division in the cortex leading to a thick short root (TSR) phenotype (Verma 1992). Rhizobia also secrete cytokinins which can produce nodulelike structures (Libbenga et al 1973). Such structures are also produced by auxin transport inhibitors (see Nap and Bisseling 1990), suggesting that Nod factors mediate their interaction by altering phytohormone levels. The Nod signals may interact with the cell cycle control mechanism so that resting cortical cells enter mitosis (see Verma 1992). Although many infections occur on the root, relatively few give rise to nodule meristem. This suggests that further titration of these factors is essential for eventual success in generating nodule primordia. Many bacterial and plant mutants are able to abort this development program, but the precise steps in the process are not known.

Nodule meristem originates from cortical cells, while lateral root primordia have their origin in the pericycle. Nodules share a part of the genetic development of lateral roots. One of the late nodulin gene promoters of soybean (nodulin-26), when fused to a reporter (GUS) gene and introduced in *Lotus corniculatus,* was found to be expressed in incipient lateral roots and nodule primordia (Verma 1992), suggesting a common requirement for the expression of this gene in both types of meristems. Although this gene is not expressed in soybean roots, a homolog of nodulin-26 is expressed in root tips of tobacco and *Arabidopsis* (Yamamoto et a1 1990). Infection of *Parasponia* (a nonlegume) by *Bradyrhizobium* gives rise to nodules that resemble modified lateral roots with a central vascular system. These nodules initiate from the pericycle, not the cortex, and may be progenitors of modern legume nodules. The continuity of a meristem leads to indeterminate nodules, while its cessation gives rise to determinate nodules. Since the same bacteria can form determinate or indeterminate nodules in different hosts, the persistence of nodule meristem is host-dependent.

Nodulelike structures formed by Nod factors or bacterial mutants that fail to enter the host cell are highly differentiated. They consist of enlarged, infected cells and small interstitial cells (Morrison and Verma 1987). This suggests that the entire nodule development program is under the control of organogenesis. Rhizobia are only released from the infection thread in newly divided cells, possibly due to the need for endoplasmic reticulum and Golgi activity to generate enough membrane to enclose the bacteria. Cell division ceases in the infected cells while nodule meristem persists in indeterminate nodules, generating new cells which continue to be infected. In determinate nodules, cell division ceases before N_2 fixation begins, while the growth of the nodule continues by cell enlargement. Cell division and N_2 fixation are incompatible processes. Prior to the commencement of N_2 fixation, the bacteria cease to divide, and only those that are differentiated into bacteroids may fix N_2.

Many early nodulin genes are expressed during nodule differentiation, and some can be induced in nodules devoid of any bacteria (Nap and Bisseling 1990). Some early nodulins are involved in the Rhizobium infection process, while others mediate root nodule morphogenesis. Expression of one of the early nodulin genes, ENOD2, occurs

due to high levels of cytokinin (Dehio and de Bruijn 1992). The late nodulin genes are expressed during or following the release of bacteria from the infection thread, but before the induction of nitrogenase and commencement of N_2 fixation (Verma and Delauney, 1988). They are regulated by a variety of factors. Many late nodulin genes are not expressed in nodules without bacteroids (Verma et al 1988).

A fully differentiated nodule contains infected cells and uninfected cells. These two cell types assume different roles in order to meet the metabolic needs of the nodule. The symbiotic zone is shielded by a cell layer, endodermis, which seems to prevent ammonia diffusion (Miao et al 1991) and may also control diffusion of O_2. Infected cells produce leghemoglobin which allows O_2 flux to be maintained under low O_2 tension. Oxygen tension differs between infected and uninfected cells, as shown by monitoring expression of an O_2-responsive promoter (alcohol dehydrogenase, ADH) in a transgenic legume (Verma et al 1992). Differentiation of infected and uninfected cells and adaptation of carbon and nitrogen metabolic pathways provide maximum energy under microaerobic conditions.

Entry of bacteria into eukaryotic cells is a fundamental problem for cell interaction. Many rhizobia1 mutants are known (including exo- and lipo-polysaccharide mutants) that uncouple entry of bacteria from nodule morphogenesis, though their specific interaction with the host is unknown. Rhizobia are refined parasites (Djordjevic et al 1987) which provide the host with an essential nutrient and have thus become symbionts. Recent studies on the interaction between pathogenic bacteria and their animal hosts suggest a possible molecular mechanism by which some prokaryotic organisms enter the eukaryotic host cell (see Falkow 1991).

Finally, rhizobia are encapsulated within the peribacteroid membrane (PBM) upon release from the infection thread. This subcellular compartment is essential for successful infection. Failure to form PBM, or its disintegration, renders the association pathogenic (Werner et al 1985). The concentration gradient between host and rhizobia across the PBM must be equilibrated with certain metabolites or ions. This is apparently accomplished by opening specific channels in the PBM. Nodulin-26 appears to be such a channel, although the compounds transported through this channel are unknown. Nodulin-26 may facilitate active transport of metabolites, such as dicarboxylic acids, across the PBM into the peribacteroid fluid for uptake by the bacteria. The presence of this type of channel in the PBM demonstrates a previously unknown dimension of symbiosis, where the "extracellular compartment" that encloses the microsymbiont must be physiologically "internalized," bringing the foreign organism spatially into closer proximity with the host. These channels seem essential to the efficiency of endosymbioses.

Future prospects

The molecular basis for several key steps that control nodule development and endocytosis of rhizobia are yet to be understood. The availability of purified Nod compounds that trigger cell division in roots provides a handle to understand the control mechanism for initiation of nodule primordia. Recent isolation of cell cycle control genes may allow understanding of meristem continuity in indeterminate nodules and

its cessation in determinate nodules. Understanding the perception and transduction of signals for nodule organogenesis is vital for progress toward widening the host range of rhizobia. It would be of great interest to know if Nod factors can trigger cell division in cereal plants such as rice.

Entry of bacteria into the host cell without provoking host defense reactions is the most crucial but least understood aspect of the legume-*Rhizobium* association. This needs to be addressed with new approaches. The factors controlling membrane production to enclose bacteria and biogenesis of this subcellular compartment need to be analyzed. Finally, understanding subcellular compartmentalization, metabolite flow, and adaptation of various biosynthetic pathways to optimize this symbiotic interaction may reveal how plants deal with the nodule environment, with respect to high pH, low O_2, and high solute concentration, while maintaining cell viability.

In light of the requirements of endosymbiosis and the role of the host in elaborating the nodule structure, recent studies to broaden interaction of rhizobia with cereal plants require careful evaluation. Specific parameters governing nodule organogenesis that allow a foreign organism to coexist within the living plant cell need to be evaluated. Some bacteria have been shown to be engulfed by plant protoplasts nonspecifically (Davey and Cocking 1972), but such entry does not ensure survival and invasive bacteria often kill the plant cells. Death of the plant cell could be a hypersensitive reaction to the invader. This appears to be the case in nodulelike structures formed on rice and wheat roots, as the contents of the infected cells disintegrate (see Cocking et al 1990). Application of new molecular tools will allow evaluation of new symbioses to widen the host range of rhizobia and to improve productivity of cereal crops such as rice.

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Developmental asects of the *Rhizobium*-legume symbiosis

T. Bisseling

Root nodule formation involves several developmental steps (Nap and Bisseling 1990). Rhizobia attach to the root hairs of their host and cause deformation and curling of root hairs. They then invade the plant via infection threads, tubular structures that start in the curl of the root hair. Concomitant with the infection process, cells in the root cortex start to divide and form the nodule primordium. Infection threads enter individual primordium cells and bacteria are released into the plant cell cytoplasm. The nodule primordium then differentiates into a nodule. Like the formation of other plant organs, root nodule formation also involves the expression of a set of organ-specific genes, the so-called nodulin genes (Van Kammen 1984). The nodulin genes expressed markedly before the onset of N_2 fixation are named early nodulin genes (Nap and Bisseling 1990). These genes are supposedly involved in root hair curling, infection, and the formation of the nodule structure. The nodulin genes expressed shortly before or concomitantly with the start of N_2 fixation are the late nodulin genes (Nap and Bisseling 1990).

This paper discusses aspects of the *Rhizobium*-legume interaction related to the development of nodule formation, with an emphasis on the bacterial signal molecules which induce plant developmental processes and the plant genes specifically involved in the different steps of nodule formation.

Root hair curling

Changes in root hair gene expression after inoculation with Rhizobium have been studied. In pea root hairs, one mRNA, *RH-42,* whose synthesis is induced by the interaction of the root hairs with *R. leguminosarum* bv. *viciae* has been identified. The *RH-42* gene is not expressed in root hairs of uninoculated plants or in developing nodules. Therefore, it is likely that *RH-42* is involved in curling or deformation of root hairs (Gloudemans et al 1989). The expression of another pea gene, *RH-44,* is stimulated markedly in pea root hairs after inoculation with *R. leguminosarum* bv. *viciae,* but this gene is already expressed at a low level in root hairs on uninoculated plants (Gloudemans et al 1989).

Nod factors of *R. leguminosarum* bv. *viciae* are able to induce root hair deformation. Exudates of *R. leguminosarum* bv. *viciae* containing Nod factors stimulate the expression of the *RH-44* gene in pea root hairs. However, transcription of the RH-42 gene is not induced (Gloudemans et a1 1989). This indicates that Nod factors cause changes in gene expression in root hairs, but other *Rhizobium* components will probably be involved in the induction of expression of particular plant genes in root hairs.

The infection process

Two early nodulin genes involved in the infection process in pea have been identified. The early nodulin cDNA clones *pPsENOD5* and *pPsENOD12* were isolated from a pea nodule cDNA library by differential screening (Scheres et a1 1990a,b). In situ hybridization studies revealed that the *PsENODI2* gene is induced in root hairs, root cortical cells, and nodule cells containing growing infection threads. Interestingly, this early nodulin gene is expressed not only in cells containing growing infection threads, but also within several cell layers in front of the infection thread tip, where cells are preparing for infection thread penetration (Scheres et a1 1990a).

The *PsENOD12* early nodulin contains a putative signal peptide at the N terminus, the rest of the protein is composed of two repeating pentapeptides each containing two prolines. This is similar to the structure of a hydroxyproline-rich cell wall protein and, therefore, *PsENOD12* is probably a cell wall component. *PsENOD12* might be part of the cell wall of the infection thread and/or of the additional cell wall formed in root cortical cells that are penetrated by an infection thread (Scheres et a1 1990a).

The spatial distribution of the *PsENOD5* mRNA in infected pea roots is strikingly different from that of the *PsENOD12* transcript. While the *PsENODl2* mRNA is present within several root cortical cell layers in front of the infection thread tip, the *PsENOD5* gene is expressed only in cells containing the growing infection thread (Spaink et a1 1991, Scheres et a1 1990b).

The *PsENOD5* early nodulin is another proline-rich protein, but it does not show the repetitive structure of *PsENOD12. PsENOD5* is rich in proline, alanine, glycine, and serine residues, and may therefore be related to arabinogalactan proteins, of which some are plasma membrane components. Thus *PsENOD5* may be a component of the infection thread plasma membrane. Both the *ENOD12* and *ENOD5* genes can be induced by the purified Nod factors of *R. leguminosarum.* However, the *ENOD5* gene can only be induced by the Nod factor containing the highly unsaturated lipid moiety (Spaink et a1 1991).

Nodule primordium and meristem

The only early nodulin shown to be involved in cortical cell division is *SmENOD40.* The soybean *SmENOD40* gene is induced in the cortical cells that divide, but when these cells are penetrated by an infection thread, this gene is switched off.

The Nod factors of *Rhizobium* play a pivotal role in the induction of root cortical cell division since both NodRm-1 and NodRlv factors can induce cortical cell division. Application of NodRm-1 to alfalfa seedlings induces the formation of genuine nodules (Truchet et a1 1991). The *R. leguminosarum* bv. *viciae* factors elicit primordium formation in vetch roots, but these primordia do not develop into nodules (Spaink et a1 1991). We showed that in these vetch nodule primordia, the *VsENOD12* early nodulin gene is induced.

Root nodule differentiation

During normal nodule development, the nodule meristem begins to differentiate into a root nodule after the release of bacteria from the infection thread. Determinate and indeterminate nodule types both have a central tissue surrounded by several peripheral tissues. The peripheral tissues include the nodule cortex, nodule endodermis, and nodule parenchyma (inner cortex), which contains the vascular bundles connecting the nodule with the root stele.

The cells of the central tissue in indeterminate nodules are of graded age, the youngest cells located near the meristem and the oldest cells found in the proximal part of the nodule. Thus a zonation of consecutive stages of development is established in the central tissue.

Vasse et al (1990) have proposed a new nomenclature to distinguish different zones of the indeterminate nodule. The bacteria-free meristematic region at the apex is zone I. Infection occurs and cell differentiation begins in the prefixation zone II. In the N_2-fixing zone III, the plant cells have reached their maximum size and bacteroids start to fix N_2. In between zones II and III, the so-called interzone II-III is located. This zone is characterized by bacteroids with a specific morphology and the start of amyloplast deposition. In older nodules, a senescent zone IV is present.

At the transition between prefixation zone II and interzone II-III, several prominent developmental changes occur. In *Rhizobium,* the *nif* genes are expressed (Yang et al 1991) and bacteroid morphology changes (Vasse et al 1990). In the plant, amyloplast accumulation begins, the concentration of the early nodulin transcript *PsENOD5* drops markedly, and the late nodulin gene *NOD6* is induced. Furthermore, the decrease in *PsENOD3* mRNA matches the end of the interzone. The molecular mechanism underlying the developmental switch at the transition of the prefixation zone into the interzone is unclear.

The majority of the nodulin genes studied so far are expressed in the central tissue. However, some early nodulin genes that are transcribed in the peripheral tissues have been characterized. The best studied example is the *ENOD2* gene, which is expressed in the nodule parenchyma (Van de Wiel et al 1990).

A special function of the nodule parenchyma was indicated by physiological studies. The groups of Witty (Witty et al 1986) and Tjepkema (Tjepkema and Yocum 1974) have shown that the nodule parenchyma regulates free O_2 concentration in the nodule. The early nodulin *ENOD2* is composed of two repeating peptapeptides

containing two proline residues each. This suggests that this early nodulin is another cell wall component. Since the cell wall is a major factor in determining cell morphology, it is likely that early nodulins like *ENOD2* contribute to the special morphology of the nodule parenchyma and consequently to the formation of the O_2 diffusion barrier.

Future directions

Despite the fact that root nodule formation is the result of a mutual interaction of both partners, research programs on plant and bacterial symbiotic genes have developed almost independently. The recent discovery that Nod factors trigger the expression of certain early nodulin genes provides a basis for integrating the achievements of both bacterial genetics and molecular studies on host plant genes.

Nod factors play a pivotal role in the induction of all three developmental processes — root hair deformation, infection, and nodule formation—induced by *Rhizobium* in legume roots. Therefore it is of major importance to discover the mechanisms by which the Nod factors induce these processes. Studies on the molecular mechanisms underlying root nodule formation will be facilitated by the availability of purified Nod factors and cloned plant genes involved in the different developmental processes that can be induced by these factors. The major questions concerning these mechanisms that must be studied are the way the Nod factors are perceived and transduced by the plant, and how a single Nod factor—or a few very closely related factors—can induce three apparently different developmental processes in the plant.

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Host genetics of the nodulation phenotype

P.M. Gresshoff

Two approaches are used to investigate the plant's involvement in the nodulation process. One focuses on the isolation, characterization, and localization of nodule-specific gene products (nodulins). Both Desh Pal Verma and Ton Bisseling in this volume have contributed significantly to this area. The second approach involves the isolation of symbiotically impaired plant mutants, their physiological and genetic characterization, and the molecular mapping, gene isolation, and transformation of putative gene sequences responsible for the nodulation phenotype. My laboratory has focused on this second approach (see Caetano-Anollés and Gresshoff 1991, for review). This extended abstract will focus on past achievements and future directions.

Present status of research

It is possible to isolate mutations in three separate genes, giving rise to either super-nodulation or non-nodulation (Carroll et al 1985a,b; 1986; Mathews et al 1989a). All mutations are recessive nuclear mutations. Allelic series are available at some loci. The developmental blockage for each mutant type was determined (Mathews et al 1989b). The non-nodulation phenotypes are similar but differ as one gene allows some cortical cell divisions to occur while the other *(nod139)* renders the host plant nonresponsive (Sutherland et al 1990). Non-nodulation was found to suppress epistatically the supernodulation phenotype (Mathews et al 1990). Supernodulation, controlled by the *nts* gene, was found to be regulated through the shoot of the plant, while non-nodulation functions were regulated through the root (Delves et al 1986). The mutant *nts* gene alters endogenous autoregulation of the nodulation control mechanism, leading to the restriction of nodule numbers. Supernodulated plants are more tolerant of the inhibitory action of nitrate on nodulation. This is a function of the supernodulation (i.e., lack of autoregulation) condition (Day et al 1989).

The *nts* locus was mapped to the pUTG-132a probe of the soybean restriction fragment length polymorphism (RFLP) map, showing 0% recombination (Landau-Ellis et al 1991). An allelic pair, *nts382* and *nts1007,* shown previously to be allelic by complementation tests (Delves et al 1988), mapped to the same position (Landau-Ellis and Gresshoff 1992). Analysis in this region of the soybean genome has helped to

explain the nature of the polymorphism (a 800 ± 50 base pair (bp) deletion in the *G. soja* genome; Kolchinsky et a1 1993). The pUTG-132a marker has helped to define a polymerase chain reaction (PCR)-based marker for the same locus (Kolchinsky et al 1993). Parallel experiments are in progress for the non-nodulation loci, a plant host range control locus, and a nematode resistance locus. Physical mapping revealed that one centimorgan of recombination can be as small as 500 kilobases (kb) in this region of the soybean genome (Funke and Gresshoff 1992). It was possible to reveal ancient genome duplication in soybean using pulsed field gel electrophoresis (PFGE)-separated fragments and linked RFLP probes (Funke et al, unpubl. data).

We generated yeast artificial chromosome (YAC) constructs carrying soybean genomic inserts. The average insert size of the YAC library is 200 kb (Funke et al, unpubl. data). We devised methods to characterize chromosome-specific telomeres in yeast using end-labeling and intend to do the same for soybean and other plants (Kolchinsky and Gresshoff, unpubl. data).

In contrast to PCR, we developed a DNA fingerprinting approach which uses short, arbitrarily chosen oligonucleotides (as small as 5 nucleotides long), as primers for the amplification of genomic DNA (Caetano-Anollés et al 1991a,b). Resulting fragments are separated by polyacrylamide gel electrophoresis (PAGE) and silver-stained (Bassam et a1 1991), or scanned in DNA sequencer equipment after preamplification labeling of the primer with fluorescent tags (in collaboration with Applied Biosystems Inc., USA). This approach develops additional sequence-tagged sites for the soybean genome, permitting molecular mapping of gene loci for which only the genetic phenotype is known. We have started to screen F_2 bulked populations of soybean, separated according to a chosen phenotypic difference, and have started to amplify the bulked DNA with primer sets designed to detect polymorphisms in repulsion to the dominant allele being investigated. So far the bulked segregant analysis has not yielded a closely linked marker. DNA amplification fingerprinting (DAF) was successful in fingerprinting subgenomic DNA fragments stemming either directly from PFGE of soybean genomic DNA or YAC inserts.

To verify the putative coding potential of a positionally cloned DNA fragment, a soybean transformation system was developed. Considerable difficulty was experienced with published protocols of soybean transformation using *Agrobacterium tumefaciens* (Monsanto) or the biolistic approach (Agracetus). We developed an efficient *Agrobacterium rhizogenes* transformation system, which allows the verification of gene function in root or nodule-expressed genes (Bond and Gresshoff, unpubl.). Transformation was confirmed by *gus* gene expression (carrying a plant intron), antibiotic selection, and Southern blotting. Antisense constructs are being tested for their phenotypic effects. The biolistic approach was optimized with the construction of our own helium switch gene gun, using a 50-millisecond microswitch to blast DNA-coated tungsten particles of grid holder in a Swinnex filter holder. Transient transformation frequencies of 6000-8000 events per gram fresh weight of cell culture tissue are recorded (Bond et al 1992). Stable transformation was also achieved in embryonic suspension cultures of soybean and its nodulation mutants. Plantlet regeneration was achieved after embryo desiccation and in vitro germination (Bond, unpubl. data). Nodulation mutants maintained their symbiotic phenotype after regeneration.

Future research

The present status of host genetics research in soybean has shown that numerous genes control the nodulation phenotype, that these genes are single Mendelian loci, and that different plant parts participate in the phenomenon of nodule pattern regulation. We showed the utility of the positional cloning approach by mapping to an RFLP, physical mapping and the determination of a conversion factor from genetic to physical distance, the construction of soybean YACs, and the first transformation of symbiotically altered soybean using both *Agrobacterium* and biolistic approaches.

Further progress requires:

1. larger YAC inserts;
2. development of a linking library to generate contiguous YACs;
3. transformation of the YAC clone (or subclone) carrying the wild-type *nts* allele into *nts* host tissue and correction of the nodulation phenotype;
4. if 3. is achieved, the search for the *nts* gene on the transforming fragment; possible application of micro-DAF or further transformation and eventual sequencing of wild-type and mutant fragment;
5. if 4. is achieved, the characterization of the allelic nts series and analysis of the promoter in chimeric reporter constructs; in situ localization of gene product and attempted isolation of the gene product and metabolite in the leaf to ascertain the function;
6. linking of the cytological and molecular soybean map;
7. discovery of metabolic homologs for the *nts* gene; and
8. mapping, cloning, and transformation of the two non-nodulation loci.

