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Root-based N_2-fixing symbioses: Legumes, actinorhizal plants, *Parasponia* sp. and cycads

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Abstract

In the mutualistic symbioses between legumes and rhizobia, actinorhizal plants and *Frankia, Parasponia* sp. and rhizobia, and cycads and cyanobacteria, the N_2-fixing microsymbionts exist in specialized structures (nodules or cyanobacterial zones) within the roots of their host plants. Despite the phylogenetic diversity among both the hosts and the microsymbionts of these symbioses, certain developmental and physiological imperatives must be met for successful mutualisms. In this review, phylogenetic and ecological aspects of the four symbioses are first addressed, and then the symbioses are contrasted and compared in regard to infection and symbio-organ development, supply of carbon to the microsymbionts, regulation of O_2 flux to the microsymbionts, and transfer of fixed-N to the hosts. Although similarities exist in the genetics, development, and functioning of the symbioses, it is evident that there is great diversity in many aspects of these root-based N_2-fixing symbioses. Each symbiosis can be admired for the elegant means by which the host plant and microsymbiont integrate to form the mutualistic relationships so important to the functioning of the biosphere.

Introduction

The ability of a plant to supply all or part of its N requirements from biological nitrogen fixation (BNF) in its roots can be a great competitive advantage over non-N_2-fixing neighbours. BNF is the conversion of atmospheric N_2 to ammonium, a form of N that can be utilized by plants. However, BNF is in the sole domain of certain bacteria (diazotrophs), which contain nitrogenase, the enzyme complex that catalyzes the conversion of N from the gaseous to the combined form. Occurrence of N_2-fixing bacteria with higher plants is not uncommon, but in most case these are only 'associations', in which relatively free-living bacteria grow in the rhizosphere, on the rhizoplane, or more rarely, in non-specialized intercellular spaces in plants (Vessey, 2003). The transfer of fixed N from the bacterium to the plant in these associations is relatively low, and the relationship between the two organisms could be viewed as opportunistic rather than mutualistic. However, in a much smaller proportion of cases across the plant world, the association between plant and bacterium is much more intimate, with the N_2-fixing bacterium being housed within specialized plant organs. In these truly mutualistic symbioses, the genetics and physiology of the plant and bacteria are integrated to the extent that the two organisms can appear to function nearly as one.

On several occasions, symbioses have evolved between terrestrial plants and N_2- fixing bacteria, leading to the existence of specialized organs on the host plants that provide excellent environments for the prokaryotes to infect, live, and fix N_2. Although the genetic backgrounds and physiological functioning of these symbioses can be seen as very diverse, there are several developmental and physiological 'imperatives'

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that must be met for successful symbioses between host plants and their N_2-fixing microsymbionts. These imperatives include:

– the ability of the microsymbiont to infect and colonize host plant organs

– the ability of the host plant to supply energy and nutrients to the microsymbiont

– the ability of the host plant and microsymbiont to regulate O_2 flux

– the ability to transfer the fixed N from the microsymbiont to the host

Four such extant plant root symbioses are the partnerships between legumes and rhizobial bacteria, actinorhizal plants and *Frankia* bacteria, *Parasponia* and rhizobial bacteria, and cycads and cyanobacteria. In this chapter we will compare and contrast these four symbioses using these criteria. However, we will first address evolutionary and ecological aspects of the symbioses. A challenge in comparing these symbioses is the unbalanced level of knowledge that exists for the four symbioses; i.e. the legume-rhizobium symbiosis is very well studied, there is a relatively good level of knowledge on the actinorhizal-*Frankia*, information on the *Parasponia*-rhizobia symbiosis is limited except on some topics (e.g., hemoglobin), and knowledge about the cycad-cyanobacterial symbiosis is scant. Nonetheless, we will see that despite quite varied genetic makeup and evolutionary backgrounds, each of the four symbioses has developed equally elegant means to meet the physiological and developmental imperatives for a successful symbiosis.

Evolutionary and ecological considerations

The four symbioses addressed here represent wide ranges in both evolutionary/phylogenetic and ecological contexts. However, the legume, *Parasponia*, and actinorhizal plant symbioses can be seen as more closely related to each other compared to the cycad symbiosis. This is quite obvious given that the legumes, *Parasponia* and actinorhizal plants are angiosperms (and may even have had a common ancestor, see below), whereas cycads are gymnosperms. From the microsymbiont perspective, there is an extreme diversity with rhizobia (gram negative) being members of the α-subgroup of the phylum Proteobacteria, *Frankia* (gram positive) from the high-GC subgroup of the phylum Actinobacteria, and the Cyanobacteria (gram negative) representing their own phylum of photoautotrophic non-proteobacteria.

Cycads represent an ancient life form, have a unique placement in terrestrial plant evolution, and are among the most primitive extant seed-plants (Brenner et al., 2003; Schneider et al., 2002). They are ever-green and have a palm-like appearance with a thick, often columnar stem and rosettes of long pinnately compound leaves (see Costa and Lindblad, 2002; Lindblad and Bergman, 1990). They may range in height from about 0.2 to 20 m. The presence of prominent reproductive cones (male and female) reveals their true gymnosperm nature. Extant cycads may be divided into three families: Cycadaceae with the genus, *Cycas*; Stangeriaceae with the genera, *Stangeria* and *Bowenia*; and Zamiaceae comprising eight genera, *Zamia, Chigua, Ceratozamia, Dioon, Encephalartos, Lepidozamia, Microcycas* and *Macrozamia*. Together, about 240 extant species have been identified within the order Cycadales. Nucleotide sequencing of the *rbcL* gene suggests that the cycad genera cluster monophyletically, and that the genus *Cycas* forms a basal group (Treutlein and Wink, 2002). Molecular data also imply that extant cycad genera and species may not have evolved until within the last 50 million years. Cycads dominated the Earth's forests from Greenland and Alaska to Antarctica about 250 to 65 Myr ago, long before the advent of angiosperms (Brenner et al., 2003; Schneider et al., 2002). During this period the climate was warmer, wetter and low in seasonality. As CO_2 levels were also higher, this may have enhanced plant growth and the N demand, which in turn may have stimulated the development and maintenance of the cycad-cyanobacterial symbiosis. Hence, the well-developed symbiotic relationship between cycads and cyanobacteria may be due to a long-lasting co-evolution between the organisms. As the cyanobacteria are ancient organisms that arose some 3 Byr ago (Schopf et al., 2002), symbiotically competent cyanobacteria may have been wide-spread long before the cycad started to dominate the global terrestrial vegetation.

Today, the ecological distribution of cycads is considerably more limited. Many cycads are endangered due to the drastic climate change, commercial exploitation, and anthropogenic activities. Species within the *Cycas, Encephalartos* and *Macrozamia* genera are still well represented, predominantly in warmer and more humid tropical and subtropical regions. Species within the genera *Macrozamia* and *Zamia* are also common in dryer soils of low fertility in Australia, and often comprise an understorey vegetation of *Eucalyptus* forests (Grove et al., 1980). More than 35 species of the genus

Encephalartos (endemic to Africa) constitute the large cycad flora in South Africa, while *Stangeria* is endemic to its coastal regions (Grobbelaar et al., 1986). Due to their highly decorative appearance, cycads are widely cultivated and used as ornamental plants in greenhouses and in private and public botanical gardens world-wide.

The capacity of cycad-cyanobacteria symbioses to fix N_2 has been confirmed in a limited number of field and laboratory studies (see Rai et al., 2000, 2002). Grobbelaar et al. (1986) also demonstrated that all 33 species of *Encephalartos* and one *Stangeria* tested, using the acetylene reduction technique as well as ^{15}N-enrichment, were capable of fixing N_2. In the field, two *Macrozamia riedlei* stands growing naturally in Western Australia in *Eucalyptus* forests fix 19 kg N ha^{-1} yr^{-1} (Halliday and Pate, 1976) and about 8 kg N ha^{-1} yr^{-1} (Grove et al., 1980), respectively. These rates are essentially similar to those of free-living cyanobacteria, while much lower than those recorded for legume-rhizobia symbioses (see below). However, the much lower growth rate of cycad tree species compared to annual legume crop species should be taken into consideration when comparing N_2 fixation rates between these two symbioses. The proportion of cycad N derived from fixation of atmospheric N (%NDFA) is not known. Additional field studies are needed to fully estimate the N_2-fixing capacities and ecological significance of cycad-cyanobacterial symbioses.

In contrast to the cycads, legumes, *Parasponia* and actinorhizal plants are flowering plants encompassing families all found within the Eurosid I clade of the Eudicots (Soltis et al., 2000). Legumes are within the order Fabales and represented by a single family, the Fabaceae (formerly the Leguminosae); however, most of the more than 650 genera in the family contain species that can form rhizobial root nodules. *Parasponia* is one of the 18 genera of the Ulmaceae (order Rosales), and is the only genus outside the legumes known to enter an N_2-fixing symbiosis with rhizobia. There are only five identified species of *Parasponia* (Becking, 1992), with *P. andersonii* appearing to be the most commonly studied. In contrast, actinorhizal plants (which are simply defined as a group by their ability to be nodulated by *Frankia* bacteria) are more taxonomically diverse. They encompass 25 genera, in eight different families, in three different orders: the Betulaceae, Casuarinaceae and Myricaceae of the order Fagales; the Rosaceae, Rhamnaceae and Elaeagnaceae of the order Rosales; and the Coriariaceae and Datiscaceae of the order Cucurbitales (Gualtieri and Bisseling, 2000).

It was once thought that apparent taxonomic diversity among legumes, *Parasponia* and actinorhizal plants indicated that N_2-fixing symbioses in flowering plants evolved independently several or possibly many times (Baker and Mullin, 1992). However, nucleotide sequencing of the *rbcL* gene of 99 taxa in the Eurosid I lineage infers that all eight families capable of N_2-fixing symbiosis exist within a 'nitrogen-fixing clade' (Soltis et al., 1995). These data suggest a single origin of the predisposition for nodulating, N_2-fixing symbioses in flowering plants. If there was only one origin, then the fact that not all extant taxa in the N_2-fixing clade are capable of N_2-fixing symbiosis implies a loss of the capacity in those taxa. This loss might have been due to high metabolic cost to the plant for N_2 fixation compared to assimilation of combined N from the soil (Layzell, 1990). However, although the potential for nodulation may have a common ancestry, analysis of symbioses-related genes appears to indicate that actual symbioses have arisen multiple times within the clade, at least among both the legumes and actinorhizal plants (Doyle, 1998; Swensen, 1996).

Origins of the legume, *Parasponia* and actinorhizal-plant symbioses are highly likely to be much more recent than the cycad-cyanobacteria symbiosis given that angiosperms did not evolve until 250 to 150 Myr ago (Sprent and Raven, 1992), a period when cycads were already the dominant flora on the planet. Today, legumes are the third-largest family of flowering plants with over 18,000 species; however, not all species are capable of forming N_2-fixing symbiosis. Legumes are an incredibly morphologically and ecologically diverse group of plants, ranging from small forbs to large trees, and occur from the Arctic tundra, to tropical rainforests, to arid deserts (Allen and Allen, 1981). The legumes represent many of our most important grain and forage crops including soybean (*Glycine max* L. Merr.), common bean (*Phaseolus vulgaris* L.) and alfalfa (*Medicago sativa* L.). Peoples et al. (2002) have shown that across a wide range of environments and species, legumes commonly fix approximately 25 kg N per tonne of above-ground dry matter. With root biomass also taken into account, the amount of N_2 fixed by legumes can be in the range of 300–400 kg N ha^{-1} yr^{-1} (Kelner et al., 1997; Peoples et al., 2002).

