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Roots and Their Symbiotic Microbes: Strategies to Obtain Nitrogen and Phosphorus in a Nutrient-Limiting Environment

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Abstract

The association between *Rhizobium* and legumes and that between arbuscular mycorrhizal (AM) fungi and most land plants display a remarkable degree of similarity. Both events involve the recognition of, entrance into, and coexistence within the plant root, with the development of a specialized interface that always separates the two partners and at which nutrient exchange occurs. Molecules produced by rhizobia during the early stages of the symbiosis are related to fungal chitin, and the plant responds to both microbes with an increase in the production of flavonoids, which may assist in recognition and development of the symbioses. Many of the same plant genes are up-regulated in the two symbiotic pathways, and notably plants that are Nod^- are often defective in the AM association as well. However, there are a number of differences between the associations, and these are important for understanding the relationship between the two symbioses. The *Rhizobium* and AM symbioses will be compared and the question of whether the nitrogen-fixing association evolved from the much more ancient AM symbiosis will be discussed.

Key words: *Rhizobium*; Legume; Arbuscular mycorrhizal fungi; Symbiosis

Introduction

The term ''symbiosis'' was first used by de Bary (1879) to refer to ''the living together of differently named organisms.'' This definition encompasses both mutualistic and parasitic associations, but today symbiosis is usually thought of in terms of a mutually beneficial association rather than one in which one partner benefits more than the other. The mycorrhizal association between fungi and more than 80% of land plants (Newman and Reddell 1987) and the nitrogen-fixing interaction between bacterial members of the Rhizobiaceae and legumes are the two most commonly studied symbioses. The microbes benefit by acquiring photosynthates from the plant, and the plant benefits by obtaining phosphorus (P) or nitrogen (N), which are both limiting in soil.

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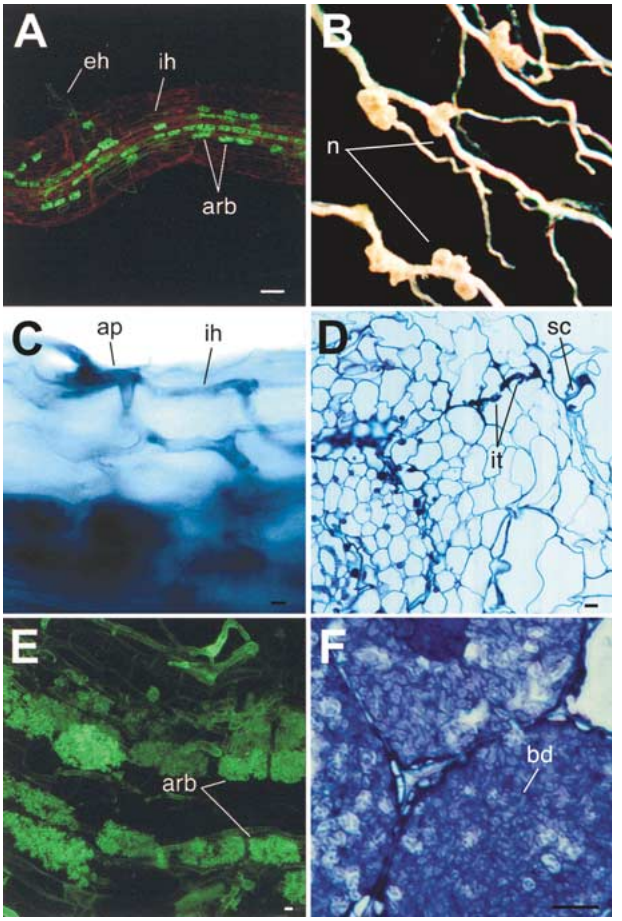

Figure 1. The *Rhizobium*-legume and arbuscular-mycorrhizal fungal symbioses. (**A**) Colonization of a white sweetclover (*Melilotus alba* Desr.) root by *Glomus intraradices*. Fungal structures stained with wheat germ agglutinin conjugated to Alexafluor 488, counterstained with acid fuchsin. arb, arbuscule; ih, intercellular hyphae; eh, external hyphae. (**B**) Indeterminate nodules of white sweetclover. n, nodule. (**C**) Appressoria formation of *G. intraradices* on white sweetclover and hyphal penetration into the root. Stained with Chlorozole Black E. ap, appressorium; ih, internal hyphae. (**D**) Infection threads travel from cell to cell within an alfalfa (*Medicago sativa* L.) nodule. sc, shepherd's crook root hair; it, infection thread. (**E**) Highly branched arbuscules of *Glomus intraradices* develop in the cortical cells of white sweetclover and are the site at which nutrient exchange is thought to occur. Stained with wheat germ agglutinin conjugated to Alexafluor 488. arb, arbuscule. (**F**) Bacteroids, which are the differentiated form of *Rhizobium* that fix atmospheric nitrogen into ammonia, in a pea (*Pisum sativum*) nodule, bd, bacteroid. Scale bars (C, D, E, F) represent 10 μM, and (A) 100 μM.

Over 80% of the P in the soil is immobile and not available to plant roots because plants preferentially assimilate inorganic phosphate (Holford 1997; Marschner 1995). However, arbuscular mycorrhizal (AM) fungi (Figure 1A) can assist the plant in obtaining mineral nutrients, primarily P, by using their extensive network of external hyphae, which branch and invade regions of the rhizosphere beyond the depletion zone formed by the root. In contrast to the rhizobia-legume symbiosis, the AM association has not been significantly exploited on a commercial basis. Nevertheless, many nitrogen-fixing symbioses fail because they are P-limited. Moreover, sources of P fertilizer are in short supply, which may result in a crisis in plant nutrition in the 21st century (Vance 2001). Thus, understanding the means by which AM fungi enable P acquisition and finding ways to employ this symbiosis for world-wide agriculture are of critical importance.