Key bottlenecks

1. Lack of a close molecular marker to the *nod* genes may require development of a jumping library.
2. YAC clones or contiguous YACs may fail to transform the *nts* embryogenic culture, and gene correction may be impossible.
3. Transformed plants may be sterile and prevent genetic analysis.
4. Resource limitation may prevent purchase of equipment such as DNA synthesizer and DNA sequencer.

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Hormones and nodule formation: cytokinin induction of the *Sesbania rostrata* early nodulin gene *Enod2*

F.J. de Bruijn, R. Chen, C. Dehio, A. Goel, S. Reiser, and K. Szczyglowski

Soil bacteria belonging to the Rhizobiaceae form N_2-fixing root or stem nodules on legume plants by a highly evolved process that requires a number of sequential steps. Each step appears to involve specific signal molecules from both symbiotic partners, which affect the expression of genes (operons, regulons) of both the rhizobia and their plant host (Nap and Bisseling 1990, de Bruijn and Downie 1991).

The early stages of the symbiotic interaction involve activation of nodulation (*nod*) genes in the bacterium by signal molecules, generally flavones and isoflavones, secreted by the plant (Peters and Verma 1990). The rhizobia1 *nod* gene products are involved in the synthesis of highly specific Nod factors, which have been purified from two *Rhizobium* species and shown to be acetylated oligoglucosamines (Lerouge et a1 1990, Spaink et al 1991, de Bruijn and Downie 1991). In the case of *Rhizobium meliloti,* the Nod factor has been found to be sulfated and is host-specific for *Medicago* species (e.g. alfalfa; Schwedock and Long 1990, Roche et al 1991). Purified *R. meliloti* Nod factor is capable of eliciting root hair deformation, cortical cell division, and formation of nodulelike structures on alfalfa roots (Lerouge et al 1990, Truchet et a1 1991), but its mode of action and the other components of the signal transduction chain responsible for nodulation remain unknown.

Hormones and nodulation

Thimann (1936) suggested that hormones were involved in nodule induction on plant roots more than 50 yr ago. This concept was extended by Arora et al (1959), who demonstrated that the cytokinin kinetin induced pseudonodules on tobacco roots. Libbenga et al (1973) suggested that cortical cell division, one of the early steps in nodule induction, could be induced by auxins and cytokinins excreted by the infecting rhizobia, acting in concert with "pre-existing transverse gradients of stimulative host factors." Cytokinin-mediated stimulation of cortical cell division in legume roots has also been demonstrated by Baueret al (1985). Long and Cooper (1988; Long 1989; S.R. Long, Stanford University, USA, 1992, pers. commun.) have presented data in support

of cytokinin involvement in nodulation by showing that a-plasmid constitutively expressing the *Agrobacterium* zeat in biosynthetic gene *tzs* enables strictly *nodABC R. meliloti* mutants to induce pseudonodules on alfalfa. Auxin transport inhibitors, such as 2,3,5-triiodobenzoic acid (TIBA), have been found to induce pseudonodules on the roots of leguminous plants (Allen et a1 1953, Hirsch et a1 1989). These observations suggest a close linkage between alterations to the plant cell hormone balance and nodule induction by rhizobia.

Hormones and rhizobia

Auxin and cytokinin production by rhizobia has been amply documented (Morris 1986, Badenoch-Jones et al 1987, Sturtevant and Taller 1989). However, a direct role in the induction of cortical cell division and other aspects of the infection process and nodule ontogeny has not yet been established for these hormones. Interestingly, it has been reported that plant compounds may influence cytokinin production by rhizobia (Sturtevant and Taller 1989). Flavonoids, potent *nod* gene inducers, have been proposed as candidates. An analogous situation has been found in the pathogenic interaction between the tumor-inducing soil bacterium *Agrobacterium tumefaciens* and plants. The cytokinin biosynthesis *tzs* gene of *A. tumefaciens* has been found to be induced by acetosyringone, which also induces the virulence genes (Rogowsky et al 1986). Studies to find if specific plant compounds involved in rhizobia1 nod gene induction also induce rhizobial hormone biosynthesis genes, and if these hormones, in turn, play a role in nodule induction, would be beneficial.

A group of flavonoids, including compounds known to be nod gene inducers, have been found to compete efficiently for receptor binding with the auxin transport inhibitor NPA, suggesting that they may act as natural auxin transport inhibitors in plants (Jacobs and Rubery 1988). Application of NPA to alfalfa roots induces nodulelike structures and the early nodulin gene *Enod2.* Thus an important connection may exist between *nod* gene induction by flavonoids, Nod signals, hormone transport/balance and nodulation (Estabrook et al 1991, A. Hirsch, pers. commun.).

Nodulin gene expression and the early nodulin gene *SrEnod2*

Plant response to rhizobial infection includes the induction of genes (nodulin genes) which play a role in nodule ontogeny and are normally silent (van Kammen 1984, Nap and Bisseling 1990, de Bruijn et al 1990). Temporal and cell-specific expression of nodulin genes is a valuable tool for dissecting the different stages of the infection process and nodule formation and elucidating the role of purified Nod factors (Nap and Bisseling 1990).

One of the best characterized "early" nodulin genes is *Enod2.* This gene is activated at an early stage of nodule development (7-10 d after infection) and has been identified in a variety of legumes (Franssen et a1 1987, Gloudemans and Bisseling 1989, Szczyglowski and Legocki 1990, Dehio and de Bruijn 1992, Govers et al 1990). The *Enod2* gene is expressed exclusively in the nodule parenchyma cell layer (Van de

Wiel et al 1990a). This tissue has been postulated to act as a barrier to limit O_2 diffusion into the central (infected) nodule tissue, where the O_2-sensitive N_2 fixation process takes place (Witty et al 1986). The *Enod2* gene encodes a (hydroxy) proline-rich (glyco) protein, which is probably targeted to the cell wall and may be involved in creating the O_2 barrier (Van de Wiel et al 1990a, Nap and Bisseling 1990).

Dehio and de Bruijn (1992) have cloned and sequenced the *Enod2* gene of the stem-nodulated legume *Sesbania rostrata,* a tropical plant which is used as green manure for rice cultivation (de Bruijn 1989, Ladha et al 1990). The *SrEnod2* gene appears to be expressed differentially in stem versus root nodules. Differential gene expression in *S. rostrata* root versus stem nodules has been reported previously (de Bruijn 1989), and may reflect the different morphology and/or physiology of the distinct nodule types. The transient expression in root nodules may be the result of early degradation of the nodule parenchyma, in which *Enod2* genes are specifically expressed (Van de Wiel et al 1990a). Alternatively, the stable *SrEnod2* expression in *S. rostrata* stem nodules may reflect a continuous need for an effective O_2 barrier in these aerial nodules (Nap and Bisseling 1990, de Bruijn 1989).

Cytokinin induction of the *SrEnod2* gene

Enod2 expression appears to be directly linked to nodule morphogenesis because successful rhizobial infection is not essential. The *Enod2* gene is expressed in "empty nodules" (lacking infection threads and intracellular bacteroids), which are induced by exopolysaccharide-deficientl *R. meliloti* mutants on alfalfa roots (Dickstein et al 1988). Using in situ hybridization, *Enod2* transcripts have been localized to parenchyma cells at the base rather than along the periphery of such nodules (Van de Wiel et al 1990b, Allen et al 1991). *Enod2* expression has also been demonstrated in "pseudonodules" induced by auxin transport inhibitors on alfalfa roots (Hirsch et al 1989, Van de Wiel et al 1990b) and in nodulelike structures that develop spontaneously on alfalfa roots in the absence of a rhizobial infection (Truchet et al 1989).

To examine the possible connection between hormones and *Enod2* expression, we have carried out RNA hybridization studies using the cloned *SrEnod2* gene as a probe. We have shown that the *SrEnod2* gene is induced in the roots of *S. rostrata* seedlings treated with cytokinins (Dehio and de Bruijn 1992). The other hormones tested neither induce *SrEnod2* expression nor interfere with cytokinin induction. Somewhat surprisingly, the auxin transport inhibitor TIBA, which is capable of inducing nodulelike structures on alfalfa roots in which the *Enod2* gene is expressed (Van de Wiel et al 1990b), does not affect *SrEnod2* expression. The cytokinin response appears to be very specific, since it is observed with a variety of cytokinins and is time- and concentration-dependent. Moreover, *SrEnod2* induction is not observed when *S. rostrata* plantlets are subjected to a variety of physical stresses.

The cytokinin effect has not only been observed after external application to *S. rostrata* roots. Alteration of the intracellular cytokinin concentration in *S. rostrata* stems via *Agrobacterium*-mediated transformation (Dehio and de Bruijn 1992) also has an effect. *S. rostrata* plants are very susceptible to *Agrobacterium* infection and

readily generate local and systemic tumors and hairy roots on the stem when infected with different *A. tumefaciens* or *A. rhizogenes* strains (Vlachova et al 1987). *SrEnod2* expression is present in tumors induced by wild-type *A. tumefaciens* strains, and mutant strains carrying a Tn5 insertion in the auxin biosynthetic T-DNA *gene 2,* but completely absent in tumors induced by an *A. tumefaciens* strain carrying a mutation in the cytokinin biosynthesis *gene 4.*

The correlation between *SrEnod2* induction and cytokinin biosynthesis in the transgenic cells supports the notion that the cytokinin effect is specific. In transgenic hairy roots induced by *A. rhizogenes* on *S. rostrata* (C. Dehio and F.J. de Bruijn, unpublished observations; de Bruijn and Schell 1992) or on white clover and *Lotus corniculatus* (Govers et al 1990), the endogenous *Enod2* genes are also highly expressed, further supporting the notion that changes in hormone balance or hormone sensitivity are responsible for the induction effect.

It is tempting to suggest a direct or indirect link between the highly specific, rapid and concentration-dependent cytokinin induction of the *SrEnod2* gene described in this report and initiation of nodule ontogeny by the infecting rhizobia. The NodRm-1 factor could be involved in inducing alterations in cytokinin concentration in root cells as part of a cascade signal transduction mechanism to initiate nodule development and early nodulin induction. Alternatively, there may be an independent cytokinin-like compound, produced by the rhizobia, which is involved in the induction of early nodulin genes, such as *Enod2.* Application of sterile culture filtrates of *Rhizobium leguminosarum* bv. *viciae* to pea seedlings results in the induction of two early nodulin genes involved in the infection process (*PsEnod12* and *PsEnod5*), but not *Enod2* (T. Bisseling, Agricultural University, Wageningen, 1992, pers. commun.).

Since the *Enod2* gene is expressed in nodulelike structures formed aseptically and spontaneously on alfalfa roots, and assuming that cytokinin is involved in *Enod2* expression in this legume as well, one could argue that rhizobial cytokinin production is not absolutely essential. In this case, one must assume that the developmental program for nodule induction and *Enod2* expression is already present in the host plant and can be self-triggered or activated by rhizobial signals, including hormones. An analysis of rhizobial mutants with disturbed cytokinin production should help to determine the role, if any, of microbial hormone production in the symbiotic process.

A series of experiments have been initiated to identify *cis*-acting elements in the soybean and *SrEnod2 5′* upstream regions, by examining the expression of chimeric *Enod2* reporter gene fusions in transgenic *Lotus corniculatus* plants. Approximately 3 kb of the *SrEnod2 5′* region and a collection of deletion fragments have been fused to the beta-glucuronidase *(uidA; gus)* gene and introduced into *Lotus.* Preliminary results suggest that the *cis*-acting elements responsible for nodule-specific expression are located within 900 bp upstream of the transcriptional start site. Moreover, histochemical GUS staining experiments of different tissues of the transgenic plants have supported the in situ hybridization results reported by Van de Wiel et al (1990a) by revealing that the *SrEnod2* promoter region directs nodule parenchyma-specific expression (Goel et al 1990).

We are presently delimiting the *cis*-acting elements responsible for nodule parenchyma-specific expression and examining whether these elements are the same

as those conferring cytokinin induction of the *SrEnod2* gene. Confirmation would strengthen the link between hormones and the developmentally controlled, cell-specific expression of this early nodulin gene, and would further support the hypothesis that hormones play an important role in rhizobial nodulation of plants. We are also trying to clone cytokinin biosynthesis genes from the *S. rostrata* symbiont *Azorhizobium caulinodans* (de Bruijn 1989). Transposon mutants in these genes would help to ascertain the importance of rhizobial cytokinin production and excretion in early nodulin induction. The tissue-specific Gus expression in transgenic Lotus and nonlegume (e.g., tobacco or *Arabidopsis*) plants carrying chimeric *SrEnod2-gus* genes will be examined after topical application of cytokinin and auxin transport inhibitors.

Prospects

The role of hormones in induction and function of nodules remains an important and poorly explored topic. A variety of studies have suggested linkages between hormone production, concentration, balance, and transport inhibition and the nodulation process. Most of these studies do not provide evidence for the direct involvement of hormones in nodule ontogenesis or for the nature of the signal transduction pathway involved in their action. It is not even clear whether the observed effects are due to rhizobial hormones produced and secreted during the infection process, or endogenous hormones which are modified, stimulated, repressed, or transport-inhibited during rhizobial infection. Several lines of research have been or should be initiated to try and establish causal links between hormone-mediated processes and nodulation.

A search for the primary receptor(s) for the Nod signals (oligogalactosamines) has been initiated and the same should be done for the cytokinin and auxin transport inhibitors. The effect of fluctuations of internal cellular hormone balance on nodulin gene induction and control of cortical cell division during nodule ontogeny should be examined in detail (Verma 1992). The process of nodule formation should be closely compared with distinct but possibly related processes, such as lateral root formation, and the role of hormones evaluated. Moreover, the relationship between spontaneous nodulation in the absence of rhizobia (Truchet et al 1989), the induction of nodulelike structures on nonlegumes such as rice by rhizobia (Simon Moffat 1990), and hormone balances should be examined. These data are essential for the understanding of existing symbiotic systems and for the development of novel, effective symbioses.

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Regulation of nodulation genes

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Signals that activate specific genes in both symbionts govern induction of nodules on leguminous plant roots by rhizobia. The first signal molecules are flavonoids, exuded by the plant roots (Peters et al 1986, Redmond et al 1986, Firmin et a1 1986, Zaat et al 1989). Specific flavonoids induce the expression of nodulation (*nod* and *nol*) genes in conjunction with the bacterial activator NodD protein (Kondorosi 1992 for review). The *nod* genes are organized into several transcriptional units and form a regulatory circuit, the *nod* regulon, ensuring their coordinate regulation. The *nod* gene products direct the synthesis of specific lipo-oligosaccharides, the Nod factors, which activate genes involved in nodule initiation in the host plant (Roche et a1 1991, Spaink et al 1991, Schultze et al 1992).

Both NodD protein and specific plant signals are essential for *nod* gene induction and nodule initiation (see Györgypal et al [1991a] for review). Various rhizobia infecting diverse host plants contain one to three copies of the *nodD* gene. On the whole, the *nodD* gene is constitutively expressed. At the same time, certain *nodD* copies are controlled by various environmental or intracellular factors, such as flavonoids, combined N (Dusha et a1 1989), the activator protein SyrM (Mulligan and Long 1989) or the *nod* repressor NolR (Kondorosi et al 1991b). In addition, the *nodD* gene is autoregulated in several species (Rossen et al 1985).

The different NodD proteins have different plant signal (flavonoid) specificity, but have the common ability to activate *nod* genes. *Nod* gene inducers isolated from host plants were shown to be flavonoids (flavones, flavanones, isoflavones, chalcones) (Györgypal et al 1991a), or, occasionally, monocyclic aromatic compounds (Le Strange et al 1990). Genetic data indicate that the signal specificity of different NodD proteins is based on specific binding of the flavonoids to the NodD protein. The NodD proteins of rhizobia with broad host ranges are responsive to a wide range of compounds, while NodD proteins of the narrow host range species interact with a limited set of flavonoid inducers, and, in many cases, isoflavones inhibit these proteins (Györgypal et al 1991b).

Several results suggest that the carboxy part of the NodD protein takes part in the interaction with the flavonoids. A helix-turn-helix motif in the N terminal part is predicted to be involved in DNA binding. Even in the absence of inducers, the NodD

protein binds to the *nod* box, a 47-base pair sequence located in the promoter of all inducible *nod* transcriptional units (Rostas et a1 1986). It has been suggested that upon binding of an inducer molecule, the *NodD* protein undergoes a conformational change which promotes RNA polymerase to start up *nod* gene transcription.

In *Rhizobium meliloti* with several copies of the *syrM* and *nodD3* genes on a plasmid, the *nod* genes are expressed constitutively (Mulligan and Long 1989) and may result in host range extension to certain nonhost plants, such as *Macroptilium* (Kondorosi et a1 1991b). It is likely that SyrM enables the NodD3 protein to attain the activatory conformation without interacting with the plant signal. By mutating the *nodD* gene and by constructing hybrid *nodD* genes from *nodD* genes of different species, *NodD* proteins that activate nod genes without inducers could be produced (Kondorosi 1992).

In several species, expression of *nod* genes was shown to be negatively regulated by the *trans*-acting factor *No1R*. In strains not containing the *nolR* gene, the *nodD* gene is autoregulated. It was found that negative control of *nod* gene expression contributes to the ability of the bacterium to obtain optimal nodulation of the plant host (Kondorosi et a1 1989).

It is unlikely that the major obstacles to rice nodulation would be problems concerning nodulation gene expression. If a *Rhizobium* strain is selected for studies on potential nodulation of rice, induction of the *nod* genes by plant extracts or exudates can be relatively easily assayed. It is encouraging that nod gene inducers have been found in extracts of seedlings of certain monocots (wheat) (Le Strange et al 1990). Numerous *nodD* genes are available which could be tested for activation by extracts of rice roots. The *nodD* genes of broad host species which can nodulate even the nonlegume woody tree *Parasponia* are promising candidates.

As an alternative, *nodD* genes whose products do not require flavonoids or the *syrM-nodD3* gene combinations for *nod* gene activation could be introduced into selected rhizobial strains. The activated form of a NodD protein can recognize the nod box sequences in different rhizobial strains and can induce *nod* gene expression, although in some *nodD*-rhizobia1 strain combinations, the *nodD* promoter may not work efficiently. The consequences of lack of other controlling elements, or their possible altered regulation in a new host, will require further investigations.

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Oligosaccharins—oligosaccharides with regulatory functions

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Plants and animals have evolved signaling mechanisms to regulate the expression of genes essential for their growth, development, and defense against pests. Research provides evidence that plants utilize the structural complexities of oligosaccharides to regulate important physiological processes. Oligosaccharides with regulatory activities are called oligosaccharins.

Oligosaccharides are ideal ligands for precise interactions with recognition sites on proteins because of the extensive stereochemistry, multiple hydroxyls and oxygen atoms, and accessible hydrophobic regions characteristic of glycosyl residues. Carbohydrate-binding proteins can distinguish among the large number of primary structures and the three-dimensional shapes oligosaccharides adopt.

Cell wall polysaccharides of plants and microbes are a rich source of oligosaccharins, and the walls also contain glycanases and glycosidases capable of generating oligosaccharins. Protein receptors can distinguish between a range of information-carrying oligosaccharides and transmit the information to the cells to which the receptors are attached.

This report will describe the structure and function of different oligosaccharins involved in plant defense, organogenesis, growth regulation, and host-plant specificity of rhizobia1 infection.

Plant defense

The biosynthesis and accumulation of antimicrobial phytoalexins is one of the best-studied plant defense mechanisms. Plants synthesize and accumulate phytoalexins in response to microbial infection or after treatment by elicitors.

Three oligosaccharin elicitors of phytoalexins have been characterized; these are a structurally defined hepta-ß-glucoside (Sharp et al 1984), a-1,4-oligogalacturonides with degrees of polymerization (DPs) from 10 to 15 (Davis et al 1986a, Jin and West 1984), and chitosan or chitin oligosaccharides of undefined length (Hadwiger and Beckman 1980). The hepta-ß-glucoside, from fungal cell wall ß-glucan, and the oligogalacturonides, from the homogalacturonan of plant cell walls, act synergistically to stimulate accumulation of phytoalexins in soybean (Davis et al 1986b). Efforts to

isolate the physiological receptors of the hepta-ß-glucoside and oligogalacturonide elicitors are in progress (Cheong and Hahn 1991, Cosio et al 1990).

Organogenesis

Oligogalacturonides are involved in plant defense, but, at a tenfold lower concentration, they can regulate organogenesis.

Most undifferentiated plant cells are totipotent, that is, each plant cell has the potential to develop into a mature plant. Oligosaccharins can act like hormones, for oligogalacturonides with DPs 10 to 15 inhibit tobacco explants from forming roots and induce the explants to form flowers when the explants are grown in media that, without the oligogalacturonides, cause roots or no organs to form (Marfà et al 1991). Evidence has been obtained suggesting that plant cell wall-derived oligosaccharides regulate other developmental processes including cell elongation and fruit ripening. Furthermore, oligogalacturonides induce a variety of rapid changes in the functions of the plasma membrane in plant cells (Farmer et al 1991, Mathieu et al 1991). These rapid effects have yet to be directly associated with any of the demonstrated biological activities of oligogalacturonides. Characterization of oligogalacturonide receptors may facilitate elucidation of the mechanisms by which oligogalacturonides can have so many biological effects.

Growth regulation

The cell walls of higher plants contain a family of highly branched polysaccharides called hemicelluloses. Hemicelluloses are functionally defined as those polysaccharides that form strong noncovalent associations with cellulose microfibrils. The predominant hemicelluloses in the primary cell walls of higher plants are arabinoxylan and xyloglucan. Xyloglucan is thought to be a load-bearing structural polymer in the primary cell wall because of its role in cross-linking cellulose microfibrils. The dynamic nature of this cross-linking is proposed as the major factor controlling the rate of cell wall expansion, thereby regulating plant cell growth.