Actinorhizal plants represent approximately 200 species of woody shrubs and trees predominantly in temperate climes but also extending into the tropics,

especially the Casuarinaceae. Like the wild (non-crop) legumes, actinorhizal plants have a propensity to grow in marginally fertile soils, and many as early-successional plants (Benson and Silvester, 1993). Hence actinorhizal plants play extremely important roles in the N cycle of forests and in the revegetation of various landscapes (Benoit and Berry, 1990; Schwencke and Caru, 2001). Rates of N_2 fixation in the range of 30 to 50 g N tree^{-1} season^{-1} are possible, but actual rates in the field are often lower due to environmental stresses such as drought, nutrient limitation, or nematode predation (Dommergues, 1995). For example, Mariotti et al. (1992) found N_2 fixation rates of only 15 kg N ha^{-1} yr^{-1} for a 3-year old *Casuarina equisetifolia* stand in Senegal. Actinorhizal plants have been used in erosion control, soil reclamation, agroforestry and dune stabilization, as well as in fuel wood, pulp and timber production. For instance, Casuarinaceae are utilized in stabilizing desert and coastal dunes (i.e. in shelter belts), and in the reclamation of salt-affected soil as well as in intercropping systems (Diem and Dommergues, 1990).

It was only 30 years ago that Trinick (1973) first reported the N_2-fixing, root-nodule-forming symbiosis between *Parasponia* (initially classified as *Trema* sp.) and rhizobia. The *Parasponia*-rhizobia symbiosis is represented by only five species of tropical tree which can grow up to 15 m and are native to the Indo-Malaysian Archipelago and the Pacific Islands, from Sumatra in the east to as far west as Polynesia (Becking, 1992; Webster et al., 1995a). As with the other symbiosis these trees are often pioneering species in very nutrient-poor soils (e.g., volcanic ash). Despite its microsymbionts being rhizobia, *Parasponia* is taxonomically and phylogenetically closer to members of the actinorhizal members of the Rosaceae, Rhamnaceae and Elaeagnaceae than to the legumes (Soltis et al., 1995). Quantification of N_2 fixation in *Parasponia* are rare and no doubt reflect the challenges in making such measurements in trees species, as is the case in many actinorhizal plants. However, acetylene reduction assays of intact and detached *Parasponia* nodules generally indicate that nitrogenase activity per unit mass of nodule is lowers than that seen in legumes by 0.5 to 1.0 orders of magnitude (see Becking, 1992). It is particularly interesting that when rhizobial isolates from *Parasponia* were used to infect the legumes *Vigna* sp. (Becking 1983a) and *Macroptilium atropurpureum* (Price et al., 1984), the nitrogenase activity per unit mass fresh weight of the legume nodules was approximately three times that of the *Parasponia* nodules. These results suggest that *Parasponia* as a host is more responsible for the lower levels of nitrogenase activity (compared to the legumes) than its microsymbiont. Nonetheless, from observations of its growth rate under optimal conditions in a glasshouse, Trinick (1981) estimated that a plantation of 6-month old *Parasponia* might fix as much as 850 kg N ha^{-1} yr^{-1}.

On the microsymbiont side, the phylogenetic relationships between rhizobia, *Frankia*, and cyanobacteria can be considered even more distant than those of their plant hosts, with each group coming from separate eubacterial phyla. Rhizobia is a generalized term referring to bacteria from the genera *Allorhizobium, Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium*, and *Sinorhizobium*. Individual legume species are often infected by a single species of rhizobia, but in some cases multiple species, even in some cases from multiple genera, infect a single host species (e.g., soybean [*Glycine max*] is infected by *Brayrhizobium japonicum, B. elkanii*, and *Sinorhizobium fredii*). *Parasponia* is infected by rhizobial strains belonging to the *Bradyrhizobium* and *Rhizobium* genera. Although there is cross inoculation between rhizobial isolates from *Parasponia* and some legumes (e.g., *Macroptilium* sp. and *Vigna* sp.) (Becking, 1992), *Parasponia* strains tend to display specificity for *Parasponia* as a host. For example, although *Bradyrhizobium* strains isolated from a wide range of legume hosts could induce nodules on *Parasponia*, the nodule morphology was commonly abnormal, and there was little or no nitrogenase activity (Becking, 1983a; Trinick and Galbraith, 1980).

Actinorhizal plants are only nodulated by strains of actinomycetes from the genus *Frankia*. Whereas 'host-specificity' (the selectivity of the plant host for specific species, or even sub-species, of microsymbiont) is useful in species designation in the rhizobia, this phenomenon is not nearly as effective in *Frankia*, and even with the abundance of molecular tools at our disposal, identification of speciation in the genus is still a difficult task (Benson and Silvester, 1993; Clawson and Benson, 1999).

All cycad genera are capable of forming well developed root-symbioses with cyanobacteria, and filamentous heterocystous species within the genus *Nostoc* are the dominant microsymbionts (Rasmussen and Nilsson, 2002), although others have been identified (e.g. *Calothrix* sp.; Grobbelaar et al., 1978). Considering the large variety of terrestrial cyanobacterial genera, the dominance of the genus *Nostoc* in cycad and other plant symbioses implies that specific sym-

biotic characters are held by this genus. In spite of the relatively narrow diversity among the cyanobacterial cycad colonizers, a recent study using PCR fingerprinting with primers derived from short tandemly repeated repetitive sequences demonstrated that a single root cluster can be colonized by several strains of the cyanobiont (Zheng et al., 2002).

The phylogenetic diversity among the microsymbiont groups in the four symbioses under discussion is not surprising. Although the structure, function, and amino acid sequence of nitrogenase is highly conserved throughout the domain, the ability to fix N_2 is 'sprinkled' in many taxa throughout the Eubacteria (Reinhold-Hurek and Hurek, 1997). This situation of a highly conservative genetic trait being widely and diversely spread among taxa is more comprehensible if horizontal (lateral) transfer of aspects of this trait were at play (Zehr et al., 2003). Another possibility is that widely spread symbiosis genes have been lost in some organisms through evolution (e.g., due to increases in N contents of soils and water), and that only those in which N_2 fixation was a competitive advantage for survival retained them. Analysis of partial *nifH* gene products (a common marker for the enzyme nitrogenase reductase) demonstrates a very broad phylogenetic range among N_2-fixing prokaryotes (Figure 1). Interestingly enough, rhizobia, *Frankia*, and cyanobacteria cluster together more closely than many other diazotrophs (Reinhold-Hurek and Hurek, 1997; Zehr et al., 2003). Many nitrogenase-encoding genes (*nif* genes) as well as genes related to enabling symbiosis with host plants (e.g., *nod* genes) are clustered in 'symbiotic islands' on the chromosome or on plasmids as in rhizobia and *Frankia* (Lavire and Cournoyer, 2003). There is strong evidence for lateral transfer of some symbiosis genes within rhizobia (Parker et al., 2002; Qian et al., 2003; Suominen et al., 2001) and some evidence for such transfer in *Frankia* (Lavire et al., 2001; Lee et al., 2001).

An interesting evolutionary convergence among rhizobia, *Frankia* and cyanobacteria is the occurrence of NiFe hydrogenase-uptake systems in all three microsymbionts. The reduction of protons to H_2 gas by nitrogenase is an obligatory process in N_2 fixation in all four symbioses. For example, in legume-rhizobia symbioses, commonly 25 to 60% of electron flow through nitrogenase may be used to produce H_2 (Layzell, 1990). The ATP and reductant utilized to form this H_2 by-product can be seen as lost energy, as there is no apparent useful function for H_2 in the microsymbiont or the host. In some diazotrophs scavenging systems have evolved, so-called uptake hydrogenases (Hup+), which oxidize the H_2 produced by nitrogenase, recovering the ATP and reducing power. Uptake hydrogenase is widespread in rhizobia (Baginsky et al., 2002), and the small and large subunits of this heterodimeric enzyme are coded for by the *hupS* and *hupL* genes, respectively. Specifics on the uptake hydrogenase of the *Parasponia*-rhizobia symbiosis are not known, but the system is known to be active due to the negligible levels of H_2 evolution from nodules as a proportion of total nitrogenase activity (Becking, 1983b). Likewise, uptake hydrogenase activity is widespread in *Frankia* (Benson et al., 1980; Mattsson and Sellstedt, 2002; Roelofsen and Akkermann, 1979). Antisera raised against the large subunit of *Bradyrhizobium japonicum* hydrogenase recognized this protein in *Frankia* KB5 (Mattsson et al., 2001). Recently the two filamentous heterocystous cyanobacteria *Anabaena* strain PCC 7120 and the symbiotically competent *Nostoc punctiforme* (Nostoc PCC 73102), have been shown to contain uptake hydrogenases coded for by the *hupSL* genes (Tamagnini et al., 2002).

Establishment of the symbioses: Signal exchange, infection, and symbio-organ development

Although all four N_2-fixing root symbioses result in specialized root organs to house the microsymbionts, the establishment of these organs and their structure is quite different. While the root nodules of *Parasponia* sp., actinorhizal plants and the symbiotic coralloid roots of cycads are modified roots, the root nodules formed in the legume-rhizobia symbiosis arise from unique zones of cell division in the root cortex. In this section we will first discuss signal exchange between the partners in each symbiosis, and then address the actual infection and symbio-organ development.

Signal exchange

The establishment of the legume-rhizobia symbioses is initiated by the exudation of flavonoid and isoflavonoid compounds (e.g., genistein, naringenin, luteolin) from the host plant (Miklashevichs et al., 2001). These substances act as both chemo-attractants to the rhizobia and inducers of the *nod* genes (i.e. the regulatory *nodD* genes and the structural *nodABC* genes) in the rhizobia (see below). Other substances implicated in chemo-attraction and proliferation of rhizobia in legume rhizosphere include nutrients (amino acids,

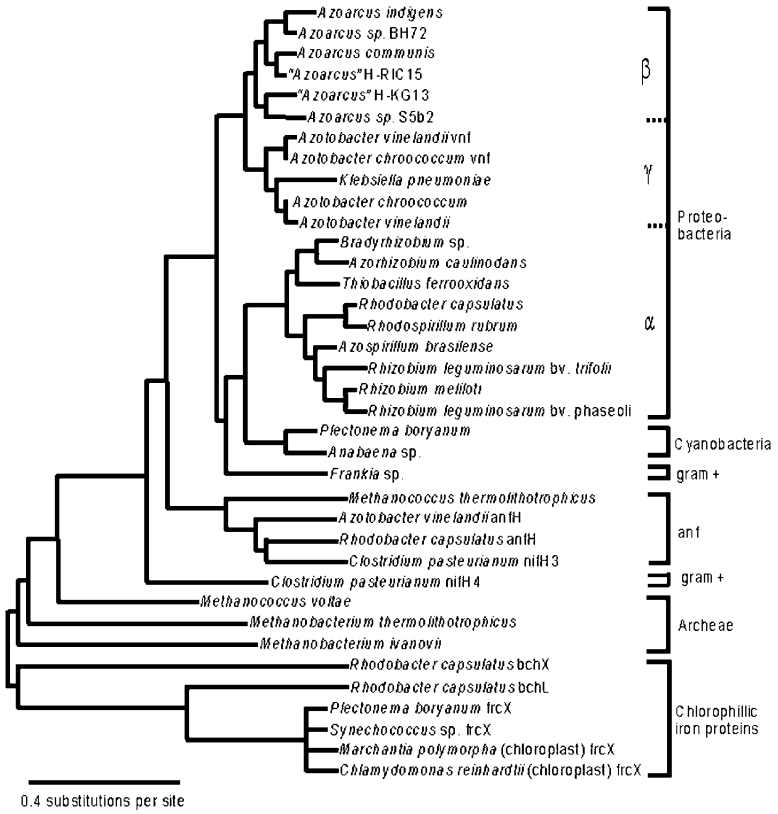

Figure 1. Phylogeny of partial NifH protein sequences from a wide range of diazotrophic bacteria and Archea. Also included are partial sequences of NifH-like iron-sulfur proteins associated with chlorophyll (bchXL and frxC). Designations 'vnf' and 'anf' refer to sequences associated with the vanadium and the iron-only (non-Mo) forms of nitrogenase, respectively. Adapted from Heinhold-Hurek and Hurek (1997).

organic acids, and sugars; e.g., Pandya et al., 1999; Robinson and Bauer, 1993) and secondary metabolites such as betaines (Phillips et al., 1995). Likewise, the non-protein amino acid toxin, mimosine, produced by some tree and shrub legumes, enhances the abundance of specific rhizobial strains in the rhizosphere (Soedarjo and Borthakur, 1998).