Molecular nitrogen (N_2) makes up approximately 78% of the atmosphere by volume; however, its very stable triple covalent bond ($N \equiv N$) makes it impossible for plants to use N_2 directly. Only prokaryotes that synthesize the oxygen-sensitive enzyme nitrogenase can reduce N_2 to ammonia (NH_3), which can later be converted to nitrate (NO_3). Those prokaryotes that fix N_2 include free-living bacteria such as *Clostridium* and *Azotobacter* as well as bacteria that live symbiotically with plants, namely, the Gram-positive *Frankia* species and members of the Gram-negative family Rhizobiaceae. In the case of the rhizobia-legume interaction, the symbiosis culminates in the formation of a novel plant organ, the nodule (Figure 1B), in which the bacteria fix atmospheric nitrogen. Fixed N can also be obtained industrially through the Haber-Bosch reaction (Smil 2001). However, the relatively high cost of producing N fertilizer, as well as some of the problems associated with its application, such as runoff into soils and water sources, provide incentive for increased exploitation of biological nitrogen fixation (BNF). BNF by the rhizobia-legume symbiosis results in approximately 30–35 Mt/year of fixed N. To ensure that enough food is produced to feed the world's increasing population, this amount of fixed N may have to be doubled or even tripled in the future (Smil 2001).

The arbuscular mycorrhizal and rhizobial symbioses, despite obvious differences, display a remarkable degree of similarity in some of the stages of their development. The object of this review is to discuss some of the central themes in these two symbioses and to highlight the similarities and differences that exist between them. Furthermore, the question of whether the rhizobial symbiosis may have evolved from the much more ancient mycorrhizal association will be considered.

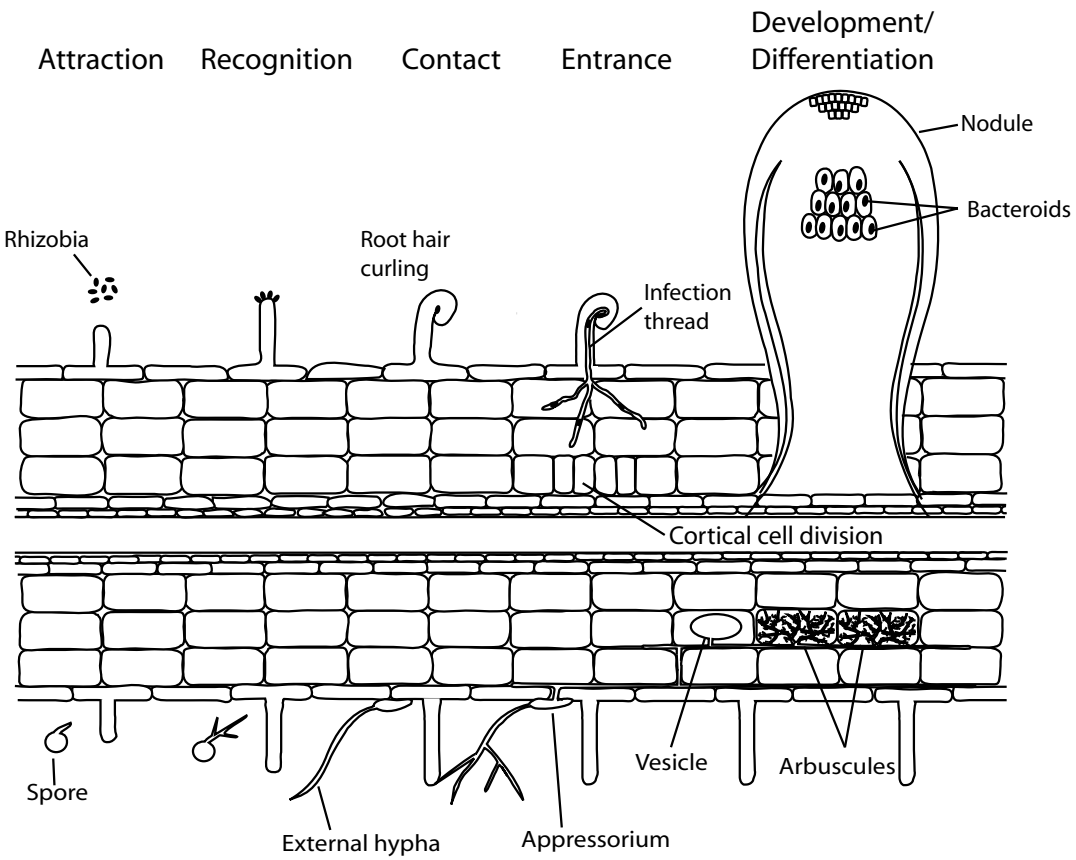

Figure 2. Progression and comparison of the AM fungal and *Rhizobium*-legume symbioses over time. Recognition by the microbes involves plant exudates, although they may not be essential in the AM fungal association. Attachment follows with rhizobia entrapment within a curled root hair and AM fungal hyphae develop appressoria on the surface of the root. Penetration of the microbes involves the invagination of the plasma membrane and deposition of cell wall material, so that the fungal structures and dividing rhizobia are always separated from the host cytoplasm. The final step illustrated is the differentiation of fungal hyphae into arbuscules and that of *Rhizobium* into nitrogen-fixing bacteroids housed in the cells of the nodule.

Overview

Arbuscular mycorrhizal (AM) fungi engage in the most common underground symbiosis that occurs with plants. They are obligate symbionts of the order Glomales (Zygomycotina), and include 149 species within 6 genera (Bentivenga and Morton 1994). Over 100 species of AM fungi are known to associate with nearly a quarter million species of plants, including angiosperms, gymnosperms, pteridophytes, and bryophytes, so this symbiosis is broadly based (Gadkar and others 2001). In contrast, the microbial players in the N_2-fixing symbiosis with legumes encompass five distinct genera in the Rhizobiaceae (*Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium*, *Rhizobium*) and also *Burkholderia* strain STM678 (Moulin and others 2001), collectively called rhizobia. Rhizobia carry *nod* (nodulation), *nif* (nitrogen fixation) and *fix* (symbiotic fixation) genes, often on pSym symbiotic plasmids, and the products of these genes are critical for establishment of a functional symbiosis. The nitrogen-fixing symbiosis with rhizobia is restricted to members of the Fabaceae with the exception of *Parasponia* (Ulmaceae). The *nod* genes in particular contribute to the particular host range that distinguishes each species.

The development of both the fungal and bacterial symbioses can be divided into an analogous progression of events: 1) attraction; 2) recognition; 3) contact; 4) entrance; and 5) development and differentiation (Figure 2). There are clear similarities between the symbioses at each of these steps, some of which prompted LaRue and Weeden (1994) to propose that the rhizobial symbiosis evolved from the much more ancient mycorrhizal symbiosis. However, not all the similarities can be attributed to evolutionary conservation, and some commonali-

ties may be due to the ubiquitous nature of certain regulatory mechanisms. In addition, there are obvious differences that are unique to each association.