Oligosaccharide fragments of xyloglucan are generated by treating the polysaccharide with a purified endo-β-l,4-glucanase. The cell wall activity of a xyloglucan-specific enzyme was increased by spraying pea seedlings with the phytohormone auxin (Byrne et a1 1975). A nonasaccharide product of the action of endo- ß-1,4-glucanase on xyloglucan inhibits the growth of pea stems (McDougall and Fry 1988, York et al 1984). This observation is consistent with a feedback control loop hypothesis in which elevated amounts of auxin promote the formation of xyloglucan-derived oligosaccharides, inhibiting the growth-promoting effect of auxin.

Host-plant specificity of rhizobial infection

The establishment of a N_2-fixing symbiosis between a *Rhizobium* and its legume host involves a complex infection process. This process requires an exchange of signal molecules between the bacterium and the cells of its eukaryote host, resulting in

coordinated regulation of gene expression in both the *Rhizobium* and the legume. The infection process produces a root nodule containing modified N_2-fixing rhizobia called bacteroids and is host symbiont-specific. For example, *Rhizobium leguminosarum* biovar *trifolii* infects clover but not alfalfa, while *Rhizobium meliloti* infects alfalfa but not clover. *Rhizobium* biovar-specific and legume species-specific signal molecules determine host-symbiont recognition and regulate differentiation processes in both the host (e.g., nodule formation) and symbiont (e.g., bacteroid formation).

The signal molecules of most *Rhizobium* biovars have been identified. Species-specific flavones or isoflavones secreted by the host legume activate nodulation genes (*nod* genes) in symbiont *Rhizobium. Rhizobium nod* genes consist of those that are common to all species of rhizobia, the *nodABCD* genes, and those that determine host specificity and are, therefore, unique to a particular *Rhizobium* biovar or species. Many of both the common and host-specific *nod* genes are now known to encode enzymes (or regulatory proteins) required for the synthesis of host-specific lipo-oligosaccharide signal molecules.

The lip-oligosaccharides that regulate nodule organogenesis are called Nodulation (Nod) factors. In the absence of *Rhizobium,* they induce nodule formation on the host legume root (Lerouge et al 1990, Schultze et al 1992, Spaink et al 1991).

Progress in this new area of biology is partly due to the development of sophisticated analytical techniques for purifying and determining the structures of complex carbohydrates and the collaborative research of physiologists, biochemists, molecular biologists, and organic chemists. The results of this interdisciplinary research are prompting plant scientists to re-evaluate their concepts of development, defense mechanisms, and functions of cell walls. These studies may also lead to biotechnology-based environmentally friendly approaches to improve resistance to microbial and insect pests and to control the growth and development of plants.

Future research

With the exception of the genetic studies of Nod factors, most of the data on the biological activities of oligosaccharins have been obtained in bioassays. Studies with intact plants are needed, perhaps using transformed plants containing genes encoding enzymes, receptors or other proteins that alter the in situ activity of oligosaccharins. Oligosaccharin activation should be elucidated through studies on the enzymes that release and process oligosaccharins, on oligosaccharin receptors, and on the effects oligosaccharins have on membranes and membrane-associated proteins. This will lead to a better understanding of the signal pathways that transduce the effect of the regulatory molecules.

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The lipo-oligosaccharidic nodulation signals of *Rhizobium* meliloti

G. Truchet, J.C. Promé, and J. Dénarié

Rhizobium, Bradyrhizobium, and *Azorhizobium* are quite distant phylogenetically. These distant genera are grouped in the same family, the Rhizobiaceae, because of their unique ability to establish a N_2-fixing symbiosis with plants of the family Leguminosae. These symbiotic associations are specific and a given rhizobial strain nodulates a limited number of leguminous hosts (Dénarié and Roche 1991, Nap and Bisseling 1990). For example, *Rhizobium meliloti* nodulates alfalfa, *Rhizobium leguminosarum* nodulates pea and vetch. Nodule induction is a complex process determined by sets of bacterial and plant genes. The following steps are usually involved: recognition between bacteria and host plant, root hair curling, infection thread formation within root hairs and the root cortex, induction of cortical cell divisions, and nodule organogenesis (Nap and Bisseling 1990, Long 1989).

The plant genetic program for nodulation

Mode of infection

The mode of infection varies and includes simple entry through cracks in the epidermis (e.g., peanut) or formation of walled infection threads that penetrate root hairs (e.g., alfalfa, clover, pea, and vetch). The mode of infection is characteristic of the host since the same rhizobial strain can penetrate different host species by different methods, but a given species is infected by the same type of process whatever the strain (Dénarié and Roche 1991).

Type of nodulation

The nodules are distinct organs, not merely tumors or deformed roots. Nodules of a given plant species have a characteristic ontogeny, anatomy, morphology, and type of development, either determinate or indeterminate. The structural characteristics of N_2-fixing nodules are specified by the host plant and not by the bacterial strain, indicating that the plant possesses the genetic information for symbiotic infection and nodulation (Dénarié and Roche 1991). The hypothesis that the plant possesses the whole genetic information for nodule information is further supported by the observation that alfalfa

can form nodules of normal ontogeny in the absence of rhizobia (Truchet et al 1989). Thus the role of the bacteria seems to be to turn on the plant genetic program.

Triggering the plant nodulation program

R. meliloti nodulation (*nod*) genes

The bacterial genes involved in this process are the nodulation (*nod*) genes (Long 1989). Regulatory NodD proteins control the expression of *nod* genes in the presence of phenolic compounds exuded by the host plants (Long 1989).

The *nodABC* genes are known as common *nod* genes. They are structurally conserved in all *Rhizobium, Bradyrhizobium,* and *Azorhizobium* species studied so far (Dénarié and Roche 1991, Martinez et al 1990). The *nodABC* genes are essential for infection and nodulation. A mutation in these genes causes a complete loss of the ability to elicit any detectable plant responses whatever the host, the type of infection, and the type or location of nodules normally produced (Dénarié and Roche 1991, Long 1989, Nap and Bisseling 1990).

Species-specific *nod* genes, such as *nodFEG, nodH,* and *nodPQ* in *R. meliloti,* are involved in defining the rhizobia1 host range (Dénarié and Roche 1991). Bacterial strains carrying mutations in these genes displayed altered infection and nodulation functions, including changes in the host range. For example, *nodH* mutants lost the ability to infect and nodulate alfalfa, but gained the ability to infect and nodulate vetch, which is not normally a host (Debellé et al 1986, Debellé and Sharma 1986, Horvath et al 1986). *nodQ* mutants have an extended host range and nodulate both alfalfa and vetch (Cervantes et al 1989).

Production of extraceltular Nod factors

The sterile supernatants of *R. leguminosarum* cultures, grown in conditions of *nod* gene expression, elicit root hair deformations (Had) and root thickening and shortening (Tsr) in vetch (van Brussel et al 1986, Zaat et al 1987). *R. meliloti* sterile filtrates elicit hair deformation on alfalfa (Faucher et al 1988). Inactivation of the common *nodABC* genes suppresses these biological activities. Thus the nod genes are involved in the production of extracellular Nod factors. These *Nod* factors are specific since the filtrates of *R. leguminosarum* are active on vetch and not on alfalfa, whereas those from *R. meliloti* are active on alfalfa and not on vetch (Faucher et al 1988).

Nature of *R. meliloti* Nod factors

Extracellular factors are produced in very low amounts in *R. meliloti.* To obtain the quantities required for structural analysis, we constructed overproducing strains by manipulating the regulatory circuits of *nod* gene expression. Increasing the copy number of the *nod* gene region or of the transcriptional activators *syrM* and *nodD3* resulted in a thousandfold increase in Nod factor production (Lerouge et al 1990, Roche et al 1991a). Using the alfalfa hair deformation bioassay, Nod factors were purified by a series of preparative and analytical reverse phase HPLC, ion exchange, and gel permeation chromatography (Lerouge et al 1990, Roche et al 1991 b). The structure of

the compounds was determined by a combination of NMR spectroscopy, mass spectrometry, and various chemical modifications.

The major Nod factors from *R. meliloti* are ß, l-4-linked tetramers or pentamers of D-glucosamine, N-acylated on the terminal nonreducing residue and N-acetylated on the other residues. In other words, Nod factors are mono-N-acylated chitin oligomers (Lerouge et al 1990, Roche et a1 1991a,b). The molecules are O-sulfated on the carbon 6 of the reducing aminosugar and may be O-acetylated on the carbon 6 of the terminal nonreducing end (Lerouge et a1 1990, Roche et a1 1991a,b). The major N-acyl group is a C16 chain with two double bonds in positions 2 and 9 (Lerouge et al 1990). Nod factors of similar general structure have recently been found in *R. leguminosarum.* They are also mono-N-acylated chitin oligomers but differ in the substituents linked to the chitin oligomer backbone. They are not sulfated and the N-acyl group is different (Spaink et al 1991).

Biological activity of Nod factors

Lipo-oligosaccharides of rhizobia1 origin elicit plant responses at different steps of the symbiotic process, including genuine plant organogenesis.

Purified Nod factors from *R. meliloti* elicit root hair deformations on lucerne at 10^{-10}–10^{-12}M (Lerouge et al 1990, Roche et al 1991a). They also elicit cortical cell divisions and the formation of genuine root nodules on lucerne at concentrations down to 10^{-9} M (Truchet et a1 1991). Nod factors at concentrations as low as 10^{-13} M have also been shown to induce transcription of the early nodulin genes, *enod5* and *enod12,* plant genes involved in the early steps of nodulation (Nap and Bisseling 1990, Scheres et al 1990). Nod factors are thus active at lower concentrations than plant hormones.

Chemical and genetic modifications of Nod factors were performed to study structure-function relationships. Removal of the O-acetate group resulted in a slight decrease of nodule formation ability (Roche et al 1991a, Truchet et al 1991). A strong decrease in the ability to induce nodules resulted from the following modifications: reduction of the anomeric carbon of the reducing sugar, removal of the sulfate group, and hydrogenation of the double bonds of the N-acyl chain (Truchet et al 1991). These results show that the anomeric carbon, the sulfate group, and at least one of the double bonds of the fatty acid chain are essential for organogenetic activity on lucerne.

Sulfation of Nod factors

The role of individual *nod* genes in the synthesis of Nod factors is subject to much attention. It has been proposed that the common *nodABC* genes determine the synthesis of Nod factor precursors, and the host-specific *nod* genes mediate the modification of the precursors to generate plant-specific signals (Faucher et a1 1988,1989). The *nodH* and *nodPQ* genes are the major host range genes of *R. meliloti* and have been shown to control the specificity of the Nod factors (Faucher et a1 1988, 1989). *nodH* mutants produce Nod factor molecules identical to those produced by the wild type, except that they are not sulfated (Roche et al 1991a). *nodP* and *nodQ* mutants produce a mixture of sulfated and nonsulfated factors. Partial sulfation of the lipo-oligosaccharides is probably due to the presence of a functional reiteration of the *nodPQ* genes (Schwedock and Long 1989, 1990). Strains which carry mutations in the two *nodPQ* copies do not

produce sulfated factors (Roche et al 1991a), indicating that both *nodH* and *nodPQ* genes are involved in the sulfation of the Nod factors.

The *nodP* and *nodQ* genes are homologous to *Escherichia coli cysD, cysN,* and *cysC* genes and encode ATP sulfurylase and APS kinase (J. Schwedock and S. Long, 1990, Stanford University, USA, pers. commun). They are responsible for the production of an activated form of sulfate, 3'-phosphoadenosine 5'-phosphosulfate (PAPS). The *nodH* product, homologous to sulfotransferases, probably transfers sulfate from PAPS to Nod lipo-oligosaccharide precursors (Roche et al 1991a).

Purified sulfated factors from *R. meliloti* elicit root hair deformation, cortical cell division, and nodule formation on lucerne but are not active on vetch, a nonhomologous host of *R. meliloti* (Roche et al 1991a). In contrast, nonsulfated compounds produced by *nodH* and *nodPQ* mutants elicit root hair deformation and root shortening on vetch and are not active on lucerne. There is thus a correlation between the symbiotic specificity of the various *R. meliloti* bacterial strains and the specificity of purified Nod factor activity (Roche et al 1991a). These results allow us to propose that *Rhizobium* determines host specificity, infection, and nodulation through the production of lipo-oligosaccharidic Nod factors.

Conclusions and perspectives

The legume hosts possess the genetic program for infection and nodulation. The rhizobia1 partner triggers this program by various signals. The major function of *nod* genes is to determine the production of N-acylated chitin oligomers. The common *nodABC* genes mediate the synthesis of core molecules and the specific *nod* genes specify the modification of these precursors.

Future work will analyze the role of individual *R. meliloti nod* genes in the synthesis and transport of Nod factors by (i) determining the structure of the factors which accumulate in each *nod* mutant, (ii) exploiting the homologies of the putative *nod* gene products with known proteins, and (iii) physiological and biochemical studies.

We will collaborate with several laboratories to study Nod factors from various rhizobia: broad host range *Rhizobium* sp. NGR234 (W. Broughton, University of Geneva), *R. tropici* (R. Palacios, University of Mexico), *R. fredii* (S. Pueppke, University of Missouri), and *Azorhizobium caulinodans* (M. van Montagu, University of Ghent).

The availability of signal molecules that elicit genuine organogenesis in plant hosts, and which can be modified at will by genetic or chemical means, will allow the study of how signals are perceived and transduced, and how they elicit a major developmental switch in the plant.

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Role of exopolysaccharides in nodulation

G.C. Walker

The succinoglycan exopolysaccharide of *R. meliloti* nodule invasion

Our laboratory has obtained strong genetic evidence that succinoglycan (EPS I), the acidic Calcofluor-binding exopolysaccharide of *Rhizobium meliloti* strain Rm 1021, is required for nodule invasion and possibly for later events in nodule development (Leigh et al 1985, Finan et a1 1985).

EPS I is a high molecular weight polymer composed of polymerized octasaccharide subunits. Each octasaccharide consists of a backbone of three glucoses and one galactose, a side chain of four glucoses, and 1-carboxyethylidene (pyruvate), acetyl, and succinyl modifications in a ratio of approximately 1:1:1 (Aman et al 1981).

We isolated a set of mutants, *exo,* of *R. meliloti* Rm 1021 on the basis of their failure to fluoresce under UV light on medium containing Calcofluor, and showed that these mutants did not synthesize EPS I (Leigh et al 1985, Long et al 1988b, Reuber et al 1991).

Alfalfa seedlings inoculated with these *exo* mutants formed ineffective (non-N_2-fixing) nodules that contained few, if any, bacteria and no bacteroids. Root hair curling was significantly delayed and infection threads formed but aborted at a very early stage, so the bacteria were never able to reach the interior of the nodule. Furthermore, these *exo* mutants could only elicit the synthesis of 2 of the 17 nodulins elicited by infection with a wild type *R. meliloti.*

Genetic analyses of the synthesis of EPS I by *R. meliloti*

A cluster of *exo* genes are located on the second symbiotic megaplasmid. Mutations in *exoA, exoB, exoF, exoM, exoL, exoP, exoQ,* and *exoT* completely abolish production of EPS I and result in mutants that form Fix- nodules (Leigh et al 1985, Finan et al 1985, Long et al 1988b, Reuber et al 1991). Analyses of the properties of translational fusions of various *exo* genes to alkaline phosphatase, which were generated using Tn*phoA,* suggest that the *exoF, exoP, exoQ,* and *exoA* gene products are membrane proteins.

To determine the roles of the *exo* gene products in the synthesis of EPS I, we have initiated in vitro studies of the synthesis of EPS I by these various mutants (T.L. Reuber and G.C. Walker, unpublished).

exoH mutants were originally identified on the basis of the failure of colonies of such mutants to form a fluorescent halo under UV light when grown on medium containing Calcofluor. Alfalfa seedlings inoculated with *exoH* mutants also form ineffective nodules that do not contain intracellular bacteria or bacteroids. Analysis of the exopolysaccharide secreted by *exoH* mutants has shown that it is identical to the Calcofluor-binding exopolysaccharide secreted by the parental $exoH^+$ strain except that it completely lacks the succinyl modification (Leigh et al 1987).

Mutations in loci originally described as *exoG, exoJ,* and *exoN* diminish the production of Calcofluor-binding material. *exoG* and *exoJ* mutants form effective nodules with decreased efficiency, whereas plants inoculated with exoN mutants fix N normally. The *exoG* and *exoJ* mutants are of particular interest because they do not produce a detectable high molecular weight exopolysaccharide, but do produce a low molecular weight Calcofluor-binding material that seems to be sufficient for the formation of some N_2-fixing nodules.

The DNA sequence of the *exoG/exoJ* region contains two divergently transcribed open reading frames, called *exoX* and *exoY,* that have homologs in other Rhizobium species. Null mutations of *exoX* cause an increase in exopolysaccharide production. However, the *exoJ*319 mutation falls in the 3'-portion of the *exoX* open reading frame, and it is probably an allele of *exoX* that results in altered function. Gene regulation studies suggest that *exoX* and *exoY* comprise a system that modulates exopolysaccharide synthesis at a post-translational level. We also found that the deduced amino acid sequence of exoY shares homology with the deduced amino acid sequence of GumD, a protein required for an early step in xanthan gum biosynthesis (Long et al 1988b, Reed et al 1991).

Regulation of EPS I synthesis during nodulation

Nodules induced by a strain carrying the *exoF*369::Tn*phoA* fusion and a plasmid which complements the *exoF* mutation allowing normal nodulation, showed staining for alkaline phosphatase activity primarily in the early symbiotic or invasion zone of the nodule, where the bacteria were invading the plant cells. In the last symbiotic zone, which contained mature bacteroids, no staining was seen. These results suggest that little or no new EPS I synthesis is needed after nodule invasion (Reuber et a1 1991).

Cells carrying either the *exoR95::*TnS or *exoS95*::Tn*5* mutations greatly overproduce EPS I in the free-living state. *exoS96*::Tn*5* mutants formed Fix^+ nodules on alfalfa. In contrast, we found that *exoR95*::TnS mutants formed both empty Fix^- nodules and also Fix^+ nodules on alfalfa that contained widely varying numbers of bacteria and bacteroids. All the bacteria isolated from the Fix+ nodules induced by the *exoR95*::Tn*5* strain had acquired unlinked suppressors that reduced the amount of exopolysaccharide produced, suggesting that the bacteria need to control either how much EPS I they synthesize, or when they synthesize it, in order to invade nodules (Doherty et a1 1988).

Production of an EPS I substitute by *R. meliloti*

The symbiotic defects of *exo* mutants can be suppresssed by the presence of a mutation, *expR101,* which causes overproduction of a second exopolysaccharide, EPS II (Glazebrook and Walker 1989).

Genetic analyses have shown that the products of a cluster of at least six *exp* genes located on the second symbiotic megaplasmid and the product of the *exoB* gene are required for EPS II synthesis. The presence of the *expR101* mutation causes overproduction of EPS II by increasing transcription of the *exp* genes.

Strains which produce EPS II but not EPS I can form Fix^+ nodules on alfalfa, indicating that EPS II is able to substitute for EPS I in the nodulation of alfalfa by *R. meliloti* (Glazebrook and Walker 1989, Zahn et al 1989).

The structure of EPS II has been determined and consists of a polymer of glucose-ß(1-3)-galactose disaccharides joined by a (1-3) linkages. The glucose carries an 0-6-acetyl modification and the galactose a 4-6 (1-carboxyethylidene) linkage (Glazebrook and Walker 1989, Her et al 1990).

Both EPS I and EPS II are acidic, contain glucose and galactose, and have acetyl and pyruvate (1 -carboxyethylidene) modifications, but the structures differ in many respects. However, it is interesting to note that each exopolysaccharide has a single glucose-ß(1-3)-galactose linkage in its backbone, and that these two rather diverse exopolysaccharides may share the common structural motif of 0-6-acetylglucose-ß(1-3)-galactose.

Evidence for the involvement of a low molecular weight form of the exopolysaccharide

exoG mutants make low molecular weight EPS I but not high molecular weight EPS I. They can still form effective nodules, at reduced efficiency relative to wild type but much better than mutants which make no exopolysaccharide. It is possible, therefore, that both the high and low molecular weight forms of EPS have symbiotic functions (Long et al 1988b, Reed et al 1991).

Both Battisti et al (1992) and Urzainqui and Walker (1992) have obtained preliminary evidence that a low molecular weight fraction of EPS I can partially suppress the symbiotic deficiencies of *R. meliloti exo* mutants.

A similar finding has been reported by Djordjevic et al (1987), who found that *exo* mutants of *Rhizobium* SP. NGR234 and *R. trifolii* form effective nodules if either high molecular weight EPS or oligosaccharide subunits of EPS are supplied exogenously. However, there appears to be some difference between the *R. meliloti*-alfalfa system and systems studied by Djordjevic et al (1987), since no group has been able to suppress the symbiotic deficiencies of *R. meliloti exo* mutants by the addition of purified high molecular weight exopolysaccharide isolated from exo^+ parent Rm 1021.

Researchable areas and prospects for success

Biosynthesis of EPS I, regulation of synthesis of EPS I, and mechanism by which the low molecular weight derivative of the exopolysacccharide is produced

- What is the biosynthetic pathway for EPS I and what is the role of each *exo* gene in this process?

- How is the synthesis of EPS I regulated?
- Is the low molecular weight form produced by direct synthesis or by degradation of the higher molecular weight form?
- Is the appearance of the low molecular weight form subject to spatial or temporal regulation during nodule invasion?
- What is the best strategy for obtaining substantial amounts of the low molecular weight form of the exopolysaccharide for subsequent investigations?

An in vitro system is being used to assign functions to the various *exo* gene products. To detect sufficient incorporation of labeled sugars using the in vitro system, we have found it necessary to construct triple mutants containing the *exoR95*::Tn5 mutation, an *exoB* mutation, and the *exo* mutation of interest. It should be possible to assign glucosyl transferase functions using this system. Others, such as export functions, will be more difficult to assign. We have sequenced much of the *exo* region and this should help us in our attempts to assign functions to various genes.