There is no information on the excretion of signalling compounds from *Parasponia* roots to its microsymbiont. However, given that the nodulation genes *nodABC* and *nodD* are highly conserved in rhizobia, including *Parasponia*-rhizobia (Marvel et al., 1987; Scott et al., 1987), we can infer that flavonoid and/or isoflavonoid compounds are exuded by the plant to induce these *nod* genes (Bender et al., 1987a).

There is some evidence of chemo-attraction and proliferation of *Frankia* in the rhizospheres of *Betula pendula* (Smolander et al., 1990) and *Alphitonia neocaledonica* (Gauthier et al., 2000), non-nodulating species of the Betulaceae and Rhamnaceae, respectively, but the nature of the exuded substances is unknown. The actinorhizal species *Alnus glutinosa* exudes flavonols (e.g., quercetin and kaempferol) that can enhance the level of nodulation; however, their exact role in the process is unknown (Hughes et al., 1999). Likewise, the process of chemo-attraction of *Nostoc* to the cycad rhizosphere is not clear, although chemo-attractants are operative in other cyanobacterial-plant symbioses (Bergman et al., 1996; Knight and Adams 1996). Symbiotically competent cyanobacteria are attracted at least to some non-cycad host plants, such as liverworts and *Gunnera*, but no signal/attractant compounds have been identified to date. A low-molecular-weight compound has been identified, but not chemically characterized; it induces the infectious form of the cyanobacteria, i.e. hormogonia (a highly motile form of the filamentous organism) in both liverworts and *Gunnera* (Adams, 2002; Bergman, 2002). Even roots of non-host plants, such as rice (*Oryza sativa* L.) and wheat (*Triticum aestivum* L.) attract cyanobacteria and re-

lease hormogonia-inducing factors (Bergman et al., 2004). Cycads are also well known for producing several secondary metabolites such as flavones, azoglucosides and neurotoxic non-protein amino acids (e.g., β-methylamino-L-alanine) (Brenner et al., 2003; Schneider et al., 2002). Whether these cycad root compounds function as chemo-attractants for cyanobacteria or only in preventing infection by other microorganisms (from incompetent cyanobacteria to bacteria and fungi) are open questions.

As referred to above, flavonoid/isoflavonoid compounds exuded by legume roots (and presumably *Parasponia*) induce the *nod* genes in rhizobia. The interaction of the rhizobial regulatory NodD protein with specific flavonoids is believed to be the first level of host-specific recognition in the symbiosis. The consequence of *nod* gene expression is the bacterial synthesis of the nodule-inducing, 'Nod factor' (see Miklashevichs et al., 2001). Nod factors are lipochito-oligosaccharides (LCOs) whose exact structure is a component of the second level of host-specific recognition in the symbiosis (i.e. only certain LCOs can initiate nodulation in certain legumes). Very recently, two putative Nod-factor receptor kinase gene (NFR1 and NFR5) have been identified in *Lotus japonicus* (Madsen et al., 2003; Radutoiu et al., 2003). Perception of Nod factor by the host legume results in numerous responses involved in infection and nodule formation, including root hair deformation, development of pre-infection threads, cortical cell divisions, and induction of nodule-specific genes expressed early in nodule development (*ENOD* genes; Miklashevichs et al., 2001; Schultze and Kondorosi, 1998). Aside from Nod factor, there are a plethora of other rhizobial cell-surface and excreted compounds implicated in symbiosis-oriented signaling functions including bacterial polysaccharides (Price, 1999), the phytohormones, IAA (indole acetic acid) and cytokinins (Hirsch et al., 1997), and most recently low-molecular-weight proteins (nodulation outer proteins or Nops) (Marie et al., 2003).

Equivalents of rhizobial Nod factors have not been identified for *Frankia*. Root-hair deformation occurs in some actinorhizal plants in response to supernatants of *Frankia* cultures induced (Van Ghelue et al., 1997) or non-induced (McEwan et al., 1992) by root exudates, but also in response to substances produced by other soil bacteria (Knowlton et al., 1980). Attempts to purify *Frankia* Nod factors using the protocol developed for rhizobial Nod factors has failed, indicating chemical differences between both types of molecules (Cérémonie et al., 1999). An *ENOD40* gene promoter, which is present in legumes and at least one actinorhizal species, *Casuarina glauca*, is induced during nodule induction in legumes by Nod factor, but not in actinorhizal plants (Santi et al., 2003b). Attempts to isolate *Frankia nod* genes by complementation of rhizobial mutants have failed as well, probably due to the fact that most *Frankia* promoters do not work in gram-negative bacteria (Lavire and Cournoyer, 2003). It will be necessary to identify a promoter from an actinorhizal plant that is induced by bacterial signal factors in order to develop a reliable bioassay. There is no evidence at this time that the production of phytohormones by *Frankia* might be involved in the initiation of host-responses. Although *Frankia* produces cytokinins (Stevens and Berry, 1988) and pseudoactinorhiza (i.e. empty nodules) can be induced by cytokinins in some cases (Rodriguez-Barrueco and de Castro 1973; C. Santi, C. Franche and E. Duhoux, personal communication), there is no evidence that cytokinins synthesized by *Frankia* are involved in nodule induction.

Homology to a few *nod* genes was detected in both compatible and non-compatible cyanobacteria as well as in cyanobacteria of the *Azolla* symbiosis (Plazinski et al., 1991; Rasmussen et al., 1996), however it was later found that an equivalent to *nodM* had no symbiotic relevance (Viterbo et al., 1999). Phenolics are also present in cycad symbiotic coralloid roots (Obukowicz et al., 1981), but it is unknown if these are involved in signalling to the microsymbiont. Extremely little is known about signals from the cyanobacterium microsymbiont which might influence development of its cycad host. Invading cyanobacteria trigger the development of a 'cyanobacterial zone' in a cell layer in the cortex or a layer underlying the root cap of coralloid roots (see below). These changes suggest that the invading cyanobacterium excretes a growth factor, which influences tissue development. There is some indirect evidence that this factor may be the phytohormone IAA (Sergeeva et al., 2002). Recent studies indicate that, like in rhizobia, surface-related or released proteins are up-regulated during hormogonium differentiation in a symbiotically competent *Nostoc*. These may be involved in aspects of symbiotic competence, such as in recognition and inter-organism signaling, in identification of target plant cells, or in camouflage to avoid plant defense responses (Klint et al., 2003).

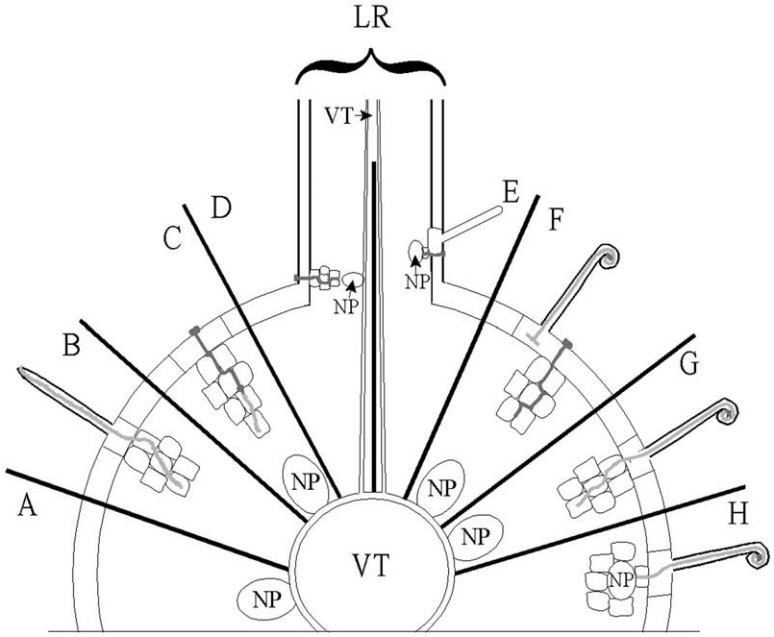

Figure 2. Diagrammatic illustration of examples of the diversity of infection and nodule-initiation patterns in legume-rhizobia symbioses. A. Spontaneous nodulation in the absence of rhizobia as seen in some genotypes of *Medicago* sp. Nodule primordia (NP) are spontaneously initiated in the inner root cortex (adjacent the vascular tissue (VT)). B. Commonly in members of the Caesalpinioideae (the oldest legume subfamily) nodules do not form. The rhizobia enter the root through root hairs, and exist within intracellular infection threads (light grey tracing). C. In some tree species of the subfamily Mimosoideae (e.g., *Mimosa scabrella*), infection occurs intercellularly (dark gray tracing) between intact epidermal cells and outer cortical cells, but an intracellular infection thread develops in the inner cortex. An NP, which will develop into an indeterminate-type nodule, is initiated in the cortex adjacent to the VT. D. In some aquatic legumes of the Mimosoideae (e.g., *Neptunia natans*), infection occurs via crack entry through the epidermis at the junction of a lateral root (LR). As the rhizobia move toward nodule primordia initiated in the inner cortex of the lateral root, an intercellular infection thread develops. The nodule primordia give rise to indeterminate nodules. E. In *Arachis hypogaea* (peanut; groundnut), a member of the subfamily Papilionoideae, infection occurs intercellularly at the base of an epidermal cell having a root hair. An infection thread does not develop. Rhizobia enter an NP initiated in the outer cortex of a LR which leads to a determinate-type nodule. F. In *Chaemaecytisus proliferus*, tagasaste, a woody species of the Papilionoideae, initially infection occurs via 'shepherd's crook' root hairs, and an infection thread which initiates a NP in the inner root cortex. However, this infection thread aborts early after initial formation and the NP receives rhizobia via a secondary, intercellular (non-infection thread) infection. The NP develops into an indeterminate-type nodule. G. In a large number of genera used in agriculture (e.g., *Lens, Medicago, Pisum, Trifolium, Vicia*) of the Papilionoideae, rhizobia enter shepherd's crook root hairs via an intracellular infection thread. The infection threads deliver the rhizobia to NP initiated in the inner cortex which gives rise to indeterminate-type nodules. H. In another large group of crop genera (e.g., *Glycine, Lotus, Phaseolus, Vigna*) of the Papilionoideae, rhizobia also enter shepherd's crook root hairs via an intracellular infection thread. However, the infection threads deliver the rhizobia to NP initiated in the outer root cortex, which gives rise to determinate-type nodules. Adapted from Guinel and Geil (2002).

Infection and symbio-organ development

The infection of the host by the microsymbiont, and the development of the root nodule/tissue to house the microsymbiont are very different among the four symbioses. Even within the legume-rhizobia symbiosis, there is considerable diversity of modes of infection and nodule development (Figure 2). Depending on the particular legume, the path of infection may be intercellular (Figure 2D, E) intracellular (Figure 2B, G, H) or a combination of the two (Figure 2C, F). Intracellular infection occurs via infection threads, tubular cell-wall-like structures constructed by the plant involving extensive cytoskeleton activity, the synthesis of which is initiated by bacterial Nod factor (Lhuissier et al., 2001).