The initiation of the AM association begins with the germination of the spore and contact of the fungal hyphae with the root epidermis. The hyphae differentiate and produce appressoria, swellings of the hyphae that develop on the surface of the root and provide a foothold upon which the fungus penetrates and enters the plant cell (Figure 1C). Upon disruption of the cell wall, the hyphae enter the root resulting in the invagination of the plant plasma membrane and the concomitant deposition of cell wall material. Some hyphae also travel intercellularly. The hyphae penetrate the cortical cell walls and highly branched, terminally differentiated hyphae known as arbuscules develop (Figure 1E). An extensive intracellular interface develops between the fungus and plant; the arbuscules are regarded as the key structure in the symbiosis and the periarbuscular membrane is thought to be the site of nutrient exchange. In some symbioses, vesicles are also formed and presumed to be storage organs (Smith and Gianinazzi-Pearson 1988). Outside the root, external hyphae play a critical role in the symbiosis in acquiring mineral nutrients for the plant, colonizing additional plants, and producing spores.

The association between *Rhizobium* and legumes is a multi-step process that begins when N-stressed plants release flavonoids, which attract the bacteria to the root and induce the transcription of rhizobial *nod* genes. The products of the *nod* genes result in the synthesis of Nod factor, considered a primary morphogen because the purified molecule can induce root hair deformation and curling, and in some legumes, cortical cell divisions. The rhizobia attach to the deformed root hairs and become entrapped in shepherd's crooks, which are 360° curled root hairs. The bacteria enter via infection threads (Figure 1D), which form from the invagination of the plant plasma membrane and the deposition of cell wall material. The infection thread extends into the dividing cortical cells. At the same time, the cortical cells divide to form the nodule primordium and the nodule meristem; the latter establishes the mature nodule (Figure 1B). Bacteria are endocytosed from the infection threads and eventually differentiate into bacteroids in the more mature regions of the nodule (Figure 1F). The bacteroids are surrounded by the peribacteroid membrane (PBM); the PBM, bacteroid, and interface between them is called the symbiosome (Roth and Stacey 1989). The plant provides a microaerobic environment in the nodule for nitrogenase to function, and nitrogen is fixed by the bacteroids.

Early Signaling Events

There is a molecular and chemical dialogue that occurs between AM fungi or rhizobia with each of their respective hosts; it is crucial for recognition and initiation of the symbioses. Plant exudates from the appropriate host are necessary to initiate the symbiosis. In the case of the nitrogen-fixing association, they induce the production of bacterial Nod factor, the bacterial molecule that dictates the specificity and progression of the symbiosis. Interestingly, there appears to be some overlap in the signals involved in both associations. For example, Nod factor application enhances mycorrhizal colonization (Xie and others 1995).

Plant Signals

AM fungal spores germinate and display a limited degree of hyphal growth in the absence of the plant host, but because the fungus is an obligate symbiont, growth is limited and the hyphae stop growing and die without a host. Plant root exudates promote germination and hyphal growth. They also induce rapid hyphal branching as the fungus approaches the vicinity of the roots. This growth-promoting effect appears to be host-specific in that *Poncirus* and *Trifolium* exudates promoted AM fungal growth and branching (Elias and Safir 1987), whereas exudates from a non-host such as *Lupinus albus* did not (Gianinazzi-Pearson and others 1989). The existence of a secreted signal was further demonstrated by the enhanced hyphal growth displayed by the fungus *Glomus mosseae*, despite its physical separation by a permeable membrane from the host-plant *Ocimum basilicum* (Giovannetti and others 1993). The signal appears to be less than 500 Da based on experiments with dialysis tubing (Giovannetti and others 1996), but is not a flavonoid (Buée and others 2000). Further research is necessary to reveal the identity of this signaling molecule and its importance to the symbiosis.

Flavonoids and phenolic compounds, present in plant root exudates, may also be involved in the early signaling by the plant to AM fungi. There are several major subgroups of flavonoids, including chalcones, flavones, flavonols, and anthocyanins, with variations in the number and position of hydroxyl groups, methyl groups, sugars, and other substituents (Figure 3A) (for a recent review see Harborne and Williams 2000). Flavonoids have

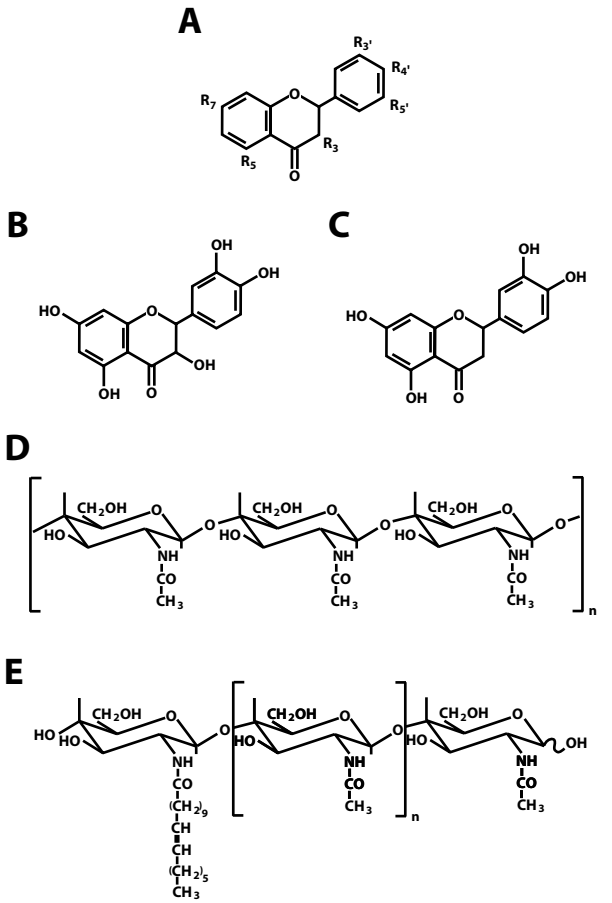

Figure 3. Signaling molecules involved in plant-microbe communication. (**A**) Generic structure of flavonoids. (**B**) Quercetin, a flavone, which has a 3'hydroxyl group, enhances AM fungal growth. (**C**) Luteolin, a flavonol, which lacks a 3'hydroxyl group, fails to promote hyphal growth, but is an inducer of the *Sinorhizobium meliloti nod* genes. (**D**) Chitin where n refers to the number of glucosamine residues in the backbone. (**E**) Generic structure of Nod factor where n is the number of glucosamine residues in the backbone. R_{3-7} refers to the various substituents.