The regulation of synthesis of EPS I is clearly complex. There may be several factors that affect the expression of the exopolysaccharide in the free-living state, as well as factors that affect the synthesis of the exopolysaccharide during the invasion process. It should be possible to analyze the transcriptional regulation of the *exo* genes by standard genetic and molecular biological approaches. Analysis of the post-translational mechanism of regulation may prove to be more difficult.

The mechanism by which the active low molecular weight form of the exopolysaccharide is generated is important but has not yet been established. We are currently characterizing mutants that are candidates for having deficiencies in exopolysaccharide degradation.

Identification of the mechanism(s) by which exopolysaccharides or their derivatives influence nodule invasion and possibly nodule development in the *R. meliloti*-alfalfa symbiosis

- Is there a role for the high molecular weight form of the exopolysaccharide as well as the low molecular weight form and, if so, are these two roles related?
- Is there a receptor in the plant membrane that recognizes some component of the exopolysaccharide or a derivative and, if so, is there a subsequent signal transduction pathway? If there is such a signal transduction pathway, what plant process or processes are ultimately affected? If a receptor in the plant membrane is not involved, how does the exopolysaccharide or low molecular weight derivative influence nodule invasion?
- What are the key structural requirements for exopolysaccharides and their derivatives to exert their effects on symbiosis?

We anticipate exploring various hypotheses concerning the possible role of exopolysaccharides in nodulation on a case-to-case basis. One particularly high priority will be to explore the hypothesis that the exopolysaccharide, or a derivative, is required to help the bacteria evade or suppress plant defense responses.

We anticipate attempting to clone the putative plant-encoded receptor for the exopolysaccharide or low molecular weight derivative.

Identification of other bacterial functions besides exopolysaccharides that are required for nodule invasion and possibly nodule development

■ In addition to investigating the roles of exopolysaccharides in nodule invasion, we are also investigating the roles of the *exoD* (Reed and Walker 1991), *bacA* (Long et al 1988a), and *trpE* (Barsomian et al 1992) gene products in nodule invasion and development.

We plan to continue using genetic approaches to identify additional mutants that have defects in nodule invasion and possibly nodule development, and to attempt to elucidate the basis of their symbiotic deficiencies. In particular we have made some progress in our characterizations of *exoD* (Reed and Walker 1991), *bacA* (Long et al 1988a), and *trpE* (Barsomian et al 1992) genes and will continue this work.

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Bacterial entry into roots

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Currently recognized modes of rhizobia1 entry into host roots and dissemination through the nodule are:

I. Penetration of root hairs and dissemination by infection threads (e.g., clover, alfalfa, pea, soybean) (Bakhuizen et al 1988; Callaham and Torrey 1981; Dazzo and Hubbell 1982; Dazzo et al 1984; Fahraeus 1957; Higashi et al 1986; Napoli and Hubbell 1975; Newcomb 1981; Nutman 1958; Turgeon and Bauer 1985; Vasse and Truchet 1984; Ward 1887)

II. Root hairs made but not infected

A. Wound entry at lateral root emergence

1. Dissemination by division within infected nodule cells, no infection thread (e.g., peanut) (Chandler 1978)

2. Dissemination by intercellular growth and infection threads (e.g., *Aeschynornene, Stylosanthes*) (Chandler et al 1982, Napoli et al 1975, Date and Roughley 1982)

B. Entry between epidermal cells, dissemination by infection threads (e.g., *Parasponia*) (Bender et a1 1987; de Faria et a1 1988; Trinick 1979; Lancelle and Torrey 1984,1985; Bender et a1 1987)

III. No root hairs made, entry between epidermal cells and wounds at lateral root emergence, dissemination by intercellular growth and then true infection threads (e.g., *Neptunia*) (Subba-Rao et al 1992)

Related morphological studies indicate that *Azospirillum* enters grass roots at lateral root emergence and at epithelial desquamation, with intercellular infection only (Umali-Garcia et al 1980, 1981).

The major "bottleneck"is the rarity of infection, and considerable effort is needed to derive quantitative data. The difficulty of the search increases with the thickness of the root and the root hair density. The methodologies to produce and interpret the results require considerable skill to avoid misinterpretations of infection threads and nodular histology, and are considered both a science and an art.

The types of microscopy used for this work include brightfield, phase contrast, fluorescence, and laser scanning confocal microscopy of stained whole roots, scanning electron microscopy of root segments, and combined brightfield and transmission

electron microscopy of embedded and sectioned root tissue. Immunofluorescence microscopy using strain-specific antibodies can be included to assist in evaluating the identity of the bacterial symbiont within the host tissue. Computer-aided image analysis is used for quantitative morphometric measurements (Dazzo and Petersen 1989).

An important area is to document the cellular events which lead to nodulelike structures on rice inoculated with rhizobia and incubated under microbiologically controlled conditions. The major points to address are the modes of entry of rhizobia into the root, initiation of the prenodule cortical cell divisions, and invasion of the nodular cells. In relation to development of a potential N_2-fixing organ, the intracellular status of the microsymbiont (endosymbiotic "symbiosome" vs free vegetative cells) should be documented. The host cell cytoplasm must be well-preserved for these studies to distinguish whether the rhizobia actively infect living host cells or enter already lysed dead host cells. Prospects for success on this proposed project are high with adequate funding. Collaborations with other investigators who wish to evaluate the morphological features of this plant-microbe interaction should be made.

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Nonlegume N_2-fixing associations

Symbiosis with *Frankia*

B.C. Mullin

Current knowledge of actinorhizal symbiosis

Host plants

Members of the actinomycete genus *Frankia* are known to infect some 165 plant species distributed among eight orders of plants (Bond 1983). These eight orders are widely distributed on the phylogenetic tree of dicotyledonous angiosperms. Host plants are mostly woody shrubs or trees. Most are temperate plants able to survive on marginal soils. No known characteristics, other than the ability to nodulate, distinguish host plants from non-nodulating close relatives.

Currently, nothing is known about factors that regulate host range among actinorhizal plants. It is not known, for example, whether *Frankia* does not recognize non-nodulating close relatives, or whether these plants are recognized but are unable to respond. Because of the phylogenetic diversity of host plants, it is unlikely that the genes involved in nodulation and N_2 fixation are totally unique to N_2-fixing plants. It is much more likely that these genes have evolved from common genes found in most plants and have simply been recruited for the highly specialized functions involved in symbiosis.

The microsymbiont

The actinorhizal endophyte *Frankia* appears to be a typical actinomycete in both morphology and molecular biology. It has an average actinomycete genome size and base composition (An et al 1985). Only sporangial morphology and the ability to produce vesicles distinguish it morphologically from other actinomycetes. Frankiae exist worldwide but are not readily cultured from the soil. Their presence is, however, easily demonstrated by baiting soils with host plants (van Dijk 1979). Despite the similar morphology exhibited by all known *Frankia* strains, we have shown a tremendous genetic diversity among the strains by DNA homology testing (An et al 1985). Isolates have been divided into affinity groups on the basis of DNA homology (An et al 1985, Fernandez et al 1989), host range (Baker 1987), gel protein patterns (Benson and Hanna 1983), and physiology (Lechevalier 1984), but as yet, no basis has been identified for the observed differences in host specificity.

The earliest attempts to determine the phylogenetic position of *Frankia* among the actinomycetes based on morphological and chemical analyses supported its alignment with the genus *Dermatophilus,* an obligate animal pathogen unable to survive in soil (Lechevalier and Lechevalier 1979). The most rudimentary molecular analysis, determination of the G+C content of *Frankia* DNA, showed that this relationship was not representative of the true phylogeny of the organisms. The G+C content of *Frankia* strains was between 10 and 15% higher than the G+C content of *Dermatophilus* (An et al 1983). Although DNA solution hybridization of *Frankia* DNA to that of a wide range of actinomycetes did not reveal any close relatives (An et al 1987), the more sensitive analyses of rRNA by oligonucleotide cataloging and sequencing identified *Geodermatophilus* as a distant but nearest relative (Fox and Stackabrandt 1987, Hahn et al 1989b). *Geodermatophilus,* a soil actinomycete, was intuitively a much better candidate for a nearest relative than the obligate animal pathogen *Dermatophilus.*

With the genus *Frankia* firmly placed among the actinomycetes, the question arises as to the origin of its N_2-fixing and symbiotic genes. Nearest relatives are not known to form any kind of association with plants, nor are they known to fix N_2. In fact few other actinomycetes, if any, have been observed to fix N_2. DNA sequence analysis of the structural genes for the enzyme nitrogenase has provided some insight into the origin of these genes in *Frankia.* We have sequenced *nifD* and *nifK,* the structural genes for the a and ß subunits of the MoFe protein of the nitrogenase complex, and found them to have relatively higher sequence similarity to *nifD* and *nifK* from *Anabaena* than to other N_2-fixing bacteria (Twigg et al 1990 and An and Mullin, unpubl.). *nifH* from two *Frankia* strains also has relatively higher sequence similarity to *nifH* from *Anabaena* sp. (Normand and Bousquet 1989). In both cases, the corresponding sequences from *Clostridium* share much less similarity with *Frankia,* despite the fact that these two genera are more closely related according to rRNA sequence data.

Further analysis of the organization and sequence of genes which function in N_2 fixation will strengthen or repudiate the speculation of Simonet et al (1990) that Frankia may have obtained its genes for N_2 fixation via horizontal gene transfer.

Root infection and nodule formation

Frankia infects host plant roots by either root hair infection (Berry et a1 1986) or intercellular penetration of the root epidermis (Miller and Baker 1986). Root hair infection is by far the most common mechanism. Root hairs become grossly deformed in the presence of frankiae and deformed hairs are the site of root hair infection.

In an electron micrographic study of root hair infection in *Alnus,* Berry et al (1986) were able to follow a *Frankia* filament from the outside to the inside of the root hair cell wall. As the microsymbiont filament grew into the cell, it was surrounded by the host cell plasma lemma and a transfer cell wall-like ingrowth. The infected cell appeared to be very active metabolically, with numerous mitochondria, ribosomes, Golgi bodies, and an extensive endoplasmic reticulum.

Other studies have shown that cortical cells beneath the infected root hair are stimulated to divide, forming a swelling on the root. Called the prenodule, this swelling is only a transitory structure and is followed by the development of modified lateral roots which become the root nodule lobes (Callaham and Torrey 1977).

In plants that are infected via an intercellular route, *Frankia* first penetrates the root cuticle and then proceeds to grow within the middle lamellar layers between the epidermal cells (Miller and Baker 1986). As the microsymbiont penetrates the intercellular spaces of the cortex, a nodule primordium is induced in the pericycle. Upon reaching the nodule cortex just behind the nodule meristem, the filaments penetrate the cell walls and become the intracellular microsymbiont.

Actinorhizal nodule physiology

Within the actinorhizal nodule, frankiae elaborate structures called vesicles, which appear to be modified filament tips. The appearance of vesicles corresponds to the onset of nitrogenase activity both *in planta* and in culture, and it is thought that N_2 fixation occurs within the vesicles (Fontaine et al 1984). In some nodules, hemoglobins are found (Tjepkema 1983) and their role may parallel that of the leghemoglobins of legume nodules. Reduced N in the form of NH_3 may be excreted by the microsymbiont and assimilated into glutamate by host plant enzymes. Indeed, elevated levels of host plant glutamine synthetase are found in *Alnus* nodules (Hirel et al 1982) and we have isolated a cDNA which codes for GS from a root-subtracted nodule cDNA library (Twigg and Mullin, unpubl.). However, *Frankia* in culture has not been found to release NH_3.

Whatever the form in which reduced N is supplied to the nodule cells, it is known that in *Alnus,* N is exported from the nodule as citrulline (Schubert and Coker 1981). In exchange for this reduced N, the host plant supplies photosynthate to the microsymbiont. The magnitude of this investment has been estimated by Tjepkema to be close to 12% of the gross photosynthate (Tjepkema et al 1986), although the form in which this photosynthate is used by frankiae is not known. Within nodule tissue, some cells remain uninfected. These cells, however, are altered by the presence of frankiae in neighboring cells, and contain large amyloplasts and vacuoles with phenolic deposits.

We are in the midst of the characterization of a large number of nodule-specific or enhanced cDNA clones from the actinorhizal host plant *Alnus.* In spite of the similarity of actinorhizal nodules to lateral roots, a large number of genes are differentially expressed in the nodule tissue. In addition to studying differential gene expression in host plants in response to *Frankia,* we are interested in the status of these genes in nonhost plants and the extent to which nonhost plants are able to recognize and respond to *Frankia.*

Betula, a nonactinorhizal genus placed in the same family with actinorhizal *Alnus* species, has been shown by restriction fragment analysis of rDNA to be very closely related to *Alnus.* In fact, cluster analysis using the unweighted pair group method using arithmetic averages (Sneath and Sokal 1973), clusters *Betula* with two *Alnus* species in a group distinct from the remaining five *Alnus* species tested (Bousquet et a1 1989). Other studies have shown that in some locations, soil under nonhost *Betula* induces nodulation in actinorhizal *Alnus incana* to a greater extent than soil under nearby *Alnus* (Smolander 1990). *Betula* has been proposed as a target for the genetic engineering of new N_2-fixing associations through gene transfer (Bousquet et al 1989), but to date only marker genes have been inserted into the *Betula* genome (Mackay et al 1988, Seguin and Lalonde 1990). Identification and study of the expression of genes found to be

important in the *Alnus* symbiosis will target sequences to be used in transformation experiments aimed at establishing new symbiotic relationships.

Despite the extreme sensitivity of *Frankia* nitrogenase proteins to molecular O_2 (Murry et al 1984, Baker and Huss-Danell 1986), N_2 fixation by *Frankia* requires a high level of aerobic metabolism to generate the ATP and NADH necessary to reduce molecular N. Contrary to the case with legume nodules, which are O_2-limited, actinorhizal nodules show maximum rates of N_2 fixation at atmospheric levels of O_2 (Tjepkema et al 1980, Gauthier et al 1981). In fact, both free-living and symbiotic frankiae are able to fix N_2 at levels of O_2 ranging from 2 to 70 kPa, provided that they have been preadapted to assay levels of O_2 (Silvester et al 1988a, Parsons et al 1987). This indicates that some protective mechanism must be operating in free-living as well as in symbiotic frankiae. In cultured and symbiotic frankiae, stepwise exposure to higher than ambient levels of O_2 results in transient drops in nitrogenase activity followed by a recovery of activity (Si1vester and Winship 1990). It is proposed that at each stepwise increase in O_2, nitrogenase undergoes a conformational change which protects it from permanent damage. Increased respiratory rates lower O_2 to permissible levels and nitrogenase activity is resumed.

In free-living aerobic cultures of *Frankia,* N, fixation occurs largely, if not solely, within spherical vesicles which are produced in response to low levels of reduced N (Noridge and Benson 1986, Tisa and Ensign 1987, Meesters 1987). The thickness of the vesicle envelope is regulated by the partial pressure of O_2 (pO_2) in the culture medium, with wall thickness increasing with increasing levels of O_2 (Parsons et al 1987). The multilaminate vesicle envelope (Torrey and Callaham 1982) has a lipid composition (Lamont et al 1988) which differs from that of noninduced cultures, and has an especially high amount of C_{22}-C_{26} polyhydroxy fatty acids or alcohols (Tunlid et al 1989). Although the structure of these long chain compounds has not been determined, they resemble the C_{26}-C_{28} hydroxyl alcohols and fatty acids found in *Anabaena* heterocysts (Lambein and Wolk 1973). High levels of triterpene hopanoids have recently been found in *Frankia* vesicles and are likely to be involved in vesicle function (Berry 1991). The vesicle envelope is thought to provide a barrier to O_2 diffusion to protect the nitrogenase within. As O_2 levels in culture increase, new nitrogenase enzyme is synthesized, and thickening of the vesicle envelope occurs on preexisting as well as newly formed vesicles (Parsons et al 1987). In free-living cultures grown at very low O_2 levels, N_2 fixation occurs in the absence of vesicle formation (Murry et al 1985). This indicates that vesicle formation is not essential for N_2 fixation and is likely to be a protection mechanism that allows *Frankia* to fix N_2 over a very wide range of O_2 concentrations. This is in sharp contrast to rhizobia, which have no analogous intrinsic O_2 protection mechanism, and is similar to the O_2 protection mechanism of the heterocystous cyanobacteria. In addition to the diffusion barrier provided by the vesicle envelope, high rates of O_2 uptake and the presence of superoxide dismutase and catalase within vesicle cells may provide additional protection for nitrogenase (Steele and Stowers 1985).

Within nodule tissue, the extent of vesicle formation and the shape of vesicles are controlled by the host plant. In *Alnus* nodules, the vesicle shape is nearly spherical and the vesicle envelope thickness appears to increase with increasing levels of O_2

surrounding the nodule, with only slight changes occurring to overall nodule morphology (Silvester et al 1988a). In *Alnus,* there are no apparent host-mediated barriers to the diffusion of O_2 and infected cells are in contact with ambient levels of O_2 as a result of numerous interconnected intercellular spaces (Wheeler et al 1979, Tjepkema 1979). In this case, it is likely that the vesicle wall envelope provides the major barrier to O_2 diffusion.

The lack of frankiae vesicles in *Casuarina* nodules is not a characteristic of the microsymbiont, which does form vesicles in aerobic culture, but probably reflects the low O_2 levels within nodule tissue. Cytological analysis of *Casuarina* nodules indicates that infected host cell walls change in composition, becoming more hydrophobic and probably less permeable to O_2, upon penetration of the cells by *Frankia* (Berg and McDowell 1988). Furthermore, infected cells are not in direct contact with air passages that connect outer and inner cortical cell layers in the nodules (Zeng et al 1989), increasing the likelihood that infected cells are not at ambient O_2 levels. Studies of respiratory rates and nitrogenase activity in response to changes in temperature and pO_2 provide physiological evidence for the presence of a host-mediated barrier to O_2 in *Casuarina* (Tjepkema and Murry 1989). However, intracellular hyphae in *Casuarina* nodules do possess a laminate surface layer which may be chemically similar to the multilaminate envelope seed in vesicles of in vitro cultured frankiae (Berg and McDowell 1987). This layer may also be involved in the regulation of O_2 concentrations within symbiotic frankiae.

In *Myrica* species that are adapted to wetland habitats, vesicle envelope structure does not appear to change with changing pO_2 levels. Instead, nodule ventilation decreases with increasing levels of O_2 and few air spaces are found adjacent to infected cells. Nodule roots increase in length and in diameter with increasing O_2 levels, providing a mechanism to channel O_2 to nodule cells (Silvester et al 1988b):

In yet another kind of O_2 response, *Coriaria* nodules respond quickly to changes in pO_2 by some mechanism that causes a rapid (5-30 min) increase in resistance above ambient O_2 levels, and a rapid decrease in resistance below ambient O_2 levels (Silvester and Harris 1989). In these nodules, infected cells are surrounded by a dense suberized periderm, the thickness of which depends on the O_2 concentration at which the nodules were grown. The variable resistance barrier is thought to be a two-cell layer gap between the endodermis and the internal periderm, adjacent to the single nodule lenticel.

Hemoglobin has been found in the nodules of some actinorhizal plants but is undetectable in the nodules of others. It is present in the highest concentrations in the nodules of *Casuarina* and *Myrica* (Tjepkema and Asa 1987) and has been purified and characterized from *C. glauca* (Fleming et al 1987, Appleby et al 1988) and *M. gale* (Pathirana 1989). Hemoglobin from *C. glauca* has 52 and 43% amino acid sequence homology with hemoglobins from *Parasponia* and soybean, respectively, providing evidence that it is evolutionarily related to other global proteins. CO-reactive heme has been found in decreasing concentrations in nodules of *Alnus, Comptonia, Ceanothus, Hippophae, Elaeagnus, Coriaria,* and *Purshia,* but is not detectable in *Datisca* (Tjepkema and Asa 1987, Silvester et al 1990). Whether the CO-reactive heme found in these actinorhizal nodules is hemoglobin remains to be determined.

The presence of elevated levels of hemoglobin in *Casuarina* and *Myrica* can be correlated with nodule structure in these two species. In both cases, a barrier to O_2 diffusion surrounds infected nodule cells, resulting in a lower O_2 concentration in infected cells. At lower O_2 levels, hemoglobin may serve to facilitate the transport of O_2 to metabolically active frankiae. This role is consistent with the role hemoglobin is thought to play in legume nodules, where all legumes are found to have a barrier to O_2 diffusion surrounding infected cells. Actinorhizal species with lower levels of hemoglobin have well-ventilated nodules and the only diffusion barrier to O_2 appears to be the vesicle envelope of the microsymbiont.

Prospects for the establishment of new symbiotic relationships

Several factors point to *Frankia* as a good candidate for the establishment of new N_2-fixing relationships:

- Host plants are phylogenetically diverse, indicating that *Frankia* can adapt to a wide range of genetic backgrounds. The diverse structure of nodules is a reflection of this.
- Nodules form by modifying a currently existing structure in the root of host plants.
- *Frankia* have an intrinsic O_2 protection mechanism, the vesicle, and therefore need not rely on host plant tissue for the protection of nitrogenase.
- *Frankia* are not released into the cytoplasm of the host plant and thus may be less likely to elicit a pathogenic response in a "nonhost" plant.
- Strains are available which can infect via root hair or by intercellular penetration mechanisms.

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Exploring new soil bacteria

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Studies on plant-bacteria interactions, particularly symbiotic N_2 fixation, are still restricted to a rather limited number of bacteria.

It is unlikely that there are naturally occurring N_2-fixing bacteria beneficial for rice, thus we will have to engineer such strains. It would help to start with bacteria whose capacity to colonize plants, especially graminaceous plants, is well established.