The most intensively studied symbioses involve crop legumes (e.g., common bean, cowpea (*Vigna sinensis* [L.] Savi ex Hassk.), fababean (*Vicia faba* L.), pea (*Pisum sativum* L.), soybean) of the Papilionoideae subfamily with intracellular infections leading to either indeterminate (Figures 2G, 3B, 4B) or determinate root nodules (Figures 2H, 3A, 4A). Nodule primordia are initiated opposite to protoxylem poles. However, primordia of indeterminate nodules are initiated within the inner-root cortex, while determinate

nodules are initiated in the outer-root cortex. Indeterminate nodules maintain a persistent apical meristem and continue to grow throughout the lifespan of the nodule, thus resulting in a developmental gradient within the nodule with a bacteroid differentiation zone, mature zone (where N_2 fixation occurs), and senescence zone (Figure 4B). Indeterminate nodules tend to be oblong in shape and, depending on the legume, may form singular or bifurcated nodules. Determinate nodules (Figures 3A, 4A) do not maintain an active meristem, are more spherically shaped, and have a defined lifespan (Vikman and Vessey, 1993). In both types of nodules, infection-thread development and initial cortical cell divisions of nodule primordia occur simultaneously. The bacteria move through the infection thread matrix and are 'released' into the infected cells of the developing nodule engulfed in a plant membrane (the peribacteroid membrane). Recently the *Sen1* gene, a plant gene involved in the differentiation of bacteria into bacteroids, has been identified in *Lotus japonicus* (Suganuma et al., 2003). The structure containing the bacteroids, the symbiosome, is analogous to a plant organelle with the peribacteroid membrane overseeing the traffic into and out of the symbiosome (Day et al., 2001; Day and Udvardi, 1993). The symbiosome may contain one or several rhizobia that have undergone certain morphological changes associated with their new symbiotic lifestyle, and in this symbiotic state are referred to as bacteroids (Oke and Long, 1999). Again depending on the legume, a single nodule may contain several strains of rhizobia, but in the majority of cases a nodule hosts a single strain of rhizobia (e.g., Martinez-Romero, 2003). However, it is common that a single plant root will contain more than one strain of rhizobia among its nodules (e.g., Bromfield et al., 2001; Denison, 2000).

A plethora of nodule-specific plant genes are expressed during the development and functioning of legume nodules (Schultze and Kondorosi, 1998; Trevaskis et al., 2002). These genes have been characterized into two groups depending on whether the proteins they code for are synthesized early or late in nodule development (the early and late nodulins, respectively). Many of the early nodulin (*ENOD*) genes are associated with induction of root-hair deformations, cortical cell division, and cell-wall modifications (including *ENODs* 2, 5, 10, 11, 12, and 40). The late nodulin genes (commonly classified *NOD* or simply *N* genes) are more closely associated with the mature N_2-fixing nodule. Genes coding for leghemoglobin are classical examples of late nodulins; however, some of their expression is as early in nodule development as some *ENOD* genes (Heidstra et al., 1997). Genes encoding proteins associated with carbon and nitrogen metabolism (e.g., Colebatch et al., 2002a; Silvente et al., 2003), and membrane transport (e.g., Kapranov et al., 2001; Szczyglowski et al., 1998) in the nodules are commonly studied late nodulins.

Like rhizobia, *Frankia* strains can enter the roots of their host plants either intracellularly, via root hairs, or intercellularly, depending on the host plant species (Miller and Baker, 1986; Racette and Torrey, 1989). Early work by Benson and Hanna (1982) using whole-cellular protein profiles indicated more than one genetically distinct *Frankia* within the same actinorhizal nodule. However, *in situ* hybridization based on a 23S rRNA insertion target which allowed for the analysis of *Frankia* populations in nodules of various *Alnus* species revealed the presence of only one *Frankia* population in every nodule homogenate (Zepp et al., 1997). However, in *Ceanothus caeruleus*, a host that *Frankia* infects intercellularly (in contrast to *Alnus*, which is infected intracellularly via root hairs), nodules also contained a non-fixing, non-nodulating actinomycete strain (Ramirez-Saad et al., 1998).

Intracellular infection takes place in the Betulaceae, Casuarinaceae and Myricaceae. When a *Frankia* hypha is trapped in a root hair curl, an infection thread develops by which the hypha enters the plant root (Berg, 1999; Berry and Torrey, 1983; Callaham et al., 1979). No equivalent of the infection thread matrix (i.e. a space encompassed by the infection thread walls) of legumes exists in actinorhizal symbioses. Instead, *Frankia* hyphae are embedded within the cell wall-like material or 'interfacial matrix' (Berg, 1999), which is the equivalent of the infection thread wall in legume nodules. As in the legume-rhizobia symbiosis, concomitant with root-hair infection, cell divisions are induced in the root cortex. The infection thread grows to the dividing cortical cells, and infects some of them by intense branching within the cells, filling them with *Frankia* hyphae from the inside outward (Burgess and Peterson, 1987; Schwintzer et al., 1982). This cortex-based structure is called the prenodule. While the prenodule develops, the formation of a nodule primordium is induced in the pericycle of the root, like in the case of lateral root primordia (Callaham and Torrey, 1977). Note that this is in contrast to legume-nodule primordia which are initiated in the inner or outer root cortex (Figure 2), not the root pericycle. However, like lateral roots and legume nodule primordia, actinorhizal nodule primordia are

usually located opposite protoxylem poles. Hyphae in the infection threads grow from the prenodule to the nodule primordium, again by cell-to-cell passage, and infect primordium cells. The nodule primordium develops into a nodule lobe. Depending on the host plant species, more than one nodule primordium can be formed per prenodule (Torrey and Callaham, 1979).

During intercellular infection, *Frankia* hyphae enter the root between epidermal cells, and colonize the root cortex intercellularly (Miller and Baker, 1985; Racette and Torrey, 1989). In contrast to rhizobia, *Frankia* does not depend on gaps in the root epidermis for entering the root. During the colonization of the cortex, the root cortical cells secrete an electron-dense pectin- and protein-rich material into the intercellular spaces, and the formation of a nodule primordium is induced in the root pericycle (Liu and Berry, 1991; Valverde and Wall, 1999). *Frankia* hyphae infect primordium cells from the apoplast by intense branching of hyphae, concomitant with continuous invagination of the plant plasma membrane. Intercellular infection takes place in host plants of the Rhamnaceae, Elaeagnaceae and Rosaceae families. In host plants of the actinorhizal Cucurbitales (*Datisca* and *Coriaria*), the infection mechanism has not been examined yet, but since no prenodules or infection threads are found in these plants, infection is assumed to follow the intercellular pathway. In contrast with all other host plants examined, infected cells of *Datisca* and *Coriaria* nodules are filled with branching *Frankia* hyphae from the periphery inward, instead of from the center outward, and they retain large central vacuoles (Hafeez et al., 1984; Newcomb and Pankhurst, 1982). Another unique feature of actinorhizal Cucurbitales is that their nodule cortical cells become multinucleate prior to infection.

Frankia induce the formation of multiple 'lobed' root nodules composed of modified lateral roots without root caps, a superficial periderm, a central vascular system (in contrast to the peripheral vasculature of legume nodules), and infected cells in the expanded cortex (Figures 3C, 4C). Due to the activity of the apical meristem, the infected cells in the expanded cortex are arranged in a developmental gradient in a similar fashion to indeterminate nodules of legumes. In the infection zone, infected calls gradually fill with branching *Frankia* hyphae. Once an infected cell is filled with branched hyphae, vesicles develop and N_2 fixation starts (Huss-Danell and Bergman, 1990). In the N_2-fixation zone, *Frankia* vesicles develop and bacterial N_2-fixation takes place. In the zone of senescence, bacterial material is degraded by the plant. In most actinorhizal nodules, infected and uninfected cells are interspersed in the cortex of the nodule lobe. In the actinorhizal Cucurbitales, however, the infected cells form a continuous patch, kidney-shaped in cross-section, at one side of the acentric stele (Hafeez et al., 1984; Newcomb and Pankhurst, 1982).

As in legume nodules, the search for, and characterization of, genes and their encoded proteins has been a focus of research in actinorhizal plants. In general, genes expressed at significantly higher levels in nodules than in roots encode products involved either in (a) nodule metabolism (e.g., symbiotic hemoglobin; Gherbi et al., 1997; Jacobson-Lyon et al., 1995), (b) the internalization of the microsymbiont (e.g., cell-wall proteins specific to infected cells; Pawlowski et al., 1997), or (c) nodule-specific development (e.g., subtilases, possibly involved in inducing infected-cell differentiation within nodules; Berger and Altmann, 2000; Svistoonoff et al., 2003). Interestingly, only one legume *Enod* gene homolog has been discovered so far in an actinorhizal plant, namely the *dg93* mRNA sequence common to soybean nodules and *Datisca glomerata* (Okubara et al., 2000).

Parasponia nodule development and structure in some ways can be seen as an intermediate between legume and actinorhizal nodules, but also has unique features. Despite early observations that inoculation effected root hair growth and morphology (Lancelle and Torrey, 1984), it is now clear that *Parasponia* rhizobia do not infect the plant through root hairs (Becking, 1992). In *P. andersonii* (Bender et al., 1987b) and *P. parviflora* (Becking, 1992), rhizobia erode the root epidermis below an area of bacterial colonization on the root surface. Bender et al. (1987a, b) referred to subsurface swelling zone as a prenodule, comparable with the prenodule development that occurs in actinorhizal plants. However, Becking (1992) argues that these swellings are not always observed and are not morphogenetically comparable to prenodules of actinorhizal plants. Others have observed 'crack entry' of rhizobia into *Parasponia* roots (Webster et al., 1995a), similar to that seen in the infection of some legumes (Figure 2C, D and E). Thick-walled infection threads of plant origin in the intercellular spaces act as a conduit for the rhizobia to nodule primordia developing from the root pericycle. In this way, *Parasponia* nodules are more similar to actinorhizal nodules than legumes, being modified lateral roots and the mature nodule having a centralized vascular system (similar to Figure 4C). The mature nodule is also

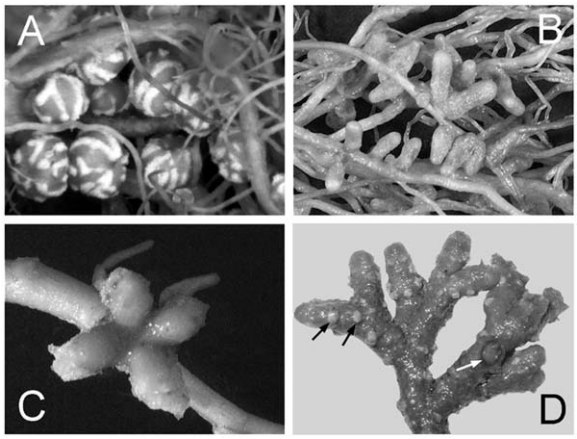

Figure 3. Examples of (A) determinate legume nodules (*Glycine max*), (B) indeterminate legume nodules (*Pisum sativum*), (C) an actinorhizal nodule (*Datisca glomerata*), and (D) a portion of a symbiotic coralloid root cluster (*Cycas revoluta*). In the coralloid root cluster (D), the black arrows indicate lenticels and the white arrow indicates where a root lobe has been cross sectioned to reveal the cyanobacterial infected zone as a darker ring.

actinorhizal in appearance being multi-lobed and indeterminate. However, the rhizobia within the infected cells of the nodule are contained in thin-walled 'fixation threads' (Price et al., 1984) which Becking (1992) contends are actually filamentous 'peribacteroid sacs' associated with, or extensions of the infected plant cell's membrane. Although cell wall-like deposits are associated with this peribacteroid membrane (Smith et al., 1986), it is not cell wall *per se*.

In the cycad-cyanobacteria symbiosis, the cyanobacterial microsymbiont, or cyanobiont, invades a particular root type, referred to as coralloid roots due to their 'coral-like' appearance. It has been speculated that the coralloid roots may have initially evolved as pneumatophores (apogeotropic roots facilitating an efficient gas exchange) that only later became cyanobacterial host-organs (Grobbelaar et al., 1986; Wittman et al., 1965). However, fossil evidence of symbiotic tissues invaded by cyanobacteria is still missing. As there are no other reports of plant pneumatophores infected by cyanobacteria, additional components only held by the cycad roots must be involved in acquiring cyanobacteria as symbionts. Root hairs, which are necessary for the infection of many legume and actinorhizal plants, are absent in cycad roots.

Coralloid roots develop from 'precoralloid' roots arising either as adventitious roots from the hypocotyl, or as secondary roots from the upper regions of the tap root. Precoralloid roots are easily recognizable by their swollen appearance (compared with normal roots), their papillose sheath, and their apogeotropic growth. As the precoralloid root matures, lenticels replace the papillose sheath at the base of the root and repeated bifurcations give rise to the extensively branched coralloid root cluster (Figure 3D). Such individual coralloid clusters can reach up to 10 cm in diameter and weigh up to 500 g, and may be found down to about 50 cm below ground. Coralloid roots are formed in the absence of cyanobacteria as has been shown using axenic cultures of *Zamia* sp. (Ow et al., 1999); however, irreversible morphological changes occur upon colonization by cyanobacteria (Ahern and Staff, 1994; Costa and Lindblad, 2002; Wittman et al., 1965).