been shown to influence the early stages of the mycorrhizal association, stimulating fungal spore germination and hyphal elongation. The flavonones hesperetin and naringenin, and the flavone apigenin were reported to stimulate hyphal growth on white clover *in vitro* (Gianinazzi-Pearson and others 1989). The efficacy of flavonols appears to reside in the hydroxyl group at position 3 (Figures 3B, 3C). For example, the flavonols quercetin and kaempferol stimulate hyphal growth of *Glomus margarita*, but luteolin and apigenin, flavones that lack the hydroxyl group, have either no effect or inhibit hyphal growth (Bécard and others 1992). There also appears to be a genus-specific relationship between the types of flavonoids that stimulate hyphal growth. For example, *Glomus* is stimulated by both flavonols and isoflavones whereas only flavonols appear to be effective inducers for *Gigaspora* (Chabot and others 1992).

Flavonoids have long been known to be involved in the *Rhizobium-legume* symbiosis and have been determined to be exuded from cells just behind the root tip (Djordjevic and others 1997). Specific flavonoids are secreted from the roots under N-stress, and are believed to attract the bacteria to the roots (Caetano-Anolles and others 1988). Additional specificity is observed in that flavonoids such as luteolin (Figure 3C) induce *Sinorhizobium meliloti nod* genes, and the isoflavones, daidzein and genistein, induce *Bradyrhizobium japonicum nod* genes (Long 1996; Phillips and others 1994). Furthermore, plants with mutations in flavonoid biosynthesis genes have fewer nodules (Hungria and Phillips 1993). Flavonoids have also been shown to increase the growth rate of *S. meliloti* (Hartwig and others 1991).

In both the bacterial and fungal symbioses, an increase in flavonoid levels is seen within the plant root in response to colonization. In the *Rhizobium* symbiosis, this is referred to as the Ini (***i***ncrease in ***n***od gene ***i***nducing flavonoids) response because there is an increase in the flavonoids, which in a positive feedback loop, induce bacterial *nod* genes (Recourt and others 1992). Such a strong correlation between flavonoids and nodule development suggested that these molecules could be involved in nodule organogenesis (Hirsch 1992; Mathesius and others 1998). Increased flavonoid biosynthesis is also detected in mycorrhizal roots. L-phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS; the first committed step in the phenylpropanoid pathway leading to flavonoid biosynthesis) transcripts were up-regulated in *Medicago truncatula* in response to colonization by *Glomus versiforme*, localizing in cells containing arbuscules (Harrison and Dixon 1993, 1994). Whether this reflects a role for flavonoids in colonization or is indicative of a defense response (as will be discussed later) is not clear. Bécard and others (1995) found that maize plants mutated in the CHS gene and therefore lacking flavonoids were colonized to the same degree as plants carrying a functional copy of the gene. These data suggest that flavonoids, functioning either as potential hormone regulators or as part of a defense response, are not essential for the establishment of the AM symbiosis. However, we have found that a *Melilotus alba* mutant with altered flavonoid production is hypercolonized by *G. intraradices*, indi-

cating that endogenous flavonoids can influence microbe colonization (M.R. Lum and A.M. Hirsch unpublished data). Furthermore, it was found that an enhancement of mycorrhizal colonization seen in response to Nod factor was correlated with an enhancement of specific flavonoids known to stimulate fungal colonization (Xie and others 1995). Clearly, more studies need to be conducted.

Microbe Signals

The rhizobial symbiosis, in contrast to the AM-fungal association, is highly specific not only in its almost complete exclusiveness to the legume family, but also in that specific strains of bacteria interact with specific species of legumes. This specificity is due, at least in part, to Nod factor. It is quite striking that the key bacterial signal molecule in the *Rhizobium* symbiosis, Nod factor, is related to chitin (Figure 3D), a molecule abundantly present in fungal cell walls (Bonfante-Fasolo and others 1990). However, Nod factor-like signal molecules have not been found in mycorrhizal fungi. van Rhijn and others (1997) were unable to detect a homologue to *nodC*, the bacterial gene encoding the enzyme responsible for producing the Nod factor core, although the *nodC* gene product does show some similarity to fungal chitin synthase.

Nod factors are monoacylated trimeric to pentameric chains of B-1, 4 linked, N-acetyl glucosamine units with a terminal non-reducing sugar N-acylated with a 16 to 18 carbon fatty acid (Figure 3E). There are varying chemical substituents on the reducing and non-reducing sugars and variations in the structure of the acyl chain; these variations are determined by the enzymes encoded by the bacterial *nod* genes. The glucosamine units are lipo-chitin oligosaccharide molecules that are absolutely essential for nodule development. Bacterial mutants deficient in Nod factor biosynthesis fail to induce root hair deformation or nodule formation (Long 1996). Purified Nod factor is able to promote root hair deformation, the initiation of pre-infection threads, and root cortical cell divisions (Lerouge and others 1990; Spaink and others 1991; Truchet and others 1991) although the latter response has been tested on only a few legumes (see references in Hirsch 1999). The chemical modifications are species-dependent and influence the specificity of the interaction. In the indeterminate nodules of alfalfa, the sulfate at the terminal reducing end is required for root hair deformation, cortical cell division, and nodulation. In addition, Nod factor application elicits the expression of many of the early nodulin (*ENOD*) genes, including *ENOD40* and *ENOD2* (Fang and Hirsch 1998). Indeed, *ENOD40* gene expression is enhanced when white sweetclover roots are inoculated with a Nod factor-overproducing *S. meliloti* compared to a typical wild-type strain (Giordano and others 2002). However, *ENOD40* and *ENOD2* are also up-regulated by cytokinin suggesting that Nod factor elicits a signal transduction cascade that involves a change in the endogenous hormone balance. Nevertheless, Nod factor's ability to function at nanomolar amounts suggests that there may be a receptor involved. The concentration of lipochitooligosaccharide molecules required to induce root hair deformation is significantly lower than that for inducing nodule primordia or *ENOD12* expression, both of which require the presence of specific Nod factor substituents (Stacey and Shibuya 1997). Whether or not there are one or multiple receptors for Nod factor to explain this differential activity is not clear at this time.