The development of molecular biology techniques has greatly facilitated research on lesser known soil bacteria. Teams involved in symbiotic or associated N_2 fixation should explore the potential of some newly isolated species. We have initiated studies of some of these "exotic" bacteria. Here, we present our progress with three of them, the Gram-positive bacterium, *Rhodococcus fascians,* and two Gram-negative N_2 fixers, *Azorhizobium caulinodans* and *Azoarcus* sp.

Rhodococcus fascians

The phytopathogen *Rhodococcus fascians* is a Gram-positive bacterium (Goodfellow 1984) assigned to the nocardioform actinomycetes (LeChevalier 1986). The development of genetic tools such as transformation (Desomer et al 1990) and insertion mutagenesis methods (Desomer et al 1991) for *R. fascians* will contribute to the study of other Gram-positive plant-associated organisms with a high G+C content genome, such as species from the N_2-fixing genera *Arthrobacter* and *Frankia.*

The interaction between *R. fascians* and plants is also of interest. *R. fascians* causes fasciation (i.e., the loss of apical dominance and appearance of several malformed shoots per internode) upon infection of a wide range of dicotyledonous and some monocotyledonous plants (Tilford 1936, Lacey 1948, Vantomme et al 1982). The abundant shoot development has the appearance of a "leafy gall" in severely infected cases.

Infected young seedlings of tobacco and sunflower remain stunted and have a thickened hypocotyl, primarily due to enlarged parenchyma cells. Preliminary results indicate that similar symptoms can be obtained by infection of *Zea mays* seedlings (J. Desomer and M. Crespi, unpublished results).

Several reports have suggested that secretion of cytokinins by the bacteria causes tumor formation, as different cytokinins have been found in the supernatants of several *R. fascians* strains (Klämbt et al 1966, Scarbrough et a1 1973, Murai et al 1980). The genes for this cytokinin production were thought to be located on a 160-kilobases (kb) plasmid (Murai et al 1980, Murai 1981).

Recent analysis has shown that essential fasciation genes are located on a large conjugative linear plasmid (approximately 200 kb), not on a circular plasmid. We have called these fasciation-inducing or Fi plasmids (Crespi et al 1992). On one of these Fi plasmids, pFiD188, we have identified three loci involved in phytopathogenicity, using an insertion mutagenesis method based on illegitimate recombination of nonreplicating constructs into different sites of the *R. fascians* genome (Desomer et al 1991).

One of these loci vis) codes for an isopentenyl transferase gene *(ipt)* and is weakly homologous to *ipt* genes from *Agrobacterium* spp. and *Pseudomonas* spp. at the amino acid level. No significant difference was found in the cytokinin secretion profiles of the wild-type and *fas* mutant strains when they were grown under isolated, axenic conditions. The *ipt* gene is, however, specifically expressed during the interaction of *R. fascians* with plants. Only extracts from fasciated tissues could induce *ipt* gene expression. Uninfected plants (tobacco, sunflower) produced no response.

Fasciation symptoms were not elicited by avirulent bacteria containing only a DNA region of the linear plasmid spanning the *fas* locus. This indicates that other genes located on the Fi plasmid are involved in the abundant shoot formation by *R. fascians* infection. These genes could modulate plant response to the hormone by producing a biologically active molecule or by mediating transport of the hormone into plant cells. Alternatively, additional DNA sequences may be present on the Fi plasmid that allow transfer and stabilization of the Fi plasmid in plant cells where expression of the fasciation genes elicits the leafy galls.

The tumor-inducing features acquired by *R. fascians* suggest that this bacterium has evolved independently from the other hyperplasia-inducing bacteria to fill a specific ecological niche. Further studies on the plant-bacterium interaction will probably uncover novel insights into cytokinin action and shoot development in plants.

Azorhizobium caulinodans

Azorhizobium caulinodans (type strain ORS571) induces the formation of root and stem nodules on the tropical leguminous plant *Sesbania rostrata.* Stem nodules are formed at predetermined sites, namely dormant root primordia that occur all along the stem. Very abundant stem nodulation can be obtained by spraying the plant with a bacterial culture. *S. rostrata* is adapted to flooding and is very fast growing. These features, together with its abundant stem nodulation and high N_2 fixation levels (fixation in stem nodules is not inhibited by relatively high concentrations of fixed N_2 in the soil), have led to its successful application as a green manure in rice culture (Alazard et al 1988, Ladha et al 1990).

The bacterium in this interaction is taxonomically distinct from *Rhizobium* and *Bradyrhizobium* (Dreyfus et al 1988) but contains essential nodulation genes that are

related to the common *nodABC* genes of the abovementioned genera (Goethals et al 1989). Further sequence analysis downstream from the *nodABC* genes has revealed the presence of open reading frames with homology to *nodS* and *nodU* found in *Bradyrhizobium* and *Rhizobium* sp. NGR234, followed by *nodI* and *nodJ* related sequences (Gottfert et al 1990, Lewin et al 1990). All the genes belong to the same operon, the expression of which is activated in the presence of the host plant. The biologically active molecule in *S. rostrata* seedling exudate was found to be 7-4'-dihydroxyflavanone (liquiritigenin) (Messens et al 1991). Besides this locus containing essential *nod* genes, a second locus with a similarly regulated gene has been identified. Inactivation of this gene had no effect on the nodulation of *S. rostrata.* Both loci are under the control of a single regulatory NodD protein (Goethals et al 1990).

The *nodABC* sequences from ORS571 diverge considerably from those of *Rhizobium* and *Bradyrhizobium* strains. The same is true for the regulatory upstream sequences that govern the controlled expression of the *nod* operon (Goethals et al 1992). Upstream from the *nodA* gene, a *nod* box-related sequence functionally equivalent to the *Rhizobium meliloti nod* box promoter is present. It was shown to be responsible for the flavonoid-inducible expression. A consensus motif A-T-C-N_9-G-A-T has been observed by comparing the nod box sequences of (brady)rhizobia with the more divergent nod promoter of *Azorhizobium.* It is proposed as the binding target of NodD dimers. Further comparison with other LysR-type promoters revealed a conserved motif T-N_{11}-A that may be involved in binding LysR-type proteins.

Our present research on *Azorhizobium* aims at elucidating the role of nod genes in the biosynthesis of the Nod factor induced by host plant exudate. In view of reports on associative N_2 fixation by *Azorhizobium* in ricefields (Ladha et al 1989), it will be interesting to study this behavior and identify functions that contribute to the interactions between *Azorhizobium* and rice plants.

Azoarcus sp.

Kallar grass (*Leptochloa fusca* L. Kunth) is grown as a pioneer plant on saline-sodic, low-fertility soils in the Punjab region of Pakistan. For more than a decade, it gives harvests of 30-40 t of hay/ha per year without application of nitrogenous fertilizer. This makes it an interesting model system for N_2 fixation in association with grasses (Reinhold et al 1988).

To define the likely microbial partners, detailed studies on diazotrophic populations were carried out with field-grown plants. Nitrogen-fixing bacteria were found to be in close, root-zone-specific association with Kallar grass. *Azospirillum* spp. dominated on the root surface, whereas diazotrophic Gram-negative rods occurred in high numbers in the root interior (approximately 10^8/g root dry weight) (Reinhold et al 1986). To assess whether the latter could be found repeatedly in this habitat, another survey was carried out in the same field 4 yr later and the strains obtained were identified. According to DNA-rRNA hybridization studies, they all belonged to the 13-subgroup of the proteobacteria. All of the strains were located on a separate rRNA branch which was linked to the baseline of this rRNA family with a $T_{m(e)}$ of 67 °C. Thus,

they had no close relatives, and we propose they belong to a new genus, *"Azoarcus."* It consists of five different species according to DNA-DNA hybridizations and SDS-PAGE patterns of cellular proteins (Reinhold et al, unpubl.).

To follow infection of grass seedlings by *Azoarcus* in gnotobiotic culture, constitutively expressed ß-glucuronidase was introduced as a reporter gene by transposon mutagenesis. The following observations were made on Kallar grass and rice seedlings 5-7 d after inoculation: (i) not all roots were penetrated, (ii) penetration was commonly observed in the elongation zone but not in the more mature parts of the root, (iii) infection apparently terminates in the formation of large inter- and intracellular colonies, mainly in the cortex region, and (iv) bacteria were rarely found to penetrate even into the stele (Hurek et al 199l), the xylem vessels being a possible site for lateral transfer. Western blot analysis with genus-specific antibodies indicates a systemic infection of rice seedlings, including the stem base and shoots. Western blot analysis with antibodies against nitrogenase (iron protein) as well as acetylene reduction assays gave no indication of significant N_2 fixation at this stage. However, the gnotobiotic system developed can be used to study early plant-bacteria interactions.

Azoarcus was found to have special features with respect to N_2 fixation. As for many other diazotrophs, microaerobic conditions are required for N_2 fixation. The metabolism is strictly respiratory, depending on O_2 as the terminal electron acceptor. Under certain culture conditions, cells develop the capacity for very high respiration and N_2 fixation activity (T. Hurek and B. Reinhold, unpubl. data), which is accompanied by profound morphological changes. Tubular arrays of internal membrane stacks are developed, which can cover almost half of the intracellular area. This membrane formation can also be induced in co-culture with a fungus isolate (ascomycete) from Kallar grass roots. Immunogold electron microscopy indicated changes in the location of nitrogenase. The iron protein of nitrogenase, which is uniformly distributed in the cytoplasm of Azoarcus when fixing N_2, is mainly associated with the membrane stacks under these conditions. This feature has so far not been demonstrated for other diazotrophs. Currently, the genetic organization of the structural genes of nitrogenase is being studied. *nijHDK* genes have been cloned and sequencing is almost completed (T. Hurek and B. Reinhold, unpubl. data).

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Rhizobium nodulation of nonlegumes

B.G. Rolfe, K.M. Ride, and R.W. Ridge

Agricultural scientists have long wished to transfer the N_2-fixing ability of *Rhizobium* to other plant groups, especially monocotyledons. The introduction of a bacterial seed inoculant system for rice would be an ecologically sound and cheap supplement for N fertilizer. Over the last few years, a number of groups have been attempting to extend the *Rhizobium*-legume symbiosis to nonlegumes by inducing nodulelike structures. They have applied various strains and growth conditions with varying degrees of success.

The only nonlegume known to form nodules with either *Rhizobium* or *Bradyrhizobium* bacteria is the wild elm genus *Parasponia* (Trinick and Galbraith 1980), and is infected close behind the growing tip of the tap root. Bacteria colonize the root surface at this infection zone. Within 24 h of inoculation, they begin to erode the mucilage layer of the primary cell wall of epidermal cells. Four days after inoculation, the overlying epidermal layer ruptures and a mass of dividing cells emerges. This region provides a site through which bacteria enter the root and colonize the root cortex. No root hair infection occurs in the invasion of *Parasponia* plants. Infection requires the initiation of cell division, bacterial colonies then form infection threads within the root cortex and a lateral root is induced below the site of cell division (Bender et al 1987a,b). The final structure is a swollen lateral root with cortical cells filled with infection threads containing bacteria actively engaged in N_2 fixation on either side of the central vascular tissue.

It was observed that not all rhizobia survive well within the cells of *Parasponia* plants or readily colonize their root surface. Studies with various rhizobia and *Parasponia* infection showed different types of strains: a) rhizobia which poorly colonized the roots of *Parasponia* plants; b) rhizobia and reconstructed strains that induced poorly developed root nodules, and often had fibrillar material surrounding the infection threads within the infected plant cells; and c) strains which induced nodules that contained healthy-looking plant cells.

Conserved nodulation genes *nodABC,* needed for legume root hair curling, were found not to be essential for the initiation of infection—i.e., not required for the induction of cell division in *Rhizobium* and *Bradyrhizobium* strains that can infect either legumes or *Parasponia* plants. However, these genes were required for *Par-*

asponia 'prenodule' development, and the regulatory *nodD* gene of *Rhizobium* strains was essential for the initiation of infection. Successful infection also required a host-specific nodulation (*hsn*) region, which is not needed for the nodulation of legumes. *Agrobacterium tumefaciens,* carrying this *Parasponia*-specific region and legume nod genes, was able to form nodules to an advanced stage of development on *Parasponia* plants. However, these nodules were non-N_2-fixing and had fibrillar material associated with the infection threads in the infected plant cells.

Present status of *Rhizobium* inoculation of rice seedlings

Results from three laboratories in the UK, China (Jing et al 1990), and Australia indicate that a) some *Rhizobium* strains can infect and induce nodulelike structures on the roots of rice seedlings, b) there appears to be a large variation in sensitivity of different rice varieties to these *Rhizobium* strains, and c) the special properties of rhizobia make them the best bacterial group for attempting to induce functional N_2-fixing 'nodules' by infecting the roots of nonlegumes with bacteria.

Genetic analysis of the different *Rhizobium* and *Parasponia* strains and microscopic investigation of the *Parasponia* symbiosis have enabled the construction of a *Rhizobium* strain which can occasionally nodulate rice without added phytohormones. This bacterial construction has the properties required by *Rhizobium* strains for the successful invasion of *Parasponia,* namely the ability to induce erosion of the root surface layer of cells, the ability to induce cell division in the subepidermal layer of root cells, and an allelic form of the regulatory gene (*nodD*) which can cause induction of the normally unexpressed *Rhizobium* nodulation genes by interacting with signal compounds produced by rice roots.

Rhizobium leguminosarum bv. *trifolii* strain ANU 843 is used in this construction. It does not induce the defense responses of *Parasponia,* but erodes the root surface epidermal cells and induces root cortical cell division. A *nodD* gene shown to respond to signals from root exudates from rice seedlings and cause root hair distortions was inserted into a derivative of this strain, ANU 851, a *nodD* mutant. The *nodD* gene was cloned into a high copy number vector to supply multiple copies of the regulatory gene and help ensure *nod* gene regulation. This genetically engineered strain, ANU 536, formed nodules on seedlings of the rice variety Calrose at a low frequency of 0.25% without any other additions (Rolfe and Bender 1990). The nodulelike structures were small, white, and contained bacteria. Sections showed that vascular bundles were associated with the root outgrowths. One of these structures was examined in some detail and had an internal structure resembling that found within legumes. It contained membrane-encapsulated bacteria which often appeared to have inclusions resembling refractile granules of poly-β-hydroxybutyrate (PHB). The infected plant cells did not appear stressed by the presence of the packaged bacteria.

So far we have been able to initiate nodulelike structures on three rice varieties readily available in Australia, Calrose and Echuca (medium grain), and Lemont (long grain). Seedling nodulation frequencies on these varieties were low, but similar in each case (between 0.10 and 0.25%), with small creamy white structures being formed.

When these induced structures contained bacteria, they were usually found between cells or in dead and dying plant cells. These bacteria also appeared to contain refractile PHB granules.

Key bottleneck

The current frequency of *Rhizobium*-induced modified root structures is too low and unpredictable for an adequate analysis of the infection process involved. Screening experiments are needed to find the rhizobia-rice variety combination which repeatedly gives nodulation frequencies of at least 20%. This frequency is close to the minimum level required for a precise study of the infection process to be made.

Researchable areas

An International Rice Nodulation Group has been established by the Rockefeller Foundation to develop a *Rhizobium* inoculant for rice. The group will a) select the best *Rhizobium*-rice cultivar combination (i.e., that which gives the highest reproducible frequency of nodulation), b) conduct a detailed light and electron microscopy study of infection of rice roots to help design a more invasive inoculum strain, c) investigate *Rhizobium* genetics and possible gene amplification processes that may be involved in the adaptation of rhizobia and their interaction with rice plants, and d) use the above findings to produce a plant-microbe association that stimulates the growth of rice plants in the field. This stimulation could be due to bacterial-derived phytohormone effects as well as to a contribution from fixed N_2.

The work is labor-intensive and progress is slow. Frequency of formation of nodulelike structures is very variable. Identification of the physiological factors that influence the induction of these root outgrowths is needed. The growth status of inoculated plants on N-free medium and the pH of the growth media appear to be important for success.

Formation of the extracellular metabolites which cause the Hac^+ phenotype on legumes requires a minimum of *nodABCD* genes. The nodulation factors are lipo-oligosaccharides (Lerouge et al 1990, Spaink et al 1991) and promote a weak Hac^+ response if applied to the roots of oat seedlings. These metabolites could be used as probes for *Rhizobium*-induced changes in the cells of nonlegumes.

One recent finding that might prove to be very important in strain development is the nodulation of oilseed rape roots by inoculation with *Rhizobium parasponia* strain RP501 (Cocking et al 1990) or *Bradyrhizobium* strain CP283 in the absence of enzyme-PEG treatment (E.C. Cocking, Apr 1991, University of Nottingham, pers. commun.).

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Nodulation genes and biosynthesis of indole acetic acid in *Azospirillum brasilense*

C. Elmerich

It has been established that plant growth-promoting rhizobacteria play a role in tropical agriculture, especially in rice crops and pasture grass where fertilizer input is very low. Numerous N_2-fixing bacterial species have been isolated from the plant rhizosphere, including *Pseudomonas*-like spp. *Enterobacteriaceae, Bacillus,* and *Azospirillum.* Nitrogen fixation occurs only under microaerobic conditions.

Effective colonization and root growth promotion depend on many parameters: survival in the soil, motility and chemotactic response to root exudates, siderophore or bacteriocin formation, phytohormone excretion, mechanism of attachment to the root system, and efficiency of transfer of fixed N_2 to the host plant. Special interest has been given to the *Azospirillum* genus, which is now a model system for studying the association between bacteria and grasses (Okon 1985, Elmerich et al 1991). Five species have been identified in this genus: *A. brasilense, A. lipoferum, A. amazonense, A. halopraeferens, and A. irakense.* The bacteria are Gram-negative, heterotrophic, aerobic, curved rods, and possess a genome of high G+C content (66-71 mol %). Many aspects of the physiology and genetics of *A. brasilense* Sp7 have been studied, including establishment of genetic tools, molecular analysis of plasmids, characterization of ammonia assimilation and N_2 fixation genes, study of indoleacetic acid (IAA) production and identification of nodulation gene homologs (for a review, see Elmerich et al 1991).

Current status of research

Azospirillum bacteria have been isolated from the roots of a large number of cereal and forage grasses. No differentiated structures are formed. The bacteria can colonize the root cortex and are observed in the intercellular spaces of the cortex. After inoculation with *Azospirillum,* the host plant root system proliferates and the number of lateral roots and root hairs increases. The interaction of the bacteria with its host plant is presumably mediated by bacterial genes. In particular, there are similarities between the root hair deformations caused by *Azospirillum* in grasses and those caused by *Rhizobium* in legumes (Patriquin et al 1983). These observations, and the report that *Azospirillum* can stimulate nodulation of *R. trifolii* on clover (Plazinski and Rolfe

1985), led us to assume that some of the bacterial genes involved in such interactions might be similar.

Occurrence of nodulation genes

The genes necessary for nodule organogenesis and host specificity are carried by megaplasmids in *Rhizobium meliloti.* To date, at least 18 genes involved in nodulation (*nod, hsn,* and *nol* genes) have been identified in *R. meliloti* (reviewed by Kondorosi 1990). These include *nodABCIJ,* referred to as the common nodulation genes; *nodD,* encoding a specific activator of the *nod* operons; and *nodQPGEFH,* referred to as the host-specific nodulation genes *(hsn* region).

Hybridization has been reported between some *R. meliloti* nodulation genes and the DNA of several *Azospirillum* strains (Fogher et al 1985). Using a DNA probe containing *nodHFEG* and part of *nodP* from *R. meliloti* 41, two DNA fragments of *A. brasilense* Sp7 have been cloned in pUC18 (Elmerich et al 1987).

A 10-kb *Eco*RI fragment sharing homology to the *nodPQ* region was isolated. The nucleotide sequence of a 3.5-kb *Eco*RI-*Sma*I fragment of the cloned insert revealed 60% homology with *R. meliloti nodP* and *nodQ* genes (Vieille and Elmerich 1990). The two genes are present on a 90-MDa plasmid of *Azospirillum* (Onyeocha et al 1990). A translational *nodP-lacZ* fusion was constructed and the fusion was constitutively expressed in *Azospirillum.* Deletion and mutations of *nodPQ* did not modify growth, N_2 fixation, or interaction with wheat seedlings. It was found that *R. meliloti nodPQ* genes are similar to *Escherichia coli's cysDN,* which encodes an ATP sulfurylase (Schwedock and Long 1990). However, deletion of *nodPQ* in *Azospirillum* does not impair sulfate assimilation.

A 4-kb *Sal*I fragment, which shares similarity with a *R. meliloti* fragment containing *nodG,* was isolated (Elmerich et al 1987). Establishment of the nucleotide sequence showed that the *Azospirillum* gene, termed *ORF3,* was closer to the *Alcaligenes eutrophus phbB* gene than to *R. meliloti nodG* (Vieille and Elmerich 1992). The *phbB* gene encodes a NADPH-linked acetoacetyl-CoA (AA-CoA) reductase, which is involved in the synthesis of the poly-ß-hydroxybutyrate (PHB) in *A. eutrophus* (reviewed by Steinbuchel and Schlegel 1991). *Azospirillum* has been shown to accumulate PHB. However, *ORF3* was not found to be involved in PHB biosynthesis in *Azospirillum.*

Nodulation factors

Activation of *nod* gene transcription by appropriate plant flavonoids leads to the synthesis of signal molecules, known as nodulation factors. The chemical structure of the major nodulation factor produced by *R. meliloti* was elucidated (Lerouge et al 1990). The molecule (NodRm-1) is a sulfated and acylated glucosamine tetrasaccharide. It has been postulated that NodG, together with NodE and NodF, is involved in the synthesis of the acyl chain, whereas NodPQ and NodH are involved in sulfation. Thus three of the structural genes for this molecule have been found in *Azospirillum.* Moreover, preliminary hybridization experiments suggested that *nodABC* genes, which are involved in the synthesis of the backbone of the molecule, were also present in *Azospirillum* (Fogher et al 1985).