Processes involved in the invasion of the coralloid roots by cyanobacteria are still largely unclear, and very few cycad genera have been examined. Nonetheless, the apogeotropic growth of the precoralloid roots brings the root caps close to the soil surface, the habitat where the photoautotrophic cyanobacterial microsymbiont would be expected. Some studies suggest that colonization takes place either at the apex, at the base, or in an intermediate position of the symbiotic coralloid root, and lenticels have often been proposed as the cyanobacterial root entry point (Figure 3D). For example, Ahern and Staff (1994) found that 95% of the infected roots of 60 *Macrozamia* seedlings had prominent apical lenticels. Other possible modes of cyanobacterial entry are through the papillose sheath, through breaks in the dermal layer, or through channels leading from the root surface to the cyanobacterial zone. In contrast to both legume and actinorhizal symbioses, there is no evidence of infection thread development in the infection process in cycads.

Upon colonization by the cyanobiont, developmental and morphological changes are initiated in symbiotic coralloid roots (Ahern and Staff, 1994; Costa and Lindblad, 2002; Lindblad et al., 1990). The production of the papillose sheath ceases, the roots become wider, and the strict apogeotropic growth of the roots ceases. The widening is due to the development of a root 'zone' which the cyanobacteria invade. This zone may be the result of the development of a persistent root cap which forms a secondary (outer) cortex overlying the original root epidermis (equivalent to the inner cortex) of the non-infected root. Another possibility is that the cyanobacterial invaded zone is the result of the development of a specialized secondary (outer) cortex overlying the original (inner) cortex. Irrespective of the origin, the cyanobacterial invaded

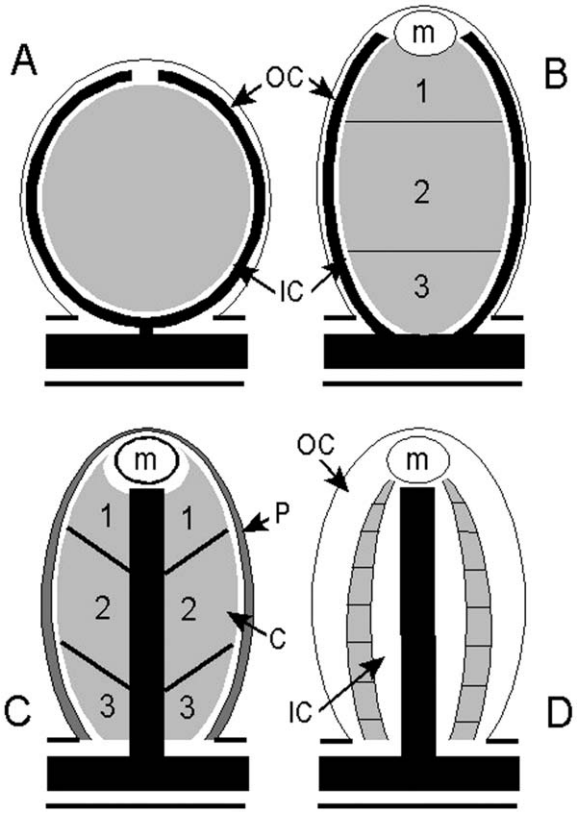

Figure 4. Diagrammatic representation of longitudinal sections through (A) a determinate legume nodule, (B) an indeterminate legume nodule, (C) an actinorhizal nodule, and (D) a lobe of a symbiotic coralloid root cluster. The grey colored regions represent the infected zones. The dark, thick lines represent vascular tissues. Cortical tissue (C), outer cortical tissue (OC), inner cortical tissue (IC) and meristems (m) and periderm tissue (P) are indicated in the various symbio-organs. In the indeterminate legume nodule (B) and the actinorhizal nodule (C), the zones of infection/differentiation (1), N_2-fixation (2) and senescence (3) are indicated.

zone develops between these two root layers (Figure 3D, 4D). The widening of the cyanobacterial zone is due to considerable host-cell elongation, radially to the long axis of the roots, and a partial separation of the host cells located between these layers.

The outcome of the developmental process in symbiotic coralloid roots is the creation of a mucilaginous extracellular space, densely filled with cyanobacteria and traversed by numerous elongated host cells (Figure 4D). These elongated cells interconnect the inner (original) and outer ('new') cortex. The cyanobacterial zone may vary in prominence, from 230 to 365 μm in diameter depending on the cycad genus (Chaudhuri and Akhtar, 1931). However, in contrast to the symbioses of legumes and actinorhizal plants, the cyanobionts always remain extracellular, although the same cyanobacterium is capable of invading plant cells in the angiosperm *Gunnera* (Johansson and Bergman, 1992). The elongated host-root cells that cross the zone filled with cyanobacteria show transfer-cell morphology (cell-wall invaginations, amyloplasts and numerous mitochondria), suggesting a role in nutrient exchange (see below) between the partners (Lindblad et al., 1985a; Obukowicz et al., 1981). The zone infected with cyanobacteria is clearly visible by the naked eye as an intensely blue-green pigmented band mid-way between the stele and the root surface on sectioning of the root (Figure 3D, 4D). The retention of pigments (chl a and phycobiliproteins) in cyanobacteria living in darkness in plant roots is due to pigment synthesis in cyanobacteria being a light-independent process, as in some lower plants. The cyanobacterial root zone is absent at the root apex, but otherwise it forms a continuous cylinder all the way down the symbiotic coralloid root, except below lenticels.

To date, no symbiosis-related genes have been identified (or searched for) in cycads, although it is obvious that the cyanobacterium influences cycad root development as evidenced by microscopic studies (see above). The search for differences in gene expression patterns of non-infected and infected coralloid roots is now desirable. More is known regarding symbiosis-related gene expression of the cyanobiont. For instance, within the host tissue, cyanobacterial cell division is slowed down to synchronize development, cell-volume increases, cell-surface structures are altered, and subcellular rearrangements are apparent (Rai et al., 2000, 2002). Host plants are also capable of dictating cell differentiation processes (that of hormogonia and heterocysts) in the cyanobacterium. For example, it has been shown in other cyanobacterial-plant symbioses that host plants may elicit or prevent the differentiation of hormogonia via the release of a hormogonia-inducing factor (HIF) or a hormogonia-repressing factor (HRF). Interestingly, a mutation in *hrmA*, a gene induced by HRF, increases the cyanobacterial infectivity several-fold, probably by prolonging the hormogonial stage (Cohen and Meeks, 1997; Meeks et al., 1999). Infectivity is also enhanced by mutations in the expression of *sigH*, which is induced by HIF. This is interesting, as it points to the possibility of obtaining 'super-infectious' cyanobionts. Such mutants may perhaps also be used to infect roots of non-host plants of great commercial interest, such as wheat and rice, which readily associ-

ate with some cyanobacteria (Gantar and Elhai, 1999; Nilsson et al., 2002).

Once inside the root, differentiation of the N_2-fixing heterocysts is stimulated, and soon reaches frequencies never seen in their free-living state (Lindblad et al., 1985b). This suggests over-expression of *hetR*, the heterocyst regulatory gene highly expressed in free-living cyanobacteria upon deprivation of combined N (Buikema and Haselkorn, 1993). Since this gene is under the control of the nitrogen-dependent global transcription factor NtcA, it has been proposed that the plant may bypass this control. However, recent data indicate that both *ntcA* and *hetR* are over-expressed *in planta* (Wang et al., 2004). Moreover, *hetR* mutants still infect liverworts while *ntcA* mutants fail to do so (Wong and Meeks, 1994). Although these studies have been performed on cyanobionts in the Gunnera and bryophyte symbioses, respectively, they are probably relevant also for cycads.

Supplying the microsymbiont with energy

An integral aspect of symbioses between host plants and their N_2-fixing microsymbionts is the supply of reduced carbon substrates from the host to the microsymbiont. Evolutionarily, it is believed that this supply of energy has played a large role in 'drawing' the microsymbionts into the symbioses (Simms and Taylor, 2000). In all four symbioses examined here, the carbon supplied to the microsymbionts ultimately derives from photosynthesis by the host. However, a great difference among the symbioses is that free-living (i.e., non-symbiotic) rhizobia and *Frankia* in the soil are carbon heterotrophs, but free-living cyanobacteria are photoautotrophic. Hence, for host-supplied carbon to play a *major* role in initiating and maintaining the cycad-cyanobacteria symbiosis through evolution, the carbon supply from the host must be superior in some fashion to the cyanobacterium supplying its own carbon from photosynthesis.

Carbon provision and assimilation to bacteroids in legume nodules has been reviewed recently by Lodwig and Poole (2003). In summary, photosynthate is supplied to nodules as sucrose in the phloem. Sucrose synthase activity in the nodule is primarily responsible for the hydrolysis of the sucrose. If carbon supply to the nodule is more than sufficient, some of the released monosaccharides can be stored as starch within the nodule. Sugars destined for utilization by the bacteroids pass through glycolysis and enzymes of the tricarboxylic acid cycle, to form malate, fumarate and succinate, the three dicarboxylic organic acids that are the primary carbon sources for bacteroids in the nodules. The transport of these dicarboxylic acids across the peribacteroid membrane and their utilization by bacteroids have been studied extensively (Day et al., 2001; Day and Udvardi, 1993; Lodwig and Poole, 2003).

Very little is known about the carbon supply to rhizobia in *Parasponia*. Mohapatra and Gresshoff (1984) found that *Parasponia-Rhizobium* strain ANU289 grew well *in vitro* on a wide range of carbon sources, but that succinate, fumarate, or pyruvate was necessary to support good levels of nitrogenase activity. It is interesting that nitrogenase activity was enhanced by more than three-fold if mannitol or sorbitol was added to succinate in the medium compared to succinate alone. The forms of carbon transported in the phloem of *Parasponia* are unknown. Micrographs of bacteroids (Becking, 1992; Trinick, 1979) within *Parasponia* nodules commonly depict abundant poly-β-hydroxybutyrate globules which Becking (1992) concluded indicated that carbon was not a limiting factor to the rhizobia. Newly infected cells contain starch-filled plastids (Lancelle and Torrey, 1985; Trinick, 1979) but the plastids of mature cells plastids contained little to no starch. It is not clear from these micrographs and others (Becking, 1979, 1992; Lancelle and Torrey, 1984, 1985; Trinick, 1979; Webster et al., 1995b) if starch accumulates in uninfected cells in the infected zone as is common in legume nodules. However, in *P. andersonii* nodules, one of the authors (K. Pawlowski, unpublished data) has observed large amyloplasts in uninfected cells in the region between the vascular tissue and the infected zone .

There are many similarities between the scenario for supplying carbon to legume bacteroids and the supply of energy to *Frankia* from its actinorhizal host. As in legumes, sucrose is believed to be the major sugar delivered to the nodules of most actinorhizal plants. Sucrose synthase expression levels are high in the nodules of *Alnus glutinosa* (Van Ghelue et al., 1996). However, sorbitol is a major phloem-transported carbohydrate in the Rosaceae (Brown and Hu, 1996), but it is not known if actinorhizal genera of this family might utilize this sugar alcohol as their major carbon supply to nodules. All actinorhizal nodules, except for those formed by *Casuarina* spp., accumulate starch, though not in infected cells (E. Duhoux, unpublished observation). It is unclear whether nod-

ule starch plays a role in carbon storage for particular growth situations; at any rate it is not used during spring flush growth (Wheeler et al., 1983). Nodules are strong carbon sinks, and starch biosynthesis may simply represent a metabolic 'safety valve' when carbon is received in excess of demand, as appears to be the case in legumes (Walsh et al., 1987).