It is thought that there may be a fungal Myc factor analogous to the rhizobial Nod factor. Whether it is a chitin-like molecule remains to be seen. However, chitin fragments from fungal cell walls are known elicitors of a defense reaction in plants, and although it is not clear what role chitin plays in the AM symbiosis, mycorrhizae-competent plants such as rice are known to respond to chitin oligosaccharides by membrane depolarization, protein phosphorylation, and the induction of unique and defense-related genes (Stacey and Shibuya 1997). One hypothesis is that most plants carry an evolutionarily related nonspecific chitin receptor, which mediates immediate responses in the plant such as a defense response to fungal pathogens, or in legumes, *ENOD40* expression, which was shown to be induced by addition of a chitin pentamer without the host-specific decorations (Minami and others 1996).

Microbial Entrance into the Plant

Attachment and penetration of AM fungi or rhizobia is a multi-step procedure that involves varying degrees of host participation. Physiologically, the details of the internalization of the fungus or bacteria show the greatest similarity.

Shortly after fungal contact with the root, the development of the mycelium is accelerated (Mosse and Hepper 1975). The secreted plant signal described above is not sufficient to induce appressoria, a process that requires physical contact between the fungal hyphae and plant cell wall. This morphogenetic event is again plant host-dependent; *Gigaspora margarita* appressoria were formed on isolated

epidermal cell walls of carrot, but not on walls of the incompatible host, sugar beet (Nagahashi and Douds 1997). The plant does not appear to assist in the penetration of the fungus through the plant cell wall and therefore, it is thought that as with pathogenic fungi, AM fungi exert mechanical pressure in order to enter; the appressorium may serve that function. In addition, degradative enzymes such as exo- and endoglucanases, cellulases, xyloglucanases, and pectolytic enzymes, are produced, all of which could assist in the degradation and penetration of the cell wall (Harrison 1999). *Rhizobium* bacteria also release hydrolytic enzymes that act on the plant cell wall (Martinez-Molina and others 1979), but the major effector of root hair change is Nod factor. The exact mechanism whereby this occurs is still unknown.

As described above, the AM and rhizobial symbioses are each characterized by the internalization of the microbe and the differentiation of the organism into a form that can carry out the processing and exchange of nutrients between fungus or bacteria and the host plant. Specialized interfaces develop between the plant and fungal hyphae or bacteroids. The periarbuscular interface carries xyloglucans, nonesterified polygalaturonans, arabinogalactans and hydroxyproline-rich glycoproteins, which are characteristic of the plant cortical cell walls and similar to components in pea bacteroid compartments (Perotto and others 1990). However, unlike the AM association, the N_2-fixing symbiosis involves not only the entrance of the microorganism into the root, but also the differentiation of the plant cells to form the nodule, a new organ that accommodates the symbiont. This suggests that although the rhizobial symbiosis shares mechanisms of internalization in common with the much older mycorrhizal symbiosis, the ability to form nodules is distinct.

Conservation of Signaling Pathways

Perception: Mutants

A number of legume mutants have been identified that are both Nod$^-$ and Myc$^-$ (unable to form normal associations with mycorrhizal fungi). These tend to be blocked in the early stages of the development of the symbiosis, that is, at signal perception or symbiont penetration. The existence of these mutants supports the hypothesis that the rhizobial association evolved from the more ancient mycorrhizal association.

Because it is relatively easy to screen for Nod$^-$ mutants, co-segregation of the defect for both associations has greatly assisted the finding of mutants that are also Myc$^-$. The first such mutants were identified by Duc and others (1989), who reported that several Nod$^-$ mutants in pea and fababean were Myc$^-$. In pea, the *sym8*, *sym9*, *sym19*, and *sym30* mutants are both Nod$^-$ and Myc$^-$ (see Provorov and others 2002). Catoira and others (2000) found that the *dmi1*, *dmi2*, and *dmi3* mutants of *M. truncatula*, which are all blocked prior to root hair deformation, cortical cell division and *ENOD* induction, are Nod$^-$Myc$^-$. There are also several *L. japonicus* mutants that are both Nod$^-$ and Myc$^-$ (Senoo and others 2000). In addition, mutants have been found in alfalfa (Bradbury and others 1991) and *Melilotus alba* (Utrup and others 1993; Lum and others 2002). Typically, mutants found to be Myc$^-$ are blocked early in the N_2-fixation symbiosis and do not form infection threads in response to rhizobia, although root hair deformation may take place. This suggests that a mechanism for the initiation of the endosymbiosis may be shared (Parniske 2001). The genes that are defective in these mutants are for the most part unknown. However, the *Sym2* gene of *L. japonicum*, which corresponds to the pea ortholog *Sym19*, and a locus in *Medicago sativa* (alfalfa), which when mutated result in a Nod$^-$Myc$^-$ phenotype, have recently been cloned (Stracke and others 2002; Endre and others 2002). The mutants show root hair swelling, but no infection thread initiation, and the mycorrhizal association is terminated in the epidermis. The genes that encode the predicted wild-type protein, a receptor kinase known as SYMRK in *Lotus* and NORK in alfalfa, are placed very early in the pathway that leads to nodulation (Figure 4).

Nevertheless, there are mutants in pea (*Pssym10*), *L. japonicus* (*Ljsym1; Ljsym5*) and *M. truncatula* (*Mtnfp*) that are Nod$^-$ and Myc$^+$, indicating that these mutants are defective in their response to a Nod factor molecule, but not to a putative signal from AM fungi. Based on their phenotypes (lack of calcium spiking/alkalinization and root hair deformation in response to Nod factor application), the mutations are suggested to be in genes upstream of the SYMRK/NORK receptor kinase. The identity of the putative upstream genes is an active area of research.