Pathways of IAA synthesis

Tryptophan (Trp) is a precursor for biosynthesis of IAA in bacteria and plants. There are several pathways for conversion of Trp into IAA. The microbial pathway which has been genetically analyzed includes two enzymes, a Trp-monoxygenase and an indoleacetamide hydrolase. The corresponding genes have been isolated and sequenced from the phytopathogen *Pseudomonas syringae,* from the pTi of *Agrobacterium tumefaciens,* and from a *Bradyrhizobium* sp. Physiological studies performed with the intermediate indoleacetamide and hybridization experiments failed to detect an analogous pathway in *Azospirillum* (Zimmer et al 1991). Consequently, a different pathway for IAA synthesis was proposed for *Azospirillum,* involving the intermediates indole-3-pyruvate and indole-3-acetaldehyde. The initial reaction, conversion of Trp into indole-3-pyruvate, can be catalyzed by aromatic aminotransferases. Four of these enzymes have been identified in *A. lipoferum.* These enzymes were found to be specific for different aromatic amino acids, and not only for Trp, so the detection of these proteins does not prove that IAA is synthesized via indole-3-pyruvate in *Azospirillum.* The structural gene for an indole-3-pyruvate decarboxylase has been cloned from *Enterobacter cloacae* (Koga et al 1991). It is not known if a similar gene is present in *Azospirillum.*

Regulation of IAA biosynthesis

Screening Trp-dependent IAA production of different *Azospirillum* species revealed that *A. irakense* KA3 released 10 times less IAA into the medium than *A. brasilense* Sp7. A cosmid library of strain Sp7 was transferred into *A. irakense* KA3 with the aim of characterizing the genes involved in IAA biosynthesis. This enabled us to characterize a 5-kb DNA region from Sp7 which carried the genetic information for increasing Trp-dependent IAA production in *A. irakense.* Nucleotide sequence analysis revealed open reading frames corresponding to TrpG, TrpD, and TrpC enzymes involved in Trp biosynthesis. Further analysis showed that the region responsible for the enhanced Trp-dependent IAA production in strain KA3 corresponded to *trpD,* which codes for the phosphoribosyl anthranilate transferase (Zimmer et al 1991).

Regulation of IAA production in the *Azospirillum* genus is probably repressed by anthranilate, which is an intermediate in Trp biosynthesis and catabolism. *A. irakense* can grow when Trp is the sole carbon and nitrogen source. It produces more anthranilate from Trp than *A. brasilense* which cannot use Trp as a carbon source for growth. Introduction of *A. brasilense trpD* into *A. irakense* resulted in a reduced release of anthranilate into the medium, due to the conversion of anthranilate by TrpD, concomitant with an increase in IAA production. The difference in Trp metabolism seems to be correlated to IAA biosynthesis. *trpD* appears to play a role in the regulation of IAA biosynthesis.

Prospects

Nodulation genes

The discovery of homologs of nodulation genes in *Azospirillum* raises the question of their involvement in the interaction with the host plant. No particular phenotype can yet be attributed to *Azospirillum nodPQ* or *nodG* (ORF3). Homologs of *nodABC* have not yet been cloned and there is no *nod*-box in the *Azospirillum* genome. It is difficult to assume that the occurrence of these genes in *Azospirillum* is fortuitous. Therefore, our research program will include further characterization of these genes and attempts to detect putative signals resembling NodRm-1 factor released into the medium.

IAA biosynthesis

As outlined above, the pathway of IAA synthesis in *Azospirillum* is unknown and there is no mutant available that is totally deficient in IAA production. Isolation of such a mutant is essential for further analysis of the role of IAA in plant promotion.

Use of artificial symbiosis

The possibility of increasing N_2 fixation in cereal crops by inoculation with wild type or engineered N_2-fixing bacteria is an extremely challenging project. It is clear that colonization of the root system results from signal exchanges between the plant and the bacteria, thus bacterial and plant genes are involved. One can postulate that the activity of some genes should be increased and that of others decreased during the association. Several *Azospirillum* genes are now characterized. In addition to *nod* and *trp* genes reported here, key regulatory genes involved in N_2 fixation have been also studied (Liang et a1 1991). These genes, fused to suitable reporters, can be used as probes to monitor what limits expression during the association with the host plant, and to tentatively identify plant signal molecules. A procedure to induce root *para*-nodules on wheat after addition of 2,4-D has recently been developed (Tchan et al 1991). Inoculation with bacteria can be achieved, and it has been established that *Azospirillum* is a good candidate for developing a N_2-fixing association. This would provide a good experimental model for pursuing analysis of the potential of *Azospirillum.*

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Ammonium excretion by *nifL* mutants of *Azotobacter vinelandii*

C. Kennedy

So far a *nifL* gene has been identified in only two genera of diazotrophic bacteria: *Klebsiella* and *Azotobacter* (Kennedy 1977, Bali et al 1991). In both *K. pneumoniae* and *A. vinelandii, nifL* is upstream of, and cotranscribed with, *nifA,* which encodes the NIFA protein, an essential activator of expression of other *nif* genes. In both species, the *nifL* gene product mediates a response to increasing levels of fixed N_2 in the environment. In *K. pneumoniae,* NIFL apparently interacts directly with NIFA to inhibit its activator function (Morett and Buck 1988); this occurs not only in the presence of fixed N_2 but also in O_2. Because of the considerable homology between the NIFAs and the NIFLs in the two organisms, it is likely that the NIFL-NIFA pair of regulatory proteins in *A. vinelandii* interact in the same way as in *K. pneumoniae.* Whether the *A. vinelandii* NIFL protein also regulates NIFA activity in response to O_2 is not yet known.

A major difference occurs in the two organisms with respect to regulation of *nifLA* operon expression. In *K. pneumoniae,* the *nifLA* promoter lying upstream of the *nifL* gene is recognized only by RNA polymerase complexed with sigma factor 54, and requires phosphorylated *ntrC* gene product (NTRC) for activation of expression. Since NTRC is dephosphorylated in cells grown in fixed N_2, *nifLA* expression does not occur if ammonium is present at greater than about 200 μM (Menick et al 1982). In *A. vinelandii,* however, the *nifLA* promoter is not recognized by sigma 54 and expression is neither activated by NTRC nor prevented by ammonium (Blanco et al 1992).

We recently found that *nifL* mutants of *A. vinelandii* synthesize nitrogenase constitutively in the presence of ammonium and, unexpectedly, excrete large amounts of ammonium during N_2 fixation. Up to 10 mM ammonium were found in the culture medium toward the end of the exponential growth phase (Bali et al 1991). In contrast, *nifL* mutants of *K. pneumoniae* excrete less than 100 μM ammonium. Constitutive *nifA* expression might be expected to result in ammonium excretion in either *K. pneumoniae* or *A. vinelandii* by titration of NIFL. However, neither high nor low copy plasmids constitutively expressing *nifA* from either organism resulted in strains excreting ammonium.

The amount of ammonium excreted by *A. vinelandii nifL* mutants growing in liquid culture is apparently limited by the increase in pH to 8.5 that occurs concomi-

tantly with excretion. If cells are resuspended in fresh medium, they again excrete ammonium to give 10 mM in the medium. Growth of the *nifL* mutants is not noticeably impaired on N-free medium compared with that of the wild-type strain, but on agar medium supplemented with ammonium, colonies of the *nifL* mutant strain are slighly smaller than wild type colonies and viability decreases more rapidly, possibly because of the detrimental effects of high pH.

Up to now, ammonium excretion in free-living, N_2-fixing bacteria has only been achieved by physiological suppression or genetic manipulation of the enzymes involved in ammonium assimilation. Examples in Cyanobacteria include treatment with glutamine synthetase (GS) inhibitors such as MSX, resulting in excretion of up to 7 mM ammonium, and isolation of MSX- or ethylenediamine-resistant mutants that excrete up to 1.6 mM ammonium (Musgrave et al 1982, Newton and Cavins 1985, Polukhina et al 1982). Among eubacteria, ammonium excretion at levels of 1-10 mM was reported for mutants of *K. pneumoniae* defective in ammonium assimilation or transport, in mutants of *Rhodobacter capsulatus* and *Azospirillum brasilense* altered in the production of GS, and in methylamine-resistant mutants of *A. vinelandii* (Andersen and Shanmugam 1977, Shanmugam and Valentine 1975, Gordon and Jacobson 1983, Wall and Gest 1979). In all cases, growth of the treated or mutated organisms was significantly impaired unless glutamine or glutamate was supplied. In contrast, the ammonium-excreting *nifL* mutants of *A. vinelandii* are unaffected in ammonium assimilation.

Researchable areas

Further studies of *A. vinelandii nifL* expression, function of gene products, and behavior of mutants are now important. Some of the scientific questions to be answered include:

- How does NIFL interact with NIFA? Which sequences on either protein are important for recognition and/or inactivation? Can interaction be prevented by strategic mutagenesis of either protein?
- How is NIFL modified in cells grown with ammonium (or oxygen)? A clue to this was recently obtained from our discovery that a regulatory gene previously identified as *nfrX* in *A. vinelandii* is structurally and functionally homologous to *glnD* in enteric bacteria (Contreras et al 1991). Furthermore, *nifL* mutations suppress the *Nif* phenotype of *nfrX* mutants; *nifL nfr-X* double mutants are *Nif*$^+$. Since the *glnD* gene product is a uridylyl-transferase/uridylyl-removing enzyme which modifies the PII protein (*glnB* gene product) in response to levels of fixed N_2, the function of *nfrX* may be to modify either directly or indirectly, possibly via PII, the NIFL protein in response to ammonium levels. Modification would occur in low ammonium concentration to convert NIFL to an inactive form. Absence of modification in the *nfrX* mutants results in constitutively active NIFL. The nature of the modification of NIFL should now be determined; attachment of phosphate or uridylyl or other nucleotidylyl groups to NIFL may occur.
- How can ammonium excretion in *nifL* mutants be maximized? Cultural conditions such as carbon source, aeration rate, and temperature should be strategically

varied. A flow-through system with immobilized (and perhaps nutritionally limited) cells might allow continuous ammonium production for some time by avoiding pH increase.

Applied aspects of work on *nifL* mutants

Another potential offered by *nifL* mutants is one of practical benefit, where ammonium produced from N_2 fixation might be used to support plant growth. The recent report of enhanced N supply to wheat from association with ethylenediamine-resistant mutants of *A. brasilense* that excrete 10-100 times less ammonium than do the *A. vinelandii nifL* mutants is encouraging (Christiansen-Weniger and Van Veen 1991). The feasibility of the following strategies could be evaluated over the next few years:

- Direct use of *A. vinelandii nifL* mutants in rhizosphere associations. This is probably unlikely to be of benefit because, although Azotobacters are widespread, they seldom achieve a high population density in soils.
- The construction of *A. vinelandii nifL* mutant derivatives with engineered affinity for binding to or association with root surfaces, e.g., through lectin binding or interaction with other adhesive molecules. Ammonium-excreting mutants that can utilize specific carbon sources provided in high amounts by plant roots could yield a high concentration of *Azotobacter* in the rhizosphere.
- The search for other associative diazotrophs with a *nifL* gene or another gene which is solely responsible for mediating ammonium repression of N_2 fixation. Mutation of such a gene could result in high amounts of ammonium excretion, as in *A. vinelandii.*

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Genetics of associative nitrogen fixation in wheat

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The interaction between N_2-fixing bacteria and nonleguminous plants was only discovered recently (Dobereiner et a1 1972, Kass et a1 1971, Larson and Neal 1978, Dommergues et a1 1973). A special term, diazotrophic symbiosis, was suggested to describe the process of N_2 fixation in the plant root zone (Vose and Ruschel 1982). The ability of nonleguminous plants to fix N_2 in the root zone is known as the nis (nitrogen fixation supportive) trait. Most cereals studied can stimulate associative N_2 fixation in the root zone.

A close relationship between associative N_2 fixation activity and photosynthesis in nonleguminous plant has been established. Part of the N_2 accumulated by nonleguminous plants was proved to have been fixed by the root zone bacteria.

The first work on associative N_2 fixation in various cultivars and varieties of wheat was carried out in the late 1970s (Dobereiner 1977, Neal and Larson 1976, Larson and Neal 1978, Rennie and Larson 1979). A great variability of nis-trait in wheat was shown.

Investigations carried out on chromosome substitution wheat lines by Canadian researchers (Neal and Larson 1976, Larson and Neal 1978, Larson and Rennie 1979, Rennie 1981) demonstrated that a change in the wheat genome results in a change in the associative N_2 fixation ability of diazotrophic strains (genus *Azospirillum, Bacillus).* These results, however, have not been confirmed (Lethbridge and Davidson 1983, Lethbridge et al 1982).

It is still not clear how many genes control N_2 fixation in wheat, the process of interaction with diazotrophic bacteria, where they are localized, and whether the associative symbiosis is specific.

According to the literature, the following bacteria are associative for wheat: *Azospirillum, Azotobacter, Bacillus, Pseudomonas, Agrobacterium,* and *Flavobacterium.* However, we lack accurate criteria for defining associative bacteria. It is also not clear whether the associative symbiosis is a primitive symbiosis or a degraded one.

Current status of research

We have

- Determined associative N_2 fixation activity in wheats with different genomes (A, AB, AG, ABD, AABG, AAGG).
- Analyzed associative N_2 fixation activity in hexaploid wheats (21 monosomic lines—i.e., lines lacking one of the following chromosomes, 1A, 2A, 3A, 4A, 5A, 6A, 7A, 1B, 2B, 3B, 4B, 5B, 6B, 7B, 1D, 2D, 3D, 4D, 5D, 6D, 7D).
- Studied the process of N_2 fixation in wild wheat *T. boeticum* (source of genome A), wild cereals *Aegilops taushii* (source of genome D) and *Aegilops speltoides* (source of genome B).
- Isolated several diazotrophic bacteria from wheat roots, among them strain *5D-1* identified as *Agrobacterium radiobacter.*
- Demonstrated the ability of *Agrobacterium radiobacter* to adsorb on wheat root surface and form fibril-like structures (Chumakov et al 1992).

The process of plasmid and vector transfer into *A. radiobacter 5D-1* and the ability of the strains isolated to fix N_2 in pure culture and in association with plants are being studied (Emtsev and Chumakov 1988, Chumakov et al 1990). The spectrum of wheat root proteins after inoculation with various diazotrophic bacteria is also being examined.

Future research

The problems raised suggest several areas for further research:

- Identification of the wheat chromosome(s) carrying genes controlling N_2 fixation and interaction with associative bacteria.
- Determination of whether nonleguminous plants contain gene sequences similar to those that control symbiosis in legumes.
- Isolation of bacteria capable of penetrating nonlegume roots and forming nodulelike structures.
- Determination of whether genomes of *Rhizobium, Agrobacterium,* and associative bacteria involved in plant-bacterium interactions are analogous.
- The possibility of obtaining transgenic cereal plants (wheat) carrying N_2 fixation genes.

We are planning to continue our work in the following areas:

- Determination of associative N_2 fixation in wheats with a rearranged genome.
- Study of *A. radiobacter 5D-1* genes controlling initial stages of plant-microbe interactions (adsorption).
- Isolation of substances which derepress nitrogenase activity in nonpathogenic agrobacteria during interaction with wheat and defining conditions under which this process proceeds.
- Study of the protein spectrum of nodulelike structures on wheat roots.
- Transformation of wheat with agrobacteria, working out a method for stable transformation of wheat, and transfer of genes involved in N_2 fixation into the plant.

Key bottlenecks

- Instability in reproducing N_2 fixation in the *Agrobacterium radiobacter*-wheat association. We are searching for substances and conditions favoring derepression of nitrogenase in *A. radiobacter*.
- Difficulties in obtaining the necessary amounts of fibril-like structures. We will study these structures during the process of plant-bacterium interaction.
  Difficulties in obtaining transformed plants from wheat callus or suspension cultures.
- It would be interesting to search for a cultivar with increased regeneration ability.

We will apply other methods of transferring agrobacterial genes into wheat, e.g., by using immature wheat germs or the pollen tube method.

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Potential for development of novel N_2-fixing associations

Potential and limitations of developing new plant-microbe interactions

J. Schell, J. Schmidt, M. John, and H. Röhrig

The submerged soil provides a suitable environment for N_2-fixing organisms, such as phototrophic diazotrophs in the floodwater and enterobacteria in the soil. These organisms require anaerobic conditions to fix N_2. The endophytic cyanobacterium *Anabaena azollae,* which forms a symbiosis with the water fern *Azolla,* is probably the best source of biological N in ricefields (Watanabe and Brotonegro 1981). The azolla/anabaena symbiosis has a N_2-fixing rate of 3 kg N/ha per day, or the potential to satisfy the entire N requirement for a high-yielding rice crop within 2 wk. *Azolla* is also an excellent fodder. It has been shown that inoculating dryland, wetland, or hybrid rice with different species of enterobacteria can lead to a N_2-fixing rate of approximately 20-30 kg/ha over a period of 2 yr (Zhang et al 1990).

More work is necessary to determine if the creation of a functional *Rhizobium*-rice interaction would be an attractive alternative or complement to these bacterial-plant associations.

Specific *Rhizobium* strains have been shown to induce root hair curling on rice (Plazinski et al 1985). Nodules have been obtained on rice by treatment with cell wall-degrading enzymes in the presence of polyethylene glycol (PEG), which facilitates rhizobia1 entry (Al-Mallah et al 1989). These nodular structures will not necessarily provide the anaerobic environment essential for nitrogenase activity. Nodules on wheat roots (Bender et al 1990) and on rice roots (Bender et al 1990, Cocking et al 1990) do not have the highly organized internal structure of legume nodules, possibly explaining why the amounts of N_2 fixed are very low (Bender et al 1990, Cocking et al 1990, Jing et al 1990). Oilseed rape seedlings can also be nodulated by rhizobia in the same way (Cocking et al 1990, Al-Mallah et al 1990), resulting in a modest rate of acetylene reduction (Cocking et al 1990). More experiments are necessary to evaluate the significance of these observations and the possibility of nodule induction on rice or wheat under field conditions.

Development of the symbiosis between rhizobia and leguminous plants is a complex multistage process requiring genes from both partners (Kondorosi et al 1991). Transfer of some legume genes may be necessary to elicit functional nodular structures on roots of nonlegumes.

The finding that the signal molecules produced by *Rhizobium nod* genes (so called Nod factors) are modified oligosaccharides (Lerouge et al 1990) supports the hypothesis that oligosaccharides are the regulatory molecules that control particular aspects of growth and development in plants (Albersheim and Darvill 1985).

The mechanism by which the Nod factor triggers a particular developmental program in the plant is unknown. It is possible that the bacterial oligosaccharide signal alters the phytohormone balance in the root tissue and that this change is necessary to elicit nodule formation. Nodulelike structures were induced on alfalfa roots by artificial auxin transport inhibitors and by *Rhizobium nod*-mutants which synthesized zeatin (a cytokinin) constitutively (Hirsch et al 1989, Long and Cooper 1988). The Nod factor alone elicits nodule formation on the roots of legumes (Truchet et al 1991) but nonlegume plants apparently do not respond. Various rhizobia1 genes (such as *nodABC* and *hsnD*) were shown to be involved in the synthesis and specific modification of Nod factors.

Recently, we have shown that NodA and NodB proteins produce compounds that stimulate the mitosis of plant protoplasts (Schmidt et al 1988). To test if this new class of plant growth factors could play a role in uninfected nonlegume plants, the *nodA* and *nodB* genes were introduced and expressed in tobacco. A comparison of control plants with transgenic tobacco plants carrying only the *nodA* gene shows that *nodA* activity leads to slightly reduced growth, a reduction in the internodal distance, and an altered leaf morphology. Expression of the single *nodB* gene is responsible for strongly reduced growth and a compact inflorescence. Many flowers had only four petals and four anthers instead of five. In all cases, we found heterostyly with increased stigma size, so the plants were unable to self-pollinate.

Transgenic tobacco plants expressing *nodA* and *nodB* under the control of the phytohormone-regulated dual TR promoter exhibit other phenotypic alterations as well. The NodAB factor, which is presumably produced in these transgenic plants, seems to have an effect on cell differentiation, leading to the formation of bifurcated leaves. This effect on organogenesis can also lead to the formation of two or more stems emerging independently from the same leaf axle. Our data indicate that the factors produced by the *nodA* and *nodB* genes alone or in combination are active in nonlegumes, but that the expression of these genes may affect the phytohormone balance in transgenic tobacco (Schmidt et al 1991). The morphological abnormalities of the transgenic plants further indicate that tobacco must contain the necessary substrates for the NodA and NodB proteins to synthesize growth-controlling factors, and the necessary receptors to respond to the presence of these regulatory molecules.

The ability of this new class of oligosaccharide signals to control plant growth and development could increase our knowledge of cellular mechanisms in plant differentiation. This knowledge could have a significant impact on plant breeding and agricultural yields and help to create a functional *Rhizobium*-crop plant symbiosis.

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Application of present knowledge on rhizobial host specificity to obtain efficient nodulation and nitrogen fixation of rice

H.P. Spaink and B.J.J. Lugtenberg

Formation of an effective N_2-fixing symbiosis through rhizobial infection of nonleguminous plants is presently restricted to plants within the *Parasponia* genus. Several molecular signaling steps between the bacteria and their host plants that determine nodulation specificity have recently been discovered. This abstract will discuss the possibility of using our present knowledge to extend the host range of rhizobia to nonleguminous plants, in particular important crops like rice. Reports on the possibility of nodule formation on rice (e.g., Al-Mallah et al 1989) are considered, and we conclude that present knowledge is sufficient to design promising research strategies for obtaining more efficient nodulation and perhaps also N_2 fixation in rice.