In the free-living state, *Frankia* strains grow on short-chain fatty acids (acetate, propionate), variably on succinate, malate or pyruvate, and (except for some living symbiotically with members of the Elaeagnaceae and Casuarinaceae) poorly or not at all on a range of sugars (Benson and Silvester, 1993). However, the *Frankia* microsymbionts of many actinorhizal plants (e.g., *Ceuthostoma* sp., *Kentrothamnus* sp., and *Chamaebatia* sp.) have not been isolated yet, so their carbon source preferences have yet to be analyzed. Studies on the metabolism of symbiotic *Frankia* have been performed using so-called vesicle clusters, consisting of symbiotic vesicles together with a part of their subtending hyphae isolated from nodules (Van Straten et al., 1977; Vikman and Huss-Danell, 1987b). Vesicle clusters from *Alnus* spp. have an aerobic metabolism (Vikman and Huss-Danell, 1987a). In agreement with the hypothesis that *Frankia* is fed dicarboxylates *in planta*, succinate, as well as a combination of malate, glutamate and NAD, were found to stimulate respiration (Akkermans et al., 1981; Vikman and Huss-Danell, 1991). Evidence for CO_2 fixation in actinorhizal nodules was found, although this might be due to reactions associated with ammonia assimilation, instead of metabolism of dicarboxylic acids (Huss-Danell, 1990; McClure et al., 1983).

An alternative hypothesis is that symbiotic *Frankia* are supplied with hexoses by the host plant. Hexoses are not the ideal carbon sources for free-living *Frankia*, but it is possible that *Frankia*'s carbon preferences in nodules do not reflect those in free culture. Sucrose, trehalose, maltose, glucose and fructose stimulated respiration in vesicle clusters from *Alnus rubra* (Lopez et al., 1986). On the other hand, these observations might simply reflect the ability of *Frankia* to metabolize its own storage carbohydrates, glycogen and trehalose (Lopez et al., 1984). Furthermore, given the dissimilarities between actinorhizal nodules formed by plants from different families, differences in the carbon source for the microsymbionts might exist amongst Fagales, Cucurbitales and Rosales. In summary, the carbon sources delivered to symbiotic *Frankia* are still not clearly known.

Compared with the legume and actinorhizal symbioses, relatively little is known about the carbon supply to cyanobionts from their cycad hosts. Free-living cyanobacteria are capable of higher-plant-type oxygenic photosynthesis; however, in cycad symbiotic coralloid roots, where the availability of light would be negligible, the normally photoautotrophic cyanobacteria would become functionally non-photosynthetic. A heterotrophic life style and a dependence on carbohydrates supported by the host plant are therefore expected. *Nostoc*, as opposed to many other cyanobacteria, can easily switch from growing photoautotrophically to growing heterotrophically (Rippka et al., 1979) which probably is a very important part of their symbiotic competence. Evidence of the heterotrophic nature of cycad cyanobionts includes the fact that externally added fructose and glucose to cycad isolates grown in darkness stimulated their nitrogenase activity (Lindblad, 1992; Martel et al., 1993). The carbon supplied by the host is still unknown, and whether the mucilage filling the extracellular space of the symbiotic coralloid roots has a role in the carbon supply to the cyanobiont, or whether it is of cyanobacterial origin, is also an open question. However, a plant-derived mucilage is apparent in organs infected by cyanobacteria of other plant symbioses (e.g., *Gunnera*) (Bergman, 2000). Amyloplasts are common in the elongated cells of the cyanobacterial zone which show transfer cell morphology. The plant carbohydrates filling the cyanobacterial zone may be made available via, or stored in, these plastids (see Rai et al., 2002).

Regulation of O_2 supply to the microsymbiont

Three types of nitrogenase (the molybdenum-iron, the vanadium-iron, and the iron-iron complexes) exist in the Eubacteria (Burris, 1991), and more recently a molybdenum-carbon monoxide nitrogenase complex was discovered in *Streptomyces thermoautotrophicus* of the Archaea (Ribbe et al., 1997). The legume-rhizobia, *Parasponia*-rhizobia, actinorhizal-*Frankia*, and cycad-cyanobacteria symbioses all contain the Mo-Fe nitrogenase complex, by far, the most abundant form of nitrogenase in the biosphere (Burris, 1991). This nitrogenase is sensitive to inhibition by molecular oxygen. The exact mechanism of the inhibition is unknown (Gallon, 1992), but it may be that O_2 itself is a substrate of nitrogenase, and its reduction leads to highly reactive oxygen species (e.g., O_2^-) which result in the denaturation of the nitrogenase complex. Re-

gardless of the mechanism, all diazotrophs containing the Mo-Fe nitrogenase complex must protect nitrogenase from O_2. In aerobic bacteria such as rhizobia, *Frankia*, and cyanobacteria, this is a particular dilemma because the organisms require an adequate O_2 flux for oxidative phosphorylation to provide the energy required for nitrogenase activity, but not so high a flux that it will result in inhibition of nitrogenase. In fact, despite existing in an aerobic milieu, it is necessary that the environment immediately surrounding the nitrogenase complex be microaerobic.

Solving this 'O_2 dilemma' in cyanobacteria is particularly interesting because in a free-living (non-symbiotic) state the bacteria can fix N_2, but also carry out photosynthesis, which generates O_2. Protection of nitrogenase from O_2 in *Nostoc* in a free-living state is accomplished by isolating nitrogenase in thick-walled, non-O_2 producing (i.e. non-photosynthetic) heterocysts in the filamentous organism (Gallon, 1992; 2001; Meeks et al., 1999). The double envelope surrounding the outer membrane of the heterocyst decreases the diffusion of O_2 into the cell. Likewise, O_2 consumption by respiration has been implicated in helping to maintain microaerobic conditions inside the heterocyst. In the heterocystous *Anabaena* PCC 7120, the *coxA* gene which codes for a subunit of cytochrome oxidase was stimulated during heterocyst differentiation, but not in a coxA mutant, which could only fix N_2 under anaerobic conditions (Haselkorn et al., 1997).

Compared with free-living, non-symbiotic forms of the cyanobacteria, it may be easier for a cyanobiont to cope with O_2. The lack of light in the symbiotic coralloid root means little or no O_2 production from cyanobacterial photosynthesis (Bergman et al., 1986). In addition, it also appears that the partial pressure of O_2 (pO_2) in the cyanobacterial zone of coralloid roots, as in other cyanobacteria-infected plant organs, is lower than levels free-living cyanobacteria may experience. Heterocyst abundance is much higher in the cyanobiont than in the free-living state (Bergman et al., 1986), but in freshly isolated cycad cyanobionts nitrogenase activity is inhibited on exposure to pO_2 above 1 kPa (Lindblad et al., 1991). This implies the maintenance of a low pO_2 in the cyanobacterial zone, that the heterocyst envelope may be impaired, or that isolation of the cyanobiont from the plant disrupts the protective conditions or prevents important interactions between the organisms (Costa and Lindblad, 2002). How the plant manages to lower the pO_2 in the symbiotic tissues is an intriguing question. However, the abundance of mucilage in the extracellular spaces of the cyanobacterial zone may play a role. Protection of nitrogenase activity from excessive O_2 flux by mucilage has been implicated in other plant-associated diazotrophs (Vessey and Pan, 2003; Zhulin et al., 1996).

In contrast to cyanobacteria, rhizobia in their free-living state in soil are incapable of N_2 fixation. Nitrogenase activity can be induced in rhizobial cultures or isolated bacteroids, but only when the pO_2 is maintained at very low levels (e.g., 0.5 to 1.5 kPa O_2) (Allen and Elkan, 1990; Karr et al., 2003). In its symbiotic state, the legume nodule creates a microaerobic environment to protect nitrogenase from O_2, but a relatively high flux of O_2 is maintained to the bacteroids for respiration. These seemingly contradictory functions (low O_2 concentration/high O_2 flux) are accomplished via an O_2-diffusion barrier in the cortex of the nodules, and facilitated diffusion of O_2 bound to the transporting hemoprotein, leghemoglobin.

The subject of the regulation of O_2 flux to bacteroids within legume nodules has been well reviewed (see Hunt and Layzell, 1993; Lodwig and Poole, 2003). In brief, an O_2-diffusion barrier exists in a region of densely packed cells in the inner cortex of legume nodules. There is some controversy whether expression of *Enod2* is related to the development of this diffusion barrier (Wycoff et al., 1998). The exact nature of this diffusion barrier is unknown, but there is evidence that the path length of intercellular water (Denison, 1992) or the abundance of intercellular glycoprotein (James et al., 2000) may play roles in establishing the diffusion resistance to O_2. A very important feature of this diffusion barrier is that it can quickly change (i.e. in seconds to minutes) its resistance to O_2 diffusion when either the external concentration of O_2 or the internal demand for O_2 changes. In fact, it appears that a number of stresses (drought, temperature, supplemental mineral N, carbohydrate limitations) decrease nitrogenase activity indirectly by decreasing O_2 diffusion into the infected zone of the nodule (Kuzma and Layzell, 1994; Serraj et al., 1999; Vessey et al., 1988). In the infected zone of the nodule, leghemoglobin acts as a shuttle, binding O_2 from the intercellular spaces within the infected zone, diffusing down the oxyleghemoglobin concentration gradient, and delivering O_2 to the sites of respiration (cytochrome oxidase) in the bacteroids (Becana and Klucas, 1992; Bergersen, 1996; Denison and Okano, 2003). Leghemoglobin represents approximately 5% of the total protein of a mature nodule and

is coded for by at least four *lb* genes, of which *lbc3* is the most intensively studied (e.g., Cvitanich et al., 2000).

In terms of regulating O_2 flux within symbio-organs, the actinorhizal-*Frankia* symbiosis can be seen as an intermediate between the cycad and legume symbioses. In contrast to rhizobia, *Frankia* can fix N_2 in the free-living state at ambient pO_2. The vesicles of *Frankia* have similarities in form and function to cyanobacterial heterocysts, but in some actinorhizal plants hemoglobins also appear to have a role in O_2 flux as in legumes. Hemoglobins have not been found in cyanobacterial symbioses; however, some cycad symbiotic coralloid roots are pink. Whether this color is due to hemoglobins or to the red water-soluble pigment phycoerythrin, abundant in symbiotic *Nostoc* (Poza-Carrion et al., 2001), has yet to be resolved.

When *Frankia* are cultured with limiting levels of mineral N and ambient pO_2, they form specialized vesicles at the end of normal hyphae or at the ends of short side hyphae (Silvester et al., 1990). Within the vesicles, nitrogenase is protected from O_2 and N_2 fixation can take place (Meesters, 1987; Parsons et al., 1987). The vesicles are surrounded by envelopes consisting of multiple layers of hopanoids, bacterial steroid lipids (Berry et al., 1993; Huss-Danell, 1997). The number of layers depends on the pO_2 (Parsons et al., 1987). While vesicles formed in the free-living state are always round and non-septate, the shape and cellular location of vesicles formed *in planta* depends on the host plant genus (Baker and Mullin, 1992), indicating that here, vesicles represent a symbiosis-specific differentiation comparable to bacteroids in legume nodules. In addition to diffusion resistance, the fast respiration rate of *Frankia* vesicles suggests that metabolic consumption of O_2 also plays a role in O_2-protection of nitrogenase (Vikman, 1992). In nodules of *Datisca*, *Frankia* forms lanceolate vesicles in radial orientation that form a ring around the central vacuole. Multiple mitochondria accumulate at the vesicle base and probably play a role in the metabolic removal of O_2 (Silvester et al., 1999). In contrast, the respiratory protection of nitrogenase at work in some actinorhizal symbioses appears to have little role in the protection of nitrogenase in legume nodules (Weisz and Sinclair, 1987).