Figure 4 summarizes a model based on data from a number of laboratories. For the *Rhizobium*-legume interaction, the *PsSYM10*, *LjSYM1*, *LjSYM5*, and *MtNFD* genes are likely to encode a *Rhizobium*-specific Nod factor binding protein or receptor (receptor kinase?) that is proposed to interact with the ligand. Although the genes are unknown, an analogous protein may exist for a putative Myc factor or al-

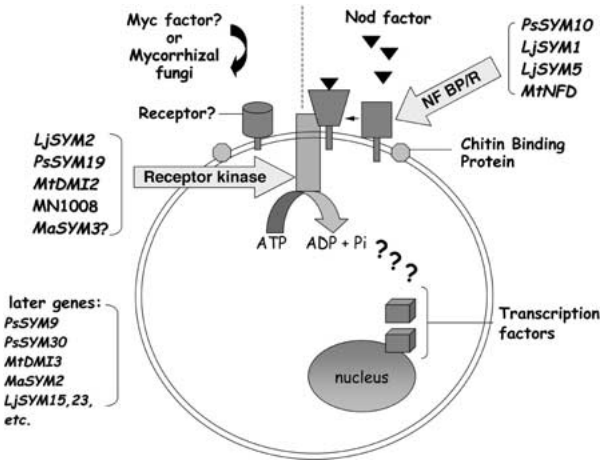

Figure 4. Model for the perception of Nod factor (right-side of dashed line) and a putative Myc factor or mycorrhizal fungi (left-side of dashed line). *PsSYM10*, *LjSYM1*, *LjSYM5*, and *MtNFD* genes are likely to encode a *Rhizobium*-specific binding protein or receptor that is proposed to interact with Nod factor. Although the identity of the analogous gene is unknown, there may be a receptor protein for a putative Myc factor. Alternatively, chitin fragments from AM fungal cell walls may attach to a chitin-binding protein. The binding proteins may then interact with a SYMRK/NORK protein, a receptor kinase, encoded by the *LjSYM2*, *PsSYM19*, *MtDMI2*, and *MN1008* genes and possibly by the *MaSYM3* gene. This interaction triggers a phosphorylation cascade (indicated by question marks) that leads to the activation of transcription factors targeting the expression of genes that are likely to be expressed later in the symbiosis, such as *PsSYM9*, *PsSYM30*, *MtDMI3*, *LjSYM15*, *LjSYM23*, and *MaSYM2* in various legumes. Based on data presented by various researchers at the 5th European Nitrogen Fixation in Norwich, UK (Sept. 6–10, 2002).

ternatively, AM fungi may interact directly with a chitin-binding protein. These proteins after a conformational change might interact with a SYMRK/NORK protein [*MaSYM3* may be a NORK ortholog based on the phenotype when mutated (see Lum and others 2002)] resulting in a phosphorylation cascade (indicated by question marks) that leads to the activation of transcription factors targeting the expression of specific genes. Although speculative, recent data support the Nod factor perception part of the model. The most tentative part is whether there is a fungal factor analogous to Nod factor and a receptor of this factor. If not, this would suggest that the initial mechanisms of rhizobia versus AM fungus perception are very different. Lastly for both symbionts, endocytosis of the microbe requires cell–cell contact, but as of yet little is known about the genes/proteins essential to this process.

Downstream Events: Gene Expression

Plant nodulin genes, originally defined because they were thought to be limited to the rhizobial symbiosis, have since been found to be expressed under non-symbiotic conditions, and/or during the mycorrhizal association. *MsENOD40* and *MsENOD2* are expressed in alfalfa mycorrhizal roots, but not in uncolonized roots or roots infected with the pathogen *Rhizoctonia solani*, indicating that the signal transduction pathway leading up to their expression is symbiosis-specific (van Rhijn and others 1997). *ENOD5* and *ENOD12* are also up-regulated by either rhizobia or AM-fungi (Albrecht and others 1998). The important role that *ENOD40* has in both associations has come from studies of transgenic *M. truncatula* plants where the gene is either over-expressed or co-suppressed. Charon and others (1999) found that over-expression of *MtENOD40* resulted in accelerated nodulation kinetics, whereas co-suppressed lines showed arrested nodule development with nodules that were small and lacking meristem organization and zonation. Subsequently, it was reported that these same over-expressing lines showed enhanced mycorrhizal colonization, whereas the co-suppressed lines showed reduced colonization by G. *mosseae* (Staehelin and others 2001).

Involvement of Plant Hormones

Plant hormone levels are affected in both symbioses. Nevertheless, their exact involvement has not been clearly defined, particularly in the case of the mycorrhizal symbiosis, where changes in hormone levels depend on the host species studied. Although there are similarities, which can correspond to similar changes in hormone-regulated gene expression, it is not clear that hormones are playing a comparable role in both associations, especially because these growth regulators are involved in all aspects of plant development.

Plants colonized by AM fungi have altered levels of auxin and cytokinin (see Barker and Tagu 2000). This alteration may account for some of the changes in gene expression that take place. For example, several cytokinin-inducible *ENOD* genes were found to be up-regulated in the alfalfa mycorrhizal symbiosis, including *ENOD2* and *ENOD40* (van Rhijn and others 1997). However, it is unclear whether the altered hormone level is due to plant or fungal production. AM fungi are known to synthesize some plant hormones (Barea and Azcon-Aguilar 1982). van Rhijn and others (1997) found that

alfalfa roots colonized by *G. intraradices* contain higher levels of trans-zeatin riboside than non-mycorrhizal roots, and this cytokinin accumulation was not observed in pathogenic interactions.