Current status of research

Determination of host specificity in the *Rhizobium* plant symbiosis

Determination of host-specific nodulation appears to involve at least two steps of molecular signaling between plant and rhizobia. In the first step, flavonoids excreted by the plant induce the transcription of bacterial nodulation genes (*nod* genes) (reviewed by Long 1989). The host specificity of this induction process (Spaink et al 1987) involves the bacterial NodD protein, which presumably interacts directly with the flavonoids. In the second step, the bacterium, by means of the *nod* genes, produces one or more lipo-oligosaccharide signals which activate plant genes (Scheres et al 1990) and trigger root responses such as root hair curling, production of new flavonoids (Van Brussel et al 1990), and nodule meristem induction (Spaink et al 1991, Truchet et al 1991). In the case of *R. meliloti,* bacterial host-specific signals have been identified as sulfated lipo-oligosaccharides (N-acyl N'N"N"'triacetyl-chitotetraose) (Lerouge et al 1990). In *R. leguminosarum* bv. *viciae,* four lipo-oligosaccharide signals were identified which differ from the *R. meliloti* signals in the absence of a sulfate group, the presence of an O-acetyl group, and a different fatty acid substituent (Spaink et al 1991). The sulfate and fatty acid substituents were shown to be major determinants of host specificity in the cases of *R. meliloti* (Lerouge et al 1990) and *R. leguminosarum* bv. *viciae* (Spaink et al 1991), respectively.

The flavonoid signals produced by the plant and the lipo-oligosaccharide signals produced by the bacterium are both crucial for effective N_2 fixation (Spaink et al 1989a, Lugtenberg et al 1990). Spaink et al (1989b) constructed Rhizobium strains which harbored a flavonoid-independent transcription activator (FITA) nodD gene. In addition to being induced in the absence of flavonoids, these strains also appeared to have improved N_2 fixation ability when present in *R. leguminosarum* bv. *trifolii* (Spaink et al 1989b). The possibility of improving symbiotic N_2 fixation through applying FITA *nodD* genes has been described in a patent application (Spaink et al 1988).

Another major determinant of host specificity of nodulation is the host-plant lectin (Diaz et al 1989). Transgenic clover plants that expressed the pea lectin gene were nodulated by the heterologous *R. leguminosarum* bv. *viciae* bacterium and substantial N_2 fixation was observed. This result indicates that introduction of the lectin gene could remove host-specific barriers in N_2 fixation.

Approaches for nodulation on rice

Nodulation of rice by rhizobia has been reported, but nodulation either occurred at very low frequency (Bender et al 1990) or required an enzymatic pretreatment of the roots (Al-Mallah et al 1989). Nitrogen fixation by nodulated rice plants was only reported by one group (Jing et al 1990). However, adequate controls were not presented. These results indicate the possibility of including rice in the *Rhizobium* host range, but also show that development of a reliable test system for nodulation of rice plants will be a major bottleneck. We propose nodulation when this is abundant. Meristem formation can be used as a phenotype for successful initiation of nodulation.

Use of FITA *nodD* genes

FITA *nodD* genes are functional with all *nod*-boxes tested so far. We anticipate that they will lead to constitutive production of lipo-oligosaccharides in all rhizobial strains. Rhizobia that produce lipo-oligosaccharides which are active on rice may be identified by testing for rice root response. The active lipo-oligosaccharides should be subsequently purified and characterized. The influence of *nodD* genes on the efficiency of N_2 fixation (Spaink et al 1989b) suggests that the presence of a FITA *nodD* gene may have a positive effect on N_2 fixation in rice.

Use of lectin genes

Transgenic rice plants containing the pea lectin gene could be investigated for their response to *R. leguminosarum* bv. *viciae*. *Rhizobium* could be allowed to produce and excrete its own lectin. Inducible expression could be achieved by cloning the lectin gene behind a *nod*-box. Preliminary experiments show promising results with this approach.

If the abovementioned approaches are not successful, the main limiting factor in the nodulation of rice may be the host-specific character of the lipo-oligosaccharide signals. A larger variety of lipo-oligosaccharides should then be tested. Isolation and identification of the endogenous lipo-oligosaccharide analog of rice may be necessary.

In vitro test systems are possible for induction of root hair deformation, flavonoid production, meristem formation, and nodulation. Although the induction of nodulation in vitro would be the most encouraging, the other effects also indicate the functionality of lipo-oligosaccharides identified in vivo.

Test effects of various known lipo-oligosaccharides on rice

Many wild-type lipo-oligosaccharide signals have been purified in our laboratory and in that of the Toulouse group. Their effects on rice, before and after enzyme treatment of roots, should be tested (Al-Mallah et al 1989). These experiments could indicate which chemical characteristics are important for effects on rice plants.

Construction of rhizobia which produce adapted lipo-oligosaccharides

This could be achieved by genetic engineering of *nod* genes, e.g., *nodE* for the fatty acid moiety, *nodABC* for oligosaccharide chain length, *nodL* for O-acetylation, and *nodH* for sulfation.

Organic synthesis of adapted Nod lipo-oligosaccharides

We are involved in the organic synthesis of lipo-oligosaccharides. Preliminary results indicate that the synthesis of such compounds, and of many analogs, is possible.

Endogenous lipo-oligosaccharide-analog of the plant

We are investigating the presence of naturally occurring lipo-oligasaccharides in *Lathyrus* plants to test the hypothesis that they are plant hormones. The role of oligosaccharides in the organogenesis of plants is consistent with this hypothesis (Darvill et al 1989). Lipo-oligosaccharides have effects on nonleguminous plants like *Nicotiana tabacum* (unpublished results), indicating that they could have a role in all plants. We propose to investigate the presence of lipo-oligosaccharides in rice. Elucidation of their structures is likely to lead to the requirements for signaling nodulation of rice.

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Genetic transformation of rice and the molecular basis of agroinfection

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Tang Ti, M. Boulton, and M.P. Gordon

We reported previously that embryo-derived cultures of two rice cultivars could be genetically transformed by *Agrobacterium* (Raineri et al 1990). Mature embryos of Nipponbare inoculated with the wide post-range strain A281 (pTibo542) formed tumorous callus tissue that grew on hormone-free medium. The transformed status of this tissue was confirmed by DNA hybridization analysis, showing transferred DNA (T-DNA) in the rice genome. Embryos of another variety, Fujisaka 5, showed no evidence of transformation when inoculated with strain A281. However, the same variety proliferated extensive roots following inoculation with a strain of *Agrobacterium* known to transform root formation in a number of different dicotyledonous plants. These roots grew on hormone-free medium and produced octopine, which is characteristic of tissue genetically transformed by this strain.

More recent studies carried out in Shanghai (People's Republic of China) and Seattle (Washington) also gave strong evidence of transformation of a variety of rice cultivars by different strains of *Agrobacterium.* As shown in Table 1, both *Agrobacterium rhizogenes* and a nopaline strain of *A. tumefaciens* apparently transformed both shoot and root explants as measured by hygromycin resistance and GUS expression. Two putatively transformed japonica cultivars, Nipponbare and Xiushui, were grown to the callus stage and plants regenerated from the callus tissue. T-DNA transfer was assayed by Southern hybridization procedures using an internal GUS fragment as a probe. Six out of nine plants of the Nipponbare and three out of seven of Xiushui, the T_0 generation, were positive by DNA hybridization. All nine plants went into the greenhouse, and five set seed. Plants were grown from the germinated seeds (T_1 generation). Eight out of eleven plants of T_1 generation Nipponbare were hygromycin-resistant and seven out of these eight were also GUS+. Of the Xiushui, five out of 18 T_1 plants were hygromycin-resistant, and four out of these five were GUS+. DNA was isolated from a number of the hygromycin-resistant, GUS+ plants and assayed for internal GUS sequences by Southern hybridization as before. A total of seven Nipponbare contained GUS T-DNA by Southern hybridization and five of the Xiushui T_1 plants were positive. We are currently analyzing these plants for junction fragments which would represent both bacterial and plant DNA sequences.

Table 1. Transformation of japonica and indica rice cultivars by *Agroacterium.*[a]

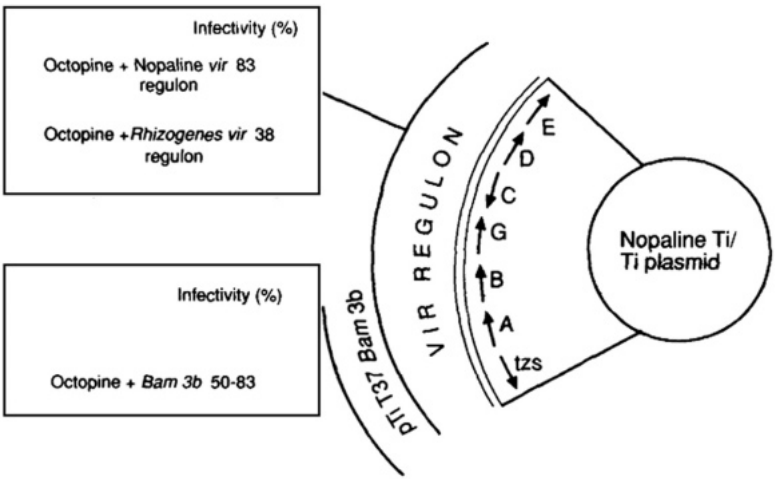
Cultivar	*Agrobacterium* strain	GUS activity of calli (no. of experiments)	Hygromycin^R	DNA hybridization
Japonica				
Nongken #58	*Rhizogenes*	+ (5)	$+_{30}$	–
Nipponbare	*Rhizogenes*	+ (8)	$+_{100}$	+
Fujisaka #5	Nopaline	+ (1)	–	–
Xiushui #4	*Rhizogenes*	Calli - shoots + (1)	$+_{30}$	+
Indica				
Gulfmont	*Rhizogenes*	+ (2)	$+_{100}$	–
Oryza glaberrima	*Rhizogenes*	+ (1)	–	–
Oryza glaberrima	"Supervirulent" octopine	+ (1)	–	–
Texmont	*Rhizogenes*	+ (2)	+	–

[a]Seeds of the rice cultivars were germinated for 3-4 d. The first shoots and roots that appeared were excised and precultured for 4-7 d on solid medium containing auxin and cytokinin . The explants were then wounded and dipped in a suspension of the appropriate *Agrobacterium* strain for 2 min. The explants were then placed on solid medium containing auxin and cytokinin for 2 d. They were then shaken in a liquid medium containing cefataxime and vancomycin for 2 d, after which they were placed onto a solid medium containing the two antibiotics and hygromycin. 4-5% of the hygromycin-resistant explants gave rise to calli. The GUS gene was under the control of a 35S CAMV promoter and the hygromycin was under the control of 19S promoter.

We have preliminary data that four different indica cultivars can also be transformed, and T0 plants have been regenerated from putatively transformed callus tissue. However, Southern hybridization blotting has not yet been carried out.

Our data strongly indicate that *Agrobacterium* can transform embryo-derived cultures and root and shoot explants of several japonica varieties of rice. The T-DNA is expressed in mature plants and can be passed on through seed. The transformed plants appear to be fertile. Preliminary data suggest that indica varieties can also be transformed by *Agrobacterium.*

We observed that different strains of *Agrobacterium* respond differently according to the rice cultivar. A paper published in 1989 demonstrated marked differences in the ability of different strains of *Agrobacterium* to transfer DNA into several monocots, including maize and wheat (Boulton et al 1989). We are collaborating with the authors of this paper at the John Innes Institute (Drs. M. Boulton and J. Davies) in order to try to identify the genes required for DNA transfer and understand the molecular basis of this difference. Recently, different strains of *Agrobacterium* have been shown to differ in their ability to transfer their T-DNA into rice. Nopaline strains of *Agrobacterium* were highly efficient; octopine strains were ineffective. Initial studies demonstrated convincingly that the tumor-inducing (Ti) plasmid of *Agrobacterium* was responsible for this difference (Boulton et al 1989). The experimental system used to study DNA transfer into monocots is agroinfection, in which a dimer of virus DNA known to induce symptoms on the particular plant is placed between the T-DNA border sequences in a binary vector system. Viral symptoms only arise when the *Agrobacterium* containing a full complement of *vir* genes is inoculated into the stem of the plant. To identify the gene(s) important for agroinfection, we have made a clone bank of Ti plasmid from a nopaline strain, and introduced it into the octopine strain to determine



1. Nopaline Ti/Ri plasmid-mediated viral DNA transfer from octopine strains.

which region of the nopaline strain can complement. Our data indicate that a 9-kb fragment can complement the octopine strain (Fig. 1). This fragment includes: 1) a *tzs* locus which interestingly is found only in nopaline and not in octopine strains, 2) the *virA* region (the sensor molecule for the plant signal), and 3) a region to the left of the *virA* gene which has not yet been identified. Our current experiments involve subcloning this 9-kb fragment to determine which gene(s) are responsible for the positive complementation.

Future research

The presence of T-DNA in junction fragments must be demonstrated to prove that it has been integrated and passed through seed. It is also important to follow the traits through several additional generations. These experiments are currently underway. Different promoters and selectable markers are required for this program. There are good reasons to think that the EMU promoter developed by Professor J. Peacock (Last et al 1991), and the ubiquitin promoter from maize, supplied to us by Dr. P. Quail, are considerably stronger than the 35S and 19S promoters currently in use.

The *bar* gene, a selectable marker under the control of maize ubiquitin promoter, confers resistance to the herbicide Bialaphos. We are currently studying *bar* genes supplied by Dr. Quail. An important area of investigation is timing Bialaphos application so that nontransformed plants are killed but transformed plants are not. The frequency of chimeric plants generated in the transformation process should be determined.

In the agroinfection study, the gene(s) involved in T-DNA transfer in nopaline strains must be identified. Once this gene has been identified and its mechanism of

action understood, it may be possible to construct a binary vector system which can transfer T-DNA into Graminae more efficiently than the wild-type strains currently in use. These experiments appear to be straightforward and we should be able to identify the gene involved soon.

All of these studies require extensive tissue culture experience, thus the collaboration of our Chinese colleagues is especially important.

We shall continue to try and improve transformation efficiency and develop the best selectable marker and the most effective promoter for selection for rice transformation. We are beginning to consider the most important genes to put into rice. Dr. Bai has suggested a *Bacillus thuringiensis* gene which is effective against certain insects harmful to rice in China. Undoubtedly, there will be many other useful genes to be introduced. We anticipate that Dr. Bai, and either his wife or another colleague, will come to Seattle at 6-mo intervals during the next 3-5 yr to continue our collaboration on rice transformations.

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Nitrogen fixation in *para*-nodulated wheat

I.R. Kennedy

In the early 1980s, Nie and his colleagues at Shandong University in China used 2,4-D to induce nodular structures on the roots of wheat, sunflower, and other plants. Although the capacity of 2,4-D and other herbicides to induce pseudonodules had been widely recognized much earlier (reviewed in Kennedy and Tchan 1992), Nie was the first to propose that these structures might harbor beneficial bacteria, such as diazotrophs. Nie has since developed an approach in which these chemically induced nodular structures are considered to emulate the root nodules of legumes (see Tchan and Kennedy [1989] for a review), suggesting that many of the features of infection and function associated with legume nodules are involved.

We readily confirmed Nie's basic findings regarding the capacity of 2,4-D to induce nodular structures and to allow their infection with rhizobia and other diazotrophs (Tchan and Kennedy 1989). However, we were unable to establish N_2-fixing capacity (acetylene reduction) with rhizobia under our conditions (Kennedy et al 1990, Kennedy and Tchan 1992). Colonization by rhizobia was erratic, with many of the bacterial cells occurring intercellularly. Chen et al (1988) also reported similar findings. Nie (1989) has conducted a number of experiments using $^{15}N_2$ to test N_2-fixing capacity, obtaining significant enrichments in the total N up to 0.06 atom percent excess ^{15}N, but this required exposures of a week or more. Acceptance of these findings as significant N_2 fixation will require higher enrichments, with better evidence of the exclusion of fixation by associated asymbiotic organisms.

An alternative approach

In considering Nie's findings, we decided that the prospects for chemically induced nodulation could be improved if these structures were not considered primarily as emulating legume nodules (Tchan and Kennedy 1989). Our previous work with wheat, using *Azospirillum* as an associative diazotroph for N_2 fixation (New and Kennedy 1989), proved disappointing (Kennedy et al 1990), possibly because of erratic infection and colonization. The chemical induction of nodular structures as suggested by Nie offered a timely alternative approach to the use of this organism.

Tchan's extensive experience with nonrhizobial diazotrophs led him to consider their potential use in constructing N_2-fixing systems with plants (Tchan 1988). To distinguish these chemically induced nodules from leguminous nodules, we coined the term '*para*-nodule' (Kennedy et al 1990, Tchan et al 1991). Since then, we have developed the *para*-nodulated wheat seedling as a laboratory model of a N_2-fixing system for a cereal (Zeman et al 1991, Kennedy et al 1991).

The present status of *para*-nodulated wheat

Para-nodulated wheat seedlings are prepared and grown in vitro hydroponically in aseptic conditions for up to 3 wk. The N_2-fixing capacity of *para*-nodulated seedlings at about 2 wk is monitored at reduced O_2 pressure. The system uses the toxicity of O_2 to minimize acetylene reduction by azospirilla exterior to the root (Tchan et al 1991). We conclude that most of the ethylene formation observed represents nitrogenase activity by azospirilla associated with *para*-nodules or in other colonies in the root cortex. Microscopy (fluorescent, confocal, and electron) indicates that the majority of the colonies of azospirilla are located intercellularly in association with the para-nodules, particularly in the basal region.

Inspection by fluorescent and confocal microscopy of plants positive for N_2-fixing activity indicates a positive correlation between high nitrogenase activity and the degree of bacterial colonization of the *para*-nodules. Acridine-stained root systems from plants with strong N_2-fixing activity show large numbers of bacteria in optical sections performed with the confocal microscope, particularly near the base of the *para*-nodules.

Obviously, there are many challenges to be negotiated before this association can be considered a true symbiosis. The *para*-nodules must either persist throughout the vegetative growth of the plant or be continuously initiated on the root system to play a significant role in nonlegume nutrition. Consequently, the effects of growth regulators (auxins) such as indoleacetic acid (IAA), naphthaleneacetic acid (NAA), and other chemical substances on *para*-nodule initiation, development, and N_2-fixing capacity were studied.

With NAA plus 2,4-D, acetylene reduction was often strongly stimulated, accompanied by some modification of *para*-nodule development. Perhaps significantly, wheat seedlings without 2,4-D but with NAA (1 mg/liter) or IAA (5 mg/liter) sometimes reduced acetylene much more rapidly than control plants without any hormone treatment (Kennedy et al 1991). Inspection by microscopy indicated that the azospirilla colonized in a similar manner to 2,4-D-treated plants, at the basal region close to the stele of the main root.

The observation that NAA and IAA can stimulate the rate of acetylene reduction stresses the positive role of auxins in the colonization of root tissues by azospirilla. Both NAA and IAA (without 2,4-D) induce strongly modified lateral roots. Although these auxin-affected structures lack the well-rounded appearance of 2,4-D-induced *para*-nodules, the process is similar to *para*-nodulation. Indeed, when the IAA concentration was raised to 10 mg/liter, the well-rounded shape of 2,4-D-induced nodules was also obtained.

Characteristics of the acetylene-reducing activity

Ethylene formation depends on inoculation with azospirilla and the presence of acetylene and shows O_2 sensitivity. There is a positive correlation between high rates of activity and the obvious presence of azospirilla in the *basal para*-nodule cells. This suggests strongly that the ethylene production observed reflects nitrogenase activity. Furthermore, our preliminary experiments with ^{15}N indicate significant enrichment of the total Kjeldahl N in *para*-nodulated root systems, which correlates with the rate of ethylene formation.

Conclusion and proposals for new research

Results so far have been obtained in the laboratory with 2-to 3-wk-old seedlings under aseptic conditions, with no competition from other organisms. We must demonstrate a direct benefit to the N nutrition and growth of the wheat seedlings to be certain of *para*-nodulation's usefulness. We are studying the transfer of newly fixed N_2 from the bacteria to the plant using techniques similar to those employed 25 yr ago, when questions regarding the site of fixation (bacteroids) and the identity of the primary stable products of symbiotic N_2 fixation (ammonia, glutamine, and glutamic acid) in legume symbiosis were answered (Kennedy 1966). Perhaps surprisingly, the question of how N is released from azospirilla in association with plants is unanswered 20 yr after these organisms were discovered to be diazotrophs.

The data obtained give grounds for optimism regarding possible benefits to the wheat plant. We need to determine if the potential benefits can be obtained throughout the growing period of the plant. At this stage, the availability of a model wheat-*Azospirillum* system that reliably fixes N_2 provides a useful basis for further progress.

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Invasion of nonlegume plants by diazotrophic bacteria

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Interest in sustainable agriculture has prompted renewed efforts to increase the amount of biologically fixed N_2 in nonlegume crops. Research includes genetic manipulation of nonlegumes to incorporate N_2-fixing genes from bacteria, studies on nonlegume-diazotroph associations, and the establishment of new nodulation symbioses between rhizobia and nonlegumes. Evidence for nodulation of rice, wheat, and oilseed rape by rhizobia from the laboratories of Rolfe (Canberra), Yuxiang Jing (Beijing), and our own at Nottingham, led to the establishment of an International Rice Nodulation Group (IRNG) by the Rockefeller Foundation. These laboratories, and that of Rafael Palacios (Cuernavaca), are conducting a coordinated program of research on the nodulation of rice and other nonlegume crops for N_2 fixation (Cocking and Davey 1991).