Actinorhizal nodule lobes are surrounded by a superficial periderm that may be impermeable to O_2. To provide the infected zone with O_2, the nodule periderm can be disrupted by lenticels (*Betulaceae, Datisca, Coriaria*), or nodule roots can be formed (*Casuarinaceae, Myricaceae, Datisca*) (Huss-Danell, 1997). Nodule roots are agravitropically growing roots with large air spaces in the cortex that are formed at the tip of nodule lobes, i.e. by a change in the activity of the nodule lobe meristem (Bond, 1952). Subsequently, new nodule lobe meristems can be induced next to the origin of the nodule root. Nodule roots provide access to O_2 for nodules formed on roots submerged in water, and their length depends on the pO_2 level (Silvester et al., 1988; Sprent and Scott, 1979). Diverse O_2 protection systems for bacterial N_2 fixation have evolved in different actinorhizal plant genera (reviewed by Silvester et al., 1990). The lack of a region of densely packed cells encompassing infected zones (i.e. an O_2-diffusion barrier) in the actinorhizal nodule lobes is inconsistent with a legume-nodule-like O_2 diffusion control system. Only *Coriaria* nodules have developed a comparable system, in that these contain a long lenticel at the non-infected side of the nodule lobe with a variable O_2-diffusion control system in the cell layers between stele and periderm that enables control of O_2 flux to the infected cells (Silvester and Harris, 1989).

In some cases, in symbioses with *Casuarina* spp. for example, only the plant seems to be responsible for O_2 protection of nitrogenase, more comparable to the situation in legume nodules. In these nodules, *Frankia* do not form vesicles (Berg and McDowell, 1987a), the walls of infected cells are lignified to prevent O_2 access (Berg and McDowell, 1987b), and large amounts of a nodule-specific hemoglobin are present in the cytosol of infected cells (Jacobson-Lyon et al., 1995). Nodules of *Myrica* spp. also contain large amounts of hemoglobin and the walls of infected cells are heavily lignified (Pathirana and Tjepkema, 1995; Zeng and Tjepkema, 1994), although in this genus, *Frankia* forms vesicles (Baker and Mullin, 1992). Even if hemoglobin is clearly plant-based in some cases (Christensen et al., 1991), recently Beckwith et al. (2002) have found production of a hemoglobin in genetically diverse strains of *Frankia*. The combined role of plant-based and *Frankia*-based hemoglobins in the regulation of O_2 flux in actinorhizal nodules has yet to be elucidated.

Although *Parasponia* nodules are morphologically similar to actinorhizal nodules, in terms of regulation of O_2 flux to bacteroids, they are more similar to legume nodules. *Parasponia* nodules have a zone of tightly packed cells surrounding the infected zone which is presumed to function similarly to the O_2-diffusion barrier of legume nodules (James et al., 1994; Tjepkema and Cartica, 1982). Hemoglobin is

probably the most intensively studied aspect of the *Parasponia*-rhizobia symbiosis (e.g., Appleby et al., 1983; Bogusz et al., 1988; Hunt et al., 2002). In a recent comparison of hemoglobin gene sequences from a wide range of symbiotic and non-symbiotic plants, it was concluded that the symbiotic hemoglobins from legumes and actinorhizal plants are related to the class 2 non-symbiotic hemoglobin genes assumed to be common to all dicots (Hunt et al., 2001). Interestingly, hemoglobin sequences from *Parasponia* nodules are more closely related to class 1 non-hemoglobin genes assumed common to both monocots and dicots. These observations lead Hunt et al. (2001) to conclude that symbiotic hemoglobin of *Parasponia* was probably recruited from non-symbiotic hemoglobins independent from the symbiotic hemoglobin in extant legume and actinorhizal plants.

Transfer of the fixed N from the microsymbiont to the host

The host's benefit from a N_2-fixing symbiosis is only realized when it receives the fixed N from the microsymbiont. The product of nitrogenase activity in the microsymbiont is NH_3; dependent upon pH, a proportion of the NH_3 will form NH_4^+ (pKa = 9.25). In legume nodules, the NH_3 quickly diffuses out of the bacteroid's alkaline protoplasm into the symbiosome's acidic peribacteroid space where it is protonated to NH_4^+. An ammonium-transporting system (Amt) present in free-living rhizobia is suppressed in bacteroids (Udvardi and Day, 1990), thereby stopping the potential for cycling the NH_4^+ back into the bacteroid. An ion channel, specific for monovalent cations, facilitates the transport of the NH_4^+ across the peribacteroid membrane and into the infected cells' cytoplasm (Tyerman et al., 1995). Once in the plant cytosol, the glutamine synthetase (GS) and glutamate-oxoglutarate aminotransferase (GOGAT) enzyme systems are the main conduits of NH_4^+ assimilation into amino acids (Cullimore and Bennett, 1992). However, additional aminotrasferase activity is required to form the main N compounds exported from the nodules, primarily amides (glutamine and aspargine) in legumes of temperate origin and ureides (allantoin and allantoic acid) in legumes of tropical origin (Parsons and Sunley, 2001; Pate, 1989).

The above scenario is the most widely accepted model for the movement of NH_3 from the sites of N_2 fixation to the cytosol of infected legume cells. However, there are alternative hypotheses. There is some evidence that alanine may be the export form of the fixed N from bacteroids (Allaway et al., 2000; Waters et al., 1998). It is still being debated as to how important this alanine may be to the overall export of fixed N from bacteroids (Li et al., 2002).

The form of N exported from *Parasponia*-rhizobia to the host cells is unknown, but there is little reason to believe it should be exceptional as compared to legume rhizobia (e.g., Udvardi et al., 1992). Early work (Becking, 1983a) on the amino acid content of *P. parviflora* nodules indicated higher levels of the glutamate, aspartate and amides in nodules with greater N_2 fixation. This would suggest that *Parasponia* nodules are amide exporters similar to legumes of temperate origin (Webster et al., 1995a) and many actinorhizal plants [see below; note that *P. parviflora* nodules did not contain citrulline (Becking, 1983a), an N-export product of *Alnus* sp. and *Casuarina* sp.]. Synthesis and transport of these amino acids in nodules of *P. andersonii* were confirmed by Baker et al. (1996), however they also demonstrated the incorporation of significant quantities of $^{15}N_2$ into 4-methylglutamate in nodules, demonstrating the *de novo* synthesis of this non-protein amino acid and suggesting a role in xylem transport of fixed N as well.

The form of fixed N exported from *Frankia* is unknown. However, similar to legume nodules, high expression levels of GS have been found in infected cells of actinorhizal nodules of *Alnus glutinosa* (Guan et al., 1996) and *Casuarina glauca* (L. Laplaze and K. Pawlowski, unpublished), supporting the proposal of NH_4^+ export by *Frankia*. However, in *Datisca glomerata* nodules, plant GS expression was restricted to the uninfected cells surrounding the infected cells (Berry et al., 2004; Pawlowski et al., 2003). Since NH_4^+ is relatively toxic, and thus unlikely to be allowed to diffuse through several plant cell layers prior to assimilation, these results suggest that within the infected cells, an assimilated form of N is exported by *Frankia* that is degraded in the uninfected cells, followed by the re-assimilation of NH_4^+ in the GS/GOGAT pathway (Berry et al., 2004). In contrast, Valverde and Wall (2003) recently demonstrated the expression of most of the enzymes involved in the synthesis of asparagine in nodules of *Discaria trinervis*, but not in roots. They hypothesized that the assimilation of fixed N, exported as NH_4^+ from the microsymbiont, followed a similar pattern to that seen in amide-exporting legume nodules (e.g., alfalfa). The above models describe amide syn-

thesis in actinorhizal nodules, and most actinorhizal plants transport amides in their xylem. However, *Alnus* spp. and *Casuarina equisetifolia* transport citrulline (Schubert, 1986; Sellstedt and Atkins, 1991). Results on the cell-specific localization of an enzyme in the citrulline biosynthetic pathway, acetylornithin aminotransferase, have led to the conclusion that citrulline biosynthesis takes place in the infected cells (Guan et al., 1996).

As opposed to other cyanobacterial-plant symbioses (i.e. Gunnera; Silvester et al., 1996) and the plant-microsymbiont systems described above, NH_4^+ may not be the fixed N transported from the cyanobiont to the cycad. This is indicated by the fact that activities, protein levels and cellular location of glutamine synthetase in cyanobionts of cycads (*Cycas, Ceratozamia* and *Zamia*) resemble that of free-living cyanobacteria (Lindblad and Bergman, 1986). Moreover, $^{15}N_2$ analyses of cycad symbiotic coralloid roots suggest that either glutamine and citrulline (Zamiaceae) or only glutamine (Cycadaceae and Stangeriaceae) are the N compounds translocated to the cycad (Costa and Lindblad, 2002; Pate et al., 1988). This implies that, although all other non-cycad cyanobionts tested so far release the fixed N as NH_4^+, by either lowering the cyanobiont GS activity or GS protein levels (Bergman et al., 2004; Rai et al., 2000), the cycad-cyanobacterial symbioses have apparently solved this crucial symbiotic issue using other mechanisms (Pate, 1989; Pate et al., 1988).

Conclusions

Despite the evolutionary, phylogenetic, and ecological diversity among the four root symbioses, there are numerous similarities in the means by which symbiotic partners in legumes, actinorhizal plants, *Parasponia* sp. and cycads facilitate the developmental and physiological imperatives that enable the symbioses to function. However, although all four symbioses meet the same symbiotic imperatives (e.g., infection, colonization, control of O_2 flux, exchange of C and N), they have not developed the same physiological and anatomical mechanisms to achieve these ends.

While there is evidence of a relatively close phylogenetic relationship between legumes, *Parasponia* and actinorhizal plants (i.e. the N_2-fixation clade) which may point to a common ancestry of these groups, the cycads, being a much more ancient order, are not closely related to the others. The three groups of microsymbionts (rhizobia, *Frankia* and cyanobacteria) are all Eubacteria, but are phylogenetically quite distant. Nonetheless, the highly conserved nature of many of the genes coding for the Mo-Fe form of nitrogenase shared by the four symbioses indicates some degree of commonality, and possibly lateral gene transfer. Ecologically, the four symbioses are dispersed across many geographical and climatic regions; however, not surprisingly, there is a propensity for distribution among low-fertility habitats.

In the infection processes there is evidence for roles of chemo-attractants, and flavonoid-type substances have been implicated in all four symbioses. There are many similarities between legumes and actinorhizal plants in the infection process, with both intercellular and intracellular infection pathways, however, the actual nature of the infection threads are quite different, and the equivalent of nod factor has not yet been identified in the actinorhizal symbiosis. *Parasponia* infection is unique as it seems to involve a degradation of the root epidermis, and two sorts of symbiotic 'threads'; a thick-walled intercellular infection thread and a 'fixation thread' that is more comparable to legume symbiosome than an infection thread in the other symbioses. Coralloid root infection is most comparable to crack-infection processes seen in some members of the other three symbioses. Little is known regarding signal exchange in the cycad- cyanobacteria symbiosis. While the symbio-organs of cycads, actinorhizal plants and *Parasponia* are modified roots, legume nodules arise from distinctly different zones of cell divisions than those that give rise to lateral roots.

In terms of energy supply to the microsymbionts, organic acids are the primary carbon supply to rhizobia and are implicated in supplying *Frankia*. Likewise, simple sugars have been proposed as substrates for *Frankia* and cyanobacteria in their respective symbioses. In legumes, most actinorhizal plants, cycads and possibly *Parasponia*, starch appears to be a common form of stored carbohydrate when supplied to the symbio-organ in surplus of the microsymbiont's requirements.

Rhizobia in both legume and *Parasponia* symbioses are extremely dependent upon the plant nodule with its O_2 diffusion barrier and leghemoglobin to provide the O_2 flux required for respiration, but without diminishing nitrogenase activity. By thickening the walls of the cells (compartments) where nitrogenase is located, *Frankia* and cyanobacteria are more independent in their regulation of O_2 flux within their respective symbioses, but still some level of con-

trol is enacted by the host tissue (e.g., hemoglobin and/or mucilage, respectively).

In terms of the means of transferring the fixed N from the microsymbiont to the host, some actinorhizal plants, legumes and possibly *Parasponia* have many similarities with NH_4^+ appearing to be the main form of N export from the bacteria, processing by GS/GOGAT, and the synthesis of amides as a form of N-transport compound out of the nodules. Less is known about the N transfer process in the cycad-cyanobacteria symbiosis, but it may be unique from the other two, with an organic-N compound (amino acid) being the potential export product from the heterocyst.