Endogenous ethylene concentrations increase upon inoculation of alfalfa, vetch, and soybean by *Rhizobium* (Ligero and others 1986; van Workum and others 1995; Suganuma and others 1995). This increase is enhanced by Nod factor application. However, ethylene can have an inhibitory effect on the rhizobial symbioses. This was first reported by Grobbelaar and others (1971), who found reduced numbers of nodules and a decrease in nitrogen fixation in the few nodules that formed on bean roots. Lee and LaRue (1992) further showed that treatment of intact pea roots with even very low amounts of ethylene, 0.007 μL/L, halved the number of nodules. In addition, 1-aminocyclopropane carboxylic acid (ACC), which is a direct precursor of ethylene, blocks nodulation (Penmetsa and Cook 1997). The inhibitory action by ethylene can be countered by the addition of ethylene inhibitors, such as aminoethoxyvinyl glycine (AVG) and silver thiosulfate, which result in an increase in nodule number. Interestingly, this inhibitory effect of ethylene appears to be species-specific. For example, soybean shows no ethylene response, although alfalfa and *Lotus* exhibit decreased nodulation in response to ethylene (see references in Geil and Guinel 2002). Expression of a mutated copy of the dominant gene *etr1-1*, which encodes an ethylene receptor, resulted in an increase in nodule number (Gresshoff and others 2001). In addition, the *sym5* and *brz* mutants in pea, which both show decreased nodulation, produced more nodules when treated with AVG (Geil and Guinel 2002). Oldroyd and others (2001) have shown that ethylene is involved early in the symbiotic pathway, regulating Nod factor signal transduction and calcium spiking. The inhibitory effect of ethylene appears to be due to its action in inhibiting infection thread progress. In the normal association, infection events far outnumber the number of threads that make it into the cortical cell layer, and so the enhanced ethylene seen in response to *Rhizobium* may be a means of regulating the infection process. In *sickle*, an ethylene-insensitive mutant of *M. truncatula*, there were almost no aborted infection threads; more than an order of magnitude in the number of persistent infections was detected (Penmetsa and Cook 1997).

Decreased colonization by *Glomus mosseae* in alfalfa in response to ethylene was first reported by Azcon-Aguilar and others (1981) by the application of ethrel. The addition of ethylene gas inhibited colonization of *Poncirus trifoliata* by *Glomus ramisporohora* (Ishii and others 1996) and of pea and leek by *Glomus aggregatum* (Geil and others 2001; Geil and Guinel 2002). However, details of the mechanism and contribution of ethylene to the mycorrhizal symbiosis is vague and contradictory. For example, it has also been found that the ethylene concentration in the host is altered. Although it was reported that potato colonized with *G. fasciculatum* had reduced ACC oxidase activity, therefore limiting ethylene production, flax roots colonized by *G. intraradices* (Dugassa and others 1996) had increased ethylene production whereas no change was observed in tomato roots colonized by *G. mosseae* (Vierheilig and others 1994).

Transport of Nutrients

The differentiation of the microbial symbionts corresponds to their becoming competent to exchange nutrients. The periarbuscular membrane and peribacteriod membrane, both of which surround the final differentiated structures, share a number of similar components, not the least of which are transporters to facilitate nutrient exchange.

The primary nutrient exchange that occurs between plants and AM fungi is phosphate for carbon. It is estimated that as much as 20% of the plant's photosynthetically fixed carbon is transferred to the fungus (Harrison 1999). Although it is assumed that this transport occurs at the arbuscular interface, the fungal arbuscular membrane itself has very low ATPase activity, which suggests that it is not involved in active transport (Gianinazzi-Pearson and others 1991; Smith 1993). In contrast, the intercellular hyphae have high ATPase activity, so it is also speculated that the transport may occur at this interface (Harrison 1999). The molecular nature of the transferred carbon remains unknown, although evidence points increasingly to it being glucose. Shachar-Hill and others (1995) demonstrated that ^{13}C was incorporated into glycogen, sucrose, and trehalose. In addition, it was shown that isolated arbuscules could use glucose for respiration when mycorrhizal root pieces were incubated with ^{14}C-glucose (Gryndler and others 1997; Solaiman and Saito 1997). The actual transporters involved are as yet unknown, although a transmembrane sugar transporter was cloned from *M. truncatula* that may be involved in glucose transport (Harrison 1996). However, it is not clear whether this glucose might then be transported into the fungus, or used by the plant cell itself.

The acquisition of P from the fungus by the plant also requires multiple steps. It is initiated with the uptake of P by the external hyphae, its transport in as-yet an unknown form to the internal fungal structures, and finally its release into plant root cells at the periarbuscular interface. Because the level of P in the soil is generally quite low (10 μM), it is generally believed that an active transporter must be involved in its uptake (Harrison 1999). A high affinity transporter that specifically localizes to the external hyphae has been cloned from *Glomus versiforme* (Harrison 1996). The proton motive force necessary for active transport of P from the fungus to the plant is likely provided by ATPases and candidates have been localized to the periarbuscular membrane (Gianinazzi-Pearson and others 2000). Recently a plant phosphate transporter has been cloned from potato, which functionally complements a yeast Pi uptake-deficient mutant, and whose transcript is specifically localized in arbuscule-containing cells, most likely the periarbuscular membrane, during symbiotic conditions (Rausch and others 2001).

In contrast to the AM association, rhizobia solely provide fixed N for the plant, which relies on their ability to produce nitrogenase. Nitrogenase is highly sensitive to oxygen and legumes provide a specialized environment in the form of a nodule for the bacteria to fix N_2. Oxygen is maintained at low levels due to a physical barrier in the inner cortex of the nodule and possibly to the presence of the O_2-scavenging enzyme leghemoglobin in the host cells. The plant-derived peribacteroid membrane surrounds the differentiated bacteroids and controls nutrient exchange between the symbionts. A monovalent cation channel with a preference for transporting NH_3 was identified in peribacteroid membrane fractions of pea and soybean (Tyerman and others 1995). Subsequently, Kaiser and others (1998) isolated *GmSat1* from soybean, which appears to be involved in transferring fixed N from the bacteria to the plant cell and was transcribed in nodules, immunolocalized to the peribacteroid membrane, and catalyzed the transport of NH_4^+. Ammonium itself is a highly toxic, reduced form of N and is immediately fed into the glutamine synthetase/glutamate synthase (GS/GOGAT) cycle, upon which N is transferred to other parts of the plant via the vascular system. Phosphoenol pyruvate carboxylase (PEPC) is a critical component in the N_2-fixing symbiosis, providing dicarboxylic acids. Import into bacteroids involves the action of two active transporters, one in the symbiosome membrane, and a rhizobial permease in the bacteroid membrane (Luyten and Vanderleyden 2000). These dicarboxylates are fed into the TCA cycle, which provide the energy and reducing power needed for N_2 fixation, and also supply carbon skeletons for amino acid synthesis.