Progress on rhizobia1 invasion of nonlegume crops, up to November 1991, was summarized at the recent International Symbiosis Congress in Jerusalem (Ridge et al 1991, Cocking et al 1991). The Canberra laboratory has focused on rhizobia isolated from *Parasponia,* the only nonlegume plant genus with species that develop N_2-fixing nodules containing rhizobia. These rhizobia have been genetically engineered and used to inoculate nonlegumes. Inoculation of rice seedlings with these bacteria occasionally caused small spherical nodules to form on the roots. The cells of these nodules contained membrane-encapsulated rhizobia. A somewhat similar approach in Beijing, using rhizobia isolated from the legume *Sesbania cannabina,* resulted in nodule development on rice roots. The nodules were spherical, and some nodule cells contained bacteria within membranelike structures (Ridge et al 1991).

Research at Nottingham was stimulated by the finding that elongate structures were produced on oilseed rape seedlings following inoculation of the roots with naturally occurring *Parasponia* rhizobia. These structures resembled swollen lateral roots and contained both inter- and intracellular rhizobia. Enzyme treatment of the seedling roots was not required (Cocking et al 1990). We then investigated whether rhizobia, which naturally nodulated *Parasponia,* would also nodulate cereals such as rice, maize, and wheat under our test conditions.

Current status of *Rhizobium* inoculation of rice, maize, and wheat seedlings

Sterilized indica rice seeds, variety IR36, were treated with *Bradyrhizobium* CP283 from *Parasponia* and germinated on a medium lacking fixed N_2. After 4 wk, lateral roots were observed that were shorter and thicker than lateral roots formed on rice seedlings in the absence of rhizobia. These short, thick lateral roots were fixed and embedded for examination using both light and electron microscopy. Cells of the lateral root cortex were stained with toluidine blue. Rhizobia were clearly visible by light microscopy between cells of the cortex. Electron microscopy showed rhizobia in membrane-bounded vesicles in the cytoplasm of cortical cells and embedded in microfibrillar material between cells (Cocking et al 1991).

Similar results have recently been reported after inoculation of surface-sterilized maize and wheat seeds with *Aeschynomene* rhizobia (ORS310). Swollen lateral roots containing both inter- and intracellular rhizobia formed on seedling roots of both maize (John Innes Hybrid) and wheat (Wembley) after 3 wk. It may be significant that in several legumes, including *Aeschynomene,* emergence of lateral roots disrupts the epidermis, allowing entry of rhizobia via intercellular spaces, and consequently the initiation of nodulation. The invasion characteristics of Parasponia rhizobia are currently being evaluated in maize and wheat roots.

Researchable areas and proposals for the future

If our earlier observations that naturally occurring rhizobia, such as those from *Parasponia* and *Aeschynomene* nodules, invade the primordia of lateral roots of rice, maize, and wheat to produce nodules are confirmed, then a secure foundation will have been laid for assessing the role of cereal nodules in any symbiotic N_2 fixation. With adequate financial support, reproducible systems will become available for field evaluation of N_2-fixing capabilities.

The IRNG, supported by the Rockefeller Foundation, is investigating the sequence of signals and responses that give rise to nodule development by applying the techniques of cytology, biochemistry, genetics, and molecular biology. In this connection, it will be interesting to determine whether sulfated lipo-oligosaccharide signals, operational in legumes (Truchet et al 1991, Spaink et al 1991), are involved in the developmental processes associated with nodule organogenesis in cereals.

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Development of nodulelike structure on rice roots

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The possibility of extending the host range of rhizobia to nonlegumes was encouraged by the discovery that nonlegumes belonging to the wild elm genus *Parasponia* formed nodules with *Rhizobium* and *Bradyrhizhobium* (Trinick 1973, 1979). In 1985, a recombinant rhizobia containing foreign *nodABCD* genes caused root hair curling and deformation in rice (Plazinski et al 1985). In 1988, a transconjugant *Azospirillum brasilense* carrying the nodulation genes of *R. meliloti* was found to induce root hair curling in maize (Piana et al 1988).

Nodulation of rice, wheat, and oilseed rape was also achieved at low frequencies by applying rhizobia to enzyme-treated roots in the presence of polyethylene glycol (PEG) and calcium chloride (Al-Mallah et al 1989, 1990). Similar results were obtained by applying rhizobia carrying multiple copies of the *nodD* gene to rice (Rolfe and Bender 1990). Pseudonodules were induced on barley roots by *R. astragali* under a permanent magnetic field (Jing et al 1990). The infection threads and bacteroids were each enclosed in a peribacteroid membrane and persisted in the infected pseudonodule cells. No plant defense response was observed. Nodulelike structures were reported on rice roots in the absence of any plant hormone treatment (Li et al 1991). This paper will describe the development of nodulelike structure on rice roots in more detail.

Current status of research

Rice root hair curling and deformation after inoculation with rice rhizobia

Except in a few cases, the initial response after rhizobial infection is root hair curling and deformation, followed by formation of infection threads.

Seedlings of rice variety Zhongxi 8408 were inoculated with wild type *Rhizobium* from either sesbania nodules (control group) or rice nodules (experimental group). No root hair curling and deformation were observed in the control group 2-3 d after inoculation. A variety of growth deformations were observed in the root-hair growth zone of the experimental group. These included swollen and intertwined growth at or near the root hair tips. These findings agree with the observation that deformation

responses occurred on oat seedling root hairs 1 wk after inoculation (Terouchi and Syono 1990). The response time of nonlegume seedlings is longer than that of legume seedlings inoculated with homologous rhizobia (Wood and Newcomb 1989). The frequency of root hair deformation in nonlegumes was about 7%, lower than that in legumes.

Development of rice nodules

Nodules form at local foci of the root cortex as a result of cell division following infection with rhizobia. Scanning electron microscopy (SEM) shows nodules at different stages of development on the root. Rice nodules, with or without root hairs, appear to be determinate. Nodules from *Sesbania cannabina* and rice were sectioned into two, fixed and dehydrated, then dried in a critical point drying apparatus with dry ice. SEM indicates a similarity between the two kinds of nodules, with infection threads and bacteroids in the infected cells.

Light and electron microscopic observation of rice nodulelike structure

One month after inoculation of the rice variety Jianongnuo, rhizobia were found to be scattered in the infected cells and have electronic transparent spaces (ETS) around them. Some infected cells contained a large amount of bacteria enclosed in a membranelike structure similar to an infection thread. In the same cell, individual bacteria with ETS were also observed. All the bacteria, scattered and enclosed, have presumptive poly-ß-hydroxybutyrate (PHB) granules present. The bacteria gathered together are clearly enclosed by extracellular mucilage similar to the peribacteroid membranelike structure. From above, host plant defense systems appear as fibril or granule materials. The rice nodules easily senesced and turned dark brown, possibly due to the defense response. In nodules obtained after enzyme treatment to remove the cell wall at the tip of rice and oilseed rape root hairs, the infected cells also showed the defense response with granular precipitates (Al-Mallah et al 1989, 1990).

Nine days after inoculation of rice variety IR36 with rice *Rhizobium* strain *Rrc2,* nodulelike structures appeared. Bacteria were present in the infected cells and intercellular spaces, but a membranelike structure like that which encloses bacteria in the nodules of rice variety Jianongnuo was not observed.

Nodules were obtained by inoculating rice variety Zhongxi 8408 with rice *Rhizobium* strain *Rrc2.* After 1.5 mo, many host cells were found to be infected under light and electron microscopic observation.

From the results mentioned above, we conclude that the methods of infection and induction of nodulelike structures on rice roots by rice rhizobia are similar to those on the roots of legumes, but that the host cell defense systems were elicited.

Effect of rhizobia on rice growth and yield

Observations on the development of rice nodules and their fine structure are not yet complete, and whether the nodules fix N_2 is still not defined. However, we put rice rhizobia into pots with rice seedlings to investigate their effect on rice growth characteristics and yield. The experiments were carried out with four rice varieties.

Rice thus inoculated produced more tillers and more productive tillers, taller plants, thicker stems, and matured several days earlier than the control group. Inoculated plants had more grains per panicle and seed fertility was higher. A yield increase of about 10% was reported for most plants. Japonica rice varieties gave better results than indica rices.

These results demonstrate that inoculating rice seedlings with rhizobia is beneficial to growth and yield production.

Future development

Nodulelike structures are obtained by inoculating rice seedlings with rhizobia in the absence of any enzyme or PEG treatment. Growth and yield are improved by the presence of these structures. However, the results are a long way from Verma's requirements for an effective symbiotic association — living infected cells, fixed N_2 secreted and utilized directly by the plant, an energy balance (i.e., fixed N_2 utilized to compensate for exhaustion of carbohydrate), and O_2 protection of nitrogenase (Verma et al 1978).

One of the key bottlenecks is nitrogenase activity, which is not defined regardless of measurements of total plant N, acetylene reduction, and $^{15}N_2$. Large nodules are sparsely distributed on rice roots and the work is labor-intensive, so the measurements cannot be done at different stages of nodule development. Most of the rice nodules picked off were aged or senesced, so naturally no nitrogenase activity was observed. Immunological procedures should be used to determine the presence or absence of the products of nitrogenase activity to resolve whether the nodulelike structures do fix N_2.

Plant defense systems were elicited by bacteria in the infected cells. However, engineered rhizobia containing the *nodD* gene induced rice nodules without appearing to stress the infected cells (Rolfe and Bender 1990), although nodulation frequency was very low. The role of the *nodD* gene in plant defense systems and its regulation in *nod* gene expression should be considered. Biochemical and genetic analyses of rice rhizobia would improve understanding of the mechanism of the interaction between both partners and growth stimulation.

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Recommendations

Summary

Key questions for the future are how to supply the crop with N during the reproductive phase, how to optimize N use efficiency, and how to reduce the undesirable competition for N that exists between grain filling and the maintenance of a functional photosynthetic apparatus. If conventional uses of BNF are inadequate and if chemical N fertilizers are undesirable for economic and environmental reasons, we must look at unconventional sources of BNF and in particular at forcing rice to nodulate, to fix its own N_2, or to associate more efficiently with a free-living diazotroph.

The challenge is to design rogorous, informative, and nontrivial experiments that could be conducted in 3-5 yr to assess the feasibility of these novel approaches. Although our aim would be to improve the N nutrition of rice, any advances would apply to other cereals. The focus of such experiments should not be exclusively on the plants that we hope to modify. We must acknowledge the need to learn more about existing nodulation in its various forms and to gain a deeper insight into the productive potential of diazotrophs already resident in rice ecosystems. By the year 2000, we should be in a much better position to see our way forward and have in hand more powerful tools to isolate, manipulate, and exploit the relevant genes.

Practical goal

Ricefields need at least 200 kg N/ha per crop season for good yields. Any new form of N_2 fixation in rice should yield at least 20% of the total requirement, 40 kg fixed N/ha per crop, to be worth the research investment.

Research program

Proposed research activities are divided into three areas: symbiosis, root biology, and tools (see table).

Possible areas of collaborative research.

Research area	Nature of work
Symbiosis	
Selection/evaluation of novel bacteria	Discussion of screening protocols (Dénarié, Walker) Classification of organisms by new molecular techniques (REP/ERIC-PCR) (de Bruijn); 16S rRNA (Dart) Measurement of N_2 fixation (Dart)
Model systems	*Frankia* in existing symbiotic systems ((Mullin) *Azotobacter* and *para*-nodulation in wheat (I. Kennedy, C. Kennedy, Dénarié, Elmerich) Interactions between roots and *Rhizobium* or *Frankia* (Rolfe. Cocking, de Bruijn) *Rhizobium* in *Lotus* for determining function of specific genes involved in membrane biogenesis (Verma) *Azorhizobium caulinodans* in rice-O_2 regulation of N_2 fixation, crack entry, free-living fixation intercellularly in plants (de Bruijn) *Azolla* (Albersheim) *Rhizobium meliloti* and lateral development (Kondorosi) Role of hormones in nodule development (Dart)
Forced associations	*Frankia*/rice (Mullin) *Rhizobium*-rice interactions (Rolfe. Cocking, de Bruijn, Chumakov) Creating a 'biased rhizosphere' using rhizopines (de Bruijn) *Rhizobium/Arabidopsis* (Kondorosi)
Host specificity	Molecular basis (Dénarié, Spaink, Walker, Albersheim)
N secretion	In *Azoarcus, Azospirillum* (C. Kennedy) To *para*-nodules of wheat (C. Kennedy, I. Kennedy) By *Azorhizobium* (de Bruijn) Nitrogen transfer to plant (Elmerich, I. Kennedy, C. Kennedy)
Signaling	*Frankia/Alnus* (Mullin) Effect of *Rhizobium* nod signals on rice root morphogenesis (Rolfe, Dazzo, Cocking) Early events in host response to *nod* signals—leghemoglobin (Verma) Role of hormones (de Bruijn) Exopolysaccharide (Walker) Receptors for *nod* factors (Kondorosi) Major interest (Spaink)
O_2 barrier	Proton NMR imaging in living roots (Gresshoff) Transfer of Azotobacter genes for O_2 tolerance to *Azospirillum* or *Azorhizobium* (de Bruijn, C. Kennedy) Involvement of early nodulin *enod2* in reaction to O_2 barrier (de Bruijn)

Table continued

Research area	Nature of work
	Improvement in O_2 tolerance in *Azospirillum* (Elmerich, C. Kennedy) Involvement of early nodulin *enod2* in reaction to O_2 barrier (de Bruijn) Improvement in O_2 tolerance in *Azospirillum* (Elmerich, C. Kennedy)
Avoidance of defense response	cDNA clones from *Alnus* (Mullin) Bacterial molecules and genes involved in eliciting and suppressing host defense (Dénarié, Walker) Testing of known compounds on rice varieties (Spaink) Analysis of elicitors, mechanisms of defense response (Albersheim) How *Agrobacterium* avoids host defenses (Nester)
Root biology	
Root hairs	Biological and structural analysis (Rolfe, de Bruijn, Cocking) Effect of *nod* factors on gene expression in rice roots (Dénarié) Analysis of cell walls (Albersheim) Genes for root hair development in legumes (Bisseling) Biology of infection in *Frankia* (Mullin, Dart)
Endocytosis	Induction of endocytosis in rice root cells (Rolfe, Dazzo, Cocking) Membrane synthesis and receptors (Verma)
Lateral roots and other meristems	In conjunction with nodulation development (Mullin) Host genetics, control of patterns and cell divisions (Gresshoff) Recruitment of lateral root meristems as new foci of dividing cells for nodule development (Rolfe, Dazzo, Cocking) *Nod* factors and the induction of cell division in rice roots (Dénarié) Cell cycle genes in control of meristematic activity (Verma) Cytokinins in induction and nodule development (de Bruijn) Gene expression in *Frankia* nodules (Bisseling) Cytokinins and *Rhizobium* leaf curl genes (Dart)
N secretion	N transfer in *para*-nodulated cereals (C. Kennedy, I. Kennedy) Enzymes of N assimilation in cereals (I. Kennedy) Glutamine synthetase, ornithine amino transferase (Verma)
Carbohydrate transport	Sucrose synthase, proline biosynthesis (Verma)
O_2 availability	Measurement in roots (I. Kennedy) Role of leghemoglobins (de Bruijn)

Table continued

Research area	Nature of work
Exoderm	Correlated varietal analysis of exodermal layers in rice roots (Rolfe, Cocking, Dazzo) Rice root structure (Dart)
Microbial ecology	Soil/bacterial populations (Dart)
Tools	
Transformation	Helium gun technology in embryonic suspension cultures (Gresshoff) *Agrobacterium* in rice (Nester, Chumakov) Protoplast methods (Cocking) Basic technology (Verma, Elmerich) Transformation of legumes (de Bruijn)
Maps and markers	Diagnostics, pathogen/symbiont classification (Gresshoff) Detection of *nif* genes (de Bruijn)
Promoters	Sucrose synthase, leghemoglobin, glutamine synthetase (Verma)
Genes	Plant genes involved in *Alnus/Frankia* symbiosis (Mullin) Teleomere-centromere diagnostics, mutant in ammonium ion uptake (Gresshoff) Genes of phenylpropanoid pathway (Rolfe) *Rhizobium nod* and avirulence genes (Spaink)
Microscopy	SEM, TEM, laser scanning confocal microscopy (Rolfe, Dazzo, Cocking, Dart, Chumakov)
Carbohydrate science	All aspects—structure, purification, synthesis, function (Albersheim)

Symbiosis

Three approaches were suggested. First, to screen the microflora of the rhizosphere to detect exogenous and endogenous bacteria already capable of fixing N_2 and colonizing rice roots. Considerable interest was expressed in using knowledge gained from studies of other symbiotic associations to design screening procedures suitable for rice.

Second, to use genetic engineering to create a symbiotic association between rice and N_2-fixing bacteria already known to be free-living in the rice rhizosphere.

Third, to strengthen our knowledge by examining model symbiotic associations and to establish why these symbionts do not nodulate rice. Among the bacterial genera mentioned in this context were *Rhizobium, Azorhizobium, Azospirillum, Azoarcus,* and *Frankia.* It was suggested that, by engineering both the rice plant and one of these bacteria, it might be possible to establish a form of symbiosis in rice.

Among the topics thought to be important in pursuing these approaches were studies on host specificity, N secretion, signaling between host and symbiont, avoid-

ance of the plant's defense responses, and erection of a barrier to O_2 diffusion from plant to bacteroid during symbiotic growth.

The fern *Azolla* and its N_2-fixing symbiont *Anabaena* were also discussed. At present, *Azolla* is widely grown in flooded ricefields as a source of N. As an alternative to engineering both rice and a symbiont, it was suggested that the *Azolla-Anabaena* association be improved by, for example, increasing *Azolla's* resistance to insect predation using the techniques of molecular biology.

Root biology

Our ignorance of the rice root system was seen as a fundamental impediment to progress in any of the above areas. It was suggested that studies be initiated on key morphological features of plant roots which could aid symbiosis (root hairs, lateral roots, other potential meristems, exoderm, storage structures). Increasing our understanding of root meristems is important because some of the nodulation factors produced by symbiotic bacteria act as signals to initiate novel meristematic activity in the host, leading to nodular differentiation.

In addition, genetic and RFLP studies should be conducted on root structure and behavior to map key genes determining root hair frequency, lateral root initiation, etc.

There are large gaps in our knowledge of N, C, and O_2 metabolism in roots. The mechanisms of N metabolism need to be understood in order to utilize fully the N secretions from the bacterial partner. Carbon metabolism should be studied in terms of energy provision for N reduction and modification of source-sink relationships. Oxygen availability is crucial: too much O_2 would inhibit nitrogenase, too little would impair energy generation.

Tools

Most rice improvement programs using molecular mechanisms will require transformation, so the development of efficient protocols for rice transformation should be given high priority. In addition to seeking specific rice promoters, every attempt should be made to exploit promoters from other sources, especially other monocots.

Transformation of bacteria with reporter genes under the control of constitutive and inducible promoters would serve to tag bacteria, to identify pathways of bacterial movement within plants, and to characterize environmental conditions required for bacterial gene expression.

New developments in gene mapping and DNA fingerprinting would permit the characterization of plant genes responsible for novel root phenotypes, and bacterial genes determining host specificity and host defense responses.

An area of growth in the future will be the carbohydrate chemistry of host and bacterial cell walls. Such studies will also be important for studying signaling between host and bacterium.

Electron and light microscopy will be key tools in the study of structure and function in rice roots. Laser scanning confocal microscopy emerged as a useful new way of seeing nondestructively into plant tissue.

Commentary

Many other points of view emerged from the discussion sessions and a sampling of these views is recorded below.

Participation of IRRI

Participants saw IRRI as the logical place to conduct and coordinate the screening program to detect bacteria capable of colonizing rice roots and fixing N_2. IRRI's past experience on N nutrition in rice should be made available to collaborators to provide background information and act as a starting point for future research. IRRI should also identify promising rice varieties for use in research, including varieties with very different root morphologies (e.g., variations in frequency of root hairs and lateral roots).

Scope of planned research effort

Some participants thought that there was no compelling scientific or practical reason to exclude other major cereals from the program. Others argued that the program should exclude other cereals at this stage, and this exclusion should be justified on the grounds that rice is emerging as the model for molecular studies on cereals. It should be noted that while the status of *Arabidopsis* as the model system for molecular studies on plants was not challenged, many participants thought that *Arabidopsis* might not be totally suitable as a model for monocots or cereals. Rice could quite convincingly be put forward as the model system for monocots and cereals.

There was also discussion of broadening the program to include important areas in addition to N_2 fixation, for example, plant nutrition as a whole, or plant protection.

Time table

Participants were reluctant to estimate the time required to achieve the above goal by genetic manipulation of plant and bacterium. They were more concerned to suggest feasible research programs that would result in considerable progress within 5 yr. There was a general consensus to initiate certain programs immediately and then to meet again in about 2 yr to reassess the situation. Priority was implicitly given to five projects: 1) screening rice varieties for colonization by N_2 fixers under conditions of soil N deficiency, 2) assessing the rice nodulation system described by Prof. Yuxiang Jing, 3) assessing the potential of the wheat/*Azospirillum para*-nodulation system described by Prof. Ivan Kennedy, 4) measuring the response of rice to nodulation signals recently identified in the *Rhizobium*-legume symbiosis, and 5) expanding upon Prof. Marc van Montagu's study of the behavior of *Azoarcus* on kallar grass and rice.

Conclusion

The workshop members acknowledged the exciting challenge of achieving nodulation and N_2 fixation in rice through genetic engineering. They agreed to work toward that goal but recognized that its achievement is unlikely in the next 10 yr. However, much progress can be made by using molecular and cellular biology to enhance the level of

N_2 fixation during rice cultivation. This will probably be gained through an endophyte process. The great progress in our understanding of N_2-fixing symbiosis suggests that further exciting discoveries in plant science will assist in reaching the goal. The participants stressed the need to support fundamental plant and microbial science, for it is discoveries in these areas that will lead to the solution of the many problems involved in achieving symbiotic N_2 fixation in rice.

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