Avoidance of Host Defense

For the symbioses to occur, there has to be some type of mechanism whereby the typical host defense response is suppressed. The plant has a number of defense responses to protect itself against invaders. These include both established and induced mechanisms, including cell wall modifications, upregulation of secondary metabolism, and the production of pathogenesis-related (PR) proteins (see Dixon and others 1994). Despite the symbiotic nature of the association between plants and AM fungi or *Rhizobium*, various mechanisms characteristic of a defense response are invoked. Gianinazzi-Pearson and others (1996) have described an increase in cell wall thickening at the site of appressorium formation, suggesting a host-defense response. In addition, it has long been observed that mycorrhizal plants display increased resistance to pathogens. Tomato plants previously colonized by *Glomus mosseae* showed reduced damage when infected with the pathogenic fungus *Phytophthora nicotianae* var. *parasitica*, and this was demonstrated using split root systems to be due to both localized and systemic resistance (Cordier and others 1998; Pozo and others 2002).

The defense response initiated by both symbionts is generally considered to be weak and transient, based on the expression of defense-related genes. After an initial induction, the symbiont appears to suppress the defense response displayed by the host. In the AM association, chitinases and B-1,3 glucanases, which hydrolyze the structural components of cell walls and have been shown to be inhibitors of fungal growth, are transiently induced (Lambais and Mehdy 1993; Volpin and others 1994). Salicylic acid (SA), which appears to have some effect on the effectiveness of both symbioses, and exogenous SA application resulted in a reduction in soybean nodulation (Sato and others 2002) and a delay, although not in appressoria formation, in mycorrhizal colonization of rice and tobacco (Blilou and others 2000). Furthermore, some of the same plant genes essential for initiating the symbiosis mediate this suppression, as shown by Nod^- Myc^- (*sym30*) pea plants, where rather than being suppressed, SA accumulation increased over time in response to either *Rhizobium* or AM fungi (Blilou and others 1999).

Evolutionary Aspects

The evolution of the mycorrhizal and rhizobial N_2-fixing symbiosis becomes interesting from an evolutionary point of view because there have been suggestions that the rhizobial symbiosis may have arisen from the much more ancient AM-fungal association. Although these two symbioses appear quite distinct, they have much in common physiologically and genetically, as has been discussed. It appears that the N_2-fixing association may have co-opted many of the mechanisms and tools already in place and streamlined them for a new type of symbiotic event.

Of the different types of mycorrhizal associations, the AM symbiosis is the most ancient; the fossil record indicates that this association may have co-evolved with land plants and shows arbuscules in association with the early Devonian land plant *Aglaophyton major* (Remy and others 1994). This and additional fossil evidence (Simon and others 1993) suggest that this association dates from 400 mya. The ancient origins of this symbiosis and the widespread and diverse presence of the association in greater than 80% of land plants, including mosses, liverworts, ferns, gymnosperms, and angiosperms (Read and others 2000), has led to the hypothesis that the association with mycorrhizal fungi was important for the evolution of land plants in the mid-Paleozoic (480–360 mya).

By contrast, the N_2-fixing symbiosis in legumes is much more recent, with nodulation presumably originating in the early Tertiary period during the Cretaceous (60–70 mya). In contrast to the AM-fungal association, the rhizobial symbiosis is specific in that, with the exception of *Parasponia*, only members of the legume family nodulate. Phylogenetic analysis suggests that a predisposition to nodulate arose in the Rosid I clade (Soltis and others 1995) because all nodulating plants, including the *Frankia*-nodulated actinorhizal plants, fall within this branch. Of the three subfamilies of legumes, the Papilionoideae, the Mimosoideae, and the Caesalpinioideae, over 90% of the first two families nodulate, whereas less than 30% nodulate in the more ancient Caesalpinioideae (Bryan and others 1996). Nodulation within legumes may have arisen independently, and evolved as many as three times, based on phylogenetic analysis (Doyle and others 1997). The latter may reflect why different nodule types exist (indeterminate vs. determinate) (see Provorov and others 2002). It may be that the production of the nodule and the infection of the plant with bacteria were two evolutionarily distinct events (Geil and Guinel 2002; Parniske 2001). Furthermore, a number of evolutionarily less advanced legumes produce nodule-like structures, reinforcing the idea that the machinery enabling nodulation was present in ancestral legumes. Additionally, there are instances of bacterial colonization without the formation of a nodule or nodule-like structure (Bryan and others 1996; de Faria and others 2000), again suggesting that nodulation and infection evolved independently.

Conclusion

In this review, we described some of the common themes that prevail in the *Rhizobium*-legume and AM fungal symbioses. It is clear that complex molecular and cellular changes occur in all partners and that mechanisms have evolved that are both common and unique for the association. The conservation of plant genes involved and required for both associations is a strong indicator that *Rhizobium* and AM fungi are utilizing some of the same machinery. The recent cloning and identification of SYMRK/NORK, a receptor kinase required for both associations, should be a prelude to a more complete understanding of what the AM fungal and rhizobial signals have in common. The early stages of colonization by each symbiont appear to have a common origin, and again, plant mutants that are defective in both associations are typically characterized by defects in infection thread formation that correlate to an early termination of hyphal proliferation within the root. The role that flavonoids and hormones may have played evolutionarily is less clear because both types of signals are ubiquitously present in plants and have diverse functions. However, it is intriguing that similar themes occur. The fact that both symbionts evoke a mild host defense response does not seem surprising, although it remains to be seen whether there may be a mechanism conserved between the two in suppressing this response. The fact that the signal initiates with early recognition seems likely because certain $Nod^{-}Myc^{-}$ mutants fail to show a repression of host defense, at least based on SA accumulation (Blilou and others 1999). However, the mechanism by which this occurs is unclear.

Although the mycorrhizal association is widespread among land plants, the *Rhizobium* symbiosis is highly specific to legumes, with the exception of *Parasponia*. The fact that the N_2-fixing symbiosis shows some commonality with the AM fungal association, even to the molecular level, is highly suggestive that *Rhizobium* co-opted and modified mechanisms generally utilized by AM fungi, but

then further refined the process to meet the demands of the N_2-fixing symbiosis via the development of the nodule. The larger question is why nonlegumes that establish mycorrhizal associations are unable to be hosts for rhizobia. Moreover, even certain legumes, for example, species of *Lupinus*, are unable to develop a mycorrhizal association although they nodulate in response to rhizobia (Vance 2001). Additional research will further elucidate the processes involved and perhaps help us understand the importance of these two ecologically and agriculturally significant symbioses.

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