As stated at the outset of this chapter, a great challenge in comparing the four symbioses is the unbalanced amount of knowledge we have of the given partnerships. It is not surprising that the amount of knowledge and research on each of the four symbioses is relative to their recognized economic importance. Agronomically important legume-rhizobia symbioses have been, and continue to be, well examined. The use of the 'model' legumes, *Lotus japonicus* and *Medicago truncatulata* (Colebatch et al., 2002b), and the complete sequencing of the genomes of a several of the rhizobial microsymbionts (Galibert et al., 2001; Kaneko et al., 2000, 2002) will further help to accelerate the investigation of these symbioses. The actinorhizal-*Frankia* symbioses are not as well characterized, despite their importance in forest succession and revegetation (Benoit and Berry, 1990). Some challenges to the rate of progress in this field is that *Frankia* cannot yet be transformed (John et al., 2003), and the proportion of the genome which has been sequenced is relatively small. Nonetheless, progress on *Frankia* genetics is being made, particularly in specific areas such as the *nif* genes (e.g., Lavire and Cournoyer, 2003; Oh et al., 2003). Transformation of actinorhizal plants is possible with *Agrobacterium rhizogenes* and *A. tumefaciens*, and progress is being made in the host's genetics, particularly in the Casuarinaceae compared with the other seven actinorhizal plant families (Franche et al., 1998; Santi et al., 2003a). Despite the fact that *Parasponia* represent only five species native to a well-defined geographical region, they have received quite a bit of attention due to the fact that they are the only nonlegume plant to establish an effective symbiosis with rhizobia. Some aspects of the symbiosis have been quite well studied (e.g., hemoglobin), but there is still an absence of much basic information on the development and maintenance of the symbiosis. Our knowledge of cycads, and in particular of their peculiar cyanobacteria root symbiosis, is still highly inadequate and fragmentary, and many questions remain, although this division dominated the Earth' flora some hundred million years ago. For example, why are cycads the only extant gymnosperms to form a symbiosis with a N_2-fixing organism, given the competitive advantages of such a trait? Likewise, why do cycads, but no other plants, accept cyanobacteria in their roots, although root colonization is dominating among symbiotic angiosperms? Cyanobacteria do colonize cells of one angiosperm family (Gunneraceae), but exclusively cells in stem glands, not its roots. Why the cycad roots are never colonized by rhizobia or *Frankia* although these N_2-fixing bacteria form distinct root symbioses with angiosperms is another open question. However, the fact that (almost) the complete genome of *Nostoc* ATCC 29133, isolated from a cycad, is known will open new research avenues.

Regardless of the genetic, evolutionary, and functional diversity of the respective hosts and microsymbionts, the outcome of all the root symbioses is equally elegant: a micro-organism finds shelter and sustenance within the host plant and compensates its host with a constant and renewable source of N.

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References

Adams D G 2002 Cyanobacteria in symbiosis with hornworts and liverworts. *In* Cyanobacteria in Symbiosis, Eds. A N Rai, B Bergman and U Rasmussen. pp. 117–135. Kluwer Academic Publishers, Dordrecht.

Ahern C P and Staff I A 1994 Symbiosis in cycads: the origin and development of corraloid roots in *Macrozamia communis* (Cycadaceae). Am. J. Bot. 81, 1559–1570.

Akkermans A D L, Huss-Danell K and Roelofsen W 1981 Enzymes of the tricarboxylic acid cycle and the malate-aspartate shuttle in the N_2 nitrogen-fixing endophyte of *Alnus glutinosa*. Physiol. Plant. 53, 289–294.

Allaway D, Lodwig E M, Crompton L A, Wood M, Parsons R, Wheeler T R and Poole P S 2000 Identification of alanine dehydrogenase and its role in mixed secretion of ammonium and alanine by pea bacteroids. Mol. Microbiol. 36, 508–515.

Allen O N and Allen E K 1981 The Leguminosae: A Source Book of Characteristics, Uses and Nodulation. The University of Wisconsin Press, Madison. 812 pp.

Allen G C and Elkan G H 1990 Growth, respiration, and polypeptide patterns of *Bradyrhizobium* sp. (*Arachis*) strain 3G4b20 from succinate- or oxygen-limited continuous cultures. Appl. Environ. Microbiol. 56, 1025–1032.

Appleby C A, Tjepkema J D and Trinick M J 1983 Hemoglobin in a nonleguminous plant, *Parasponia*: possible genetic origin and function in nitrogen fixation. Science 220, 951–953.

Baginsky C, Brito B, Imperial J, Palacios J M and Ruiz-Argueso T 2002 Diversity and evolution of hydrogenase systems in rhizobia. Appl. Environ. Microbiol. 68, 4915–4924.

Baker A, Dodd C D and Parsons R 1996 Identification of amino compounds synthesized and translocated in symbiotic *Parasponia*. Plant Cell Environ. 19, 1249–1260.

Baker D D and Mullin B C 1992 Actinorhizal symbiosis. *In* Biological Nitrogen Fixation. Eds. G Stacey, H Evans and R Burris. pp. 259–292. Chapman and Hall, New York.

Becana M and Klucas R V 1992 Oxidation and reduction of leghemoglobin in root nodules of leguminous plants. Plant Physiol. 98, 1217–1221.

Becking J H 1979 Root-nodule symbiosis between Rhizobium and *Parasponia parviflora* (Ulmaceae). Plant Soil 51, 289–296.

Becking J H 1983a The *Parasponia parviflora-Rhizobium* symbiosis. Host specificity, growth and nitrogen fixation under various conditions. Plant Soil. 75, 309–342.

Becking J H 1983b The *Parasponia parviflora-Rhizobium* symbiosis. Isotopic nitrogen fixation, hydrogen evolution and nitrogen-fixation, hydrogen evolution and nitrogen-fixation efficiency, and oxygen relations. Plant Soil 75, 343–360.

Becking J H 1992 The Rhizobium symbiosis of the nonlegume *Parasponia*. *In* Biological Nitrogen Fixation: Achievements and Objectives. Eds. G. Stacey, R H Burris and H J Evans. pp. 497–559. Chapman and Hall, New York.

Beckwith J, Tjepkema J D, Cashon R E, Schwintzer C R and Tisa L S 2002 Hemoglobin in five genetically diverse *Frankia* strains. Can. J. Microbiol. 48, 1048–1055.

Bender G L, Goydych W, Rolfe B G and Nayudu M 1987a The role of *Rhizobium* conserved and host specific nodulation genes in the infection of the non-legume *Parasponia andersonii*. Mol. Gen. Gen. 210, 299–306.

Bender G L, Nayudu M, Goydych W and Rolfe B G 1987b Early infection events in nodulation of the non-legume *Parasponia andersonii* by *Bradyrhizobium*. Plant Sci. 51, 285–293.

Benoit L F and Berry A M 1990 Methods for production and use of actinorhizal plants in forestry, low maintenance landscapes and revegetation. *In* The Biology of *Frankia* and Actinorhizal Plants. Eds. C R Schwintzer and J D Tjepkema. pp. 281–297. Academic Press, New York.

Benson D R, Arp D J and Burris R H 1980 Hydrogenase in actinorhizal root nodules and root nodule homgenates. J. Bacteriol. 142, 138–144.

Benson D R and Hanna D 1983 *Frankia* diversity in an alder stand as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of whole-cell proteins [Nitrogen-fixing symbiosis with actinomycetes]. Can. J. Bot. 61, 2919–2923.

Benson D R and Silvester W B 1993 Biology of *Frankia* strains, actinomycete symbionts of actinorhizal plants. Microbiol. Rev. 57, 293–319.

Berg R H 1999 *Frankia* forms infection threads. Can. J. Bot. 77, 1327–1333.

Berg R H and McDowell L 1987a Endophyte differentiation in *Casuarina* actinorhizae. Protoplasma 136, 104–117.

Berg R H and McDowell L 1987b Cytochemistry of the wall of infected cells in *Casuarina* actinorhizae. Can. J. Bot. 66, 2038–2047.

Berger D and Altmann T 2000 A subtilisin-like serine protease involved in the regulation of stomatal density and distribution in *Arabidopsis thaliana*. Genes Dev. 14, 1119–1131.

Bergersen F J 1996 Delivery of O_2 to bacteroids in soybean nodule cells: Consideration of gradients of concentration of free, dissolved O_2 in a near symbiosomes and beneath intercellular spaces. Protoplasma 191, 9–20.

Bergman B 2002 The *Nostoc-Gunnera* symbiosis. *In* Cyanobacteria in Symbiosis. Eds. A N Rai, B Bergman and U Rasmussen. pp. 207–232. Kluwer Academic Publishers, Dordrecht.

Bergman B, Lindblad P and Rai A N 1986 Nitrogenase in free-living and symbiotic cyanobacteria: immunoelectron microscopic localization. FEMS Microbiol. Lett. 35, 75–78.

Bergman B, Matveyev A and Rasmussen U 1996 Chemical signalling in cyanobacterial-plant symbioses. Trends Plant Sci. 1, 191–197.

Bergman B, Rasmussen U and Rai A N 2004 Cyanobacterial Associations. *In* Associative Nitrogen-fixing Bacteria and Cyanobacterial Associations. Eds. C Elmerich and W E Newton. 41 pp. Kluwer Academic Publishers (in press).

Berry A M, Harriott O T, Moreau R A, Osman S F, Benson D R and Jones A D 1993 Hopanoid lipids compose the *Frankia* vesicle envelope, presumptive barrier of oxygen diffusion to nitrogenase. Proc. Natl. Acad. Sci., USA 90, 6091–6094.

Berry A M, Murphy T M, Okubara P A, Jacobsen K R, Swensen S M and Pawlowski K 2004 Novel expression pattern of cytosolic glutamine synthetase in nitrogen-fixing root nodules of the actinorhizal host, *Datisca glomerata*. Plant Physiol. (in review).

Berry A M and Torrey J G 1983 Root hair deformation in the infection process of *Alnus rubra* [Actinomycete *Frankia*]. Can. J. Bot. 61, 2863–2876.

Bogusz D, Llewellyn D J, Craig S, Dennis E S, Appleby C A and Peacock W J 1988 Nonlegume hemoglobin genes retain organ-specific expression in heterologous transgenic plants. Plant Cell 2, 633–641.

Bond G 1952 Some features of the root growth in nodulated plants of *Myrica gale* L. Ann. Bot. 16, 467–475.

Brenner E D, Stevenson D W and Twigg R W 2003 Cycads: Evolutionary innovations and the role of plant-derived neurotoxins. Trends Plant Sci. 8, 446–452

Bromfield E S P, Butler G and Barran L R 2001 Temporal effects on the composition of a population of *Sinorhizobium meliloti* associated with *Medicago sativa* and *Melilotus alba*. Can. J. Microbiol. 47, 567–573.

Brown P H and Hu H 1996 Phloem mobility of boron is species dependent: evidence for phloem mobility in sorbitol-rich species. Ann. Bot. 77, 497–505.

Buikema W J and Haselkorn R 1993 Molecular genetics of cyanobacterial development. Annu. Rev. Plant Physiol. Plant Mol. Biol. 44, 33–52.

Burgess D and Peterson R L 1987 Development of *Alnus japonica* root nodules after inoculation with *Frankia* strain HFPArI3. Can. J. Bot. 65, 1647–1657.

Burris R H 1991 Nitrogenases. J. Biol. Chem. 266, 9339–9342.

Callaham D, Newcomb W, Torrey J G and Peterson R L 1979 Root hair infection in actinomycete-induced root nodule initiation in

Casuarina cunninghamiana Myrica gale, Myrica cerifera and *Comptonia peregrina*. Bot. Gaz. 140, S1–S9.

Callaham D and Torrey J G 1977 Prenodule formation and primary nodule development in roots of *Comptonia* (Myricaceae). Can. J. Bot. 55, 2306–2318.

Cérémonie H, Debelle F and Fernandez M P 1999 Structural and functional comparison of *Frankia* root hair deforming factor and rhizobia Nod factor. Can. J. Bot. 77, 1293–1301.

Chaudhuri H and Akhtar A R 1931 The coral-like roots of *Cycas revoluta, Cycas circinalis* and *Zamia floridana* and the alga inhabiting them. J. Ind. Bot. Soc. 10, 43–59

Colebatch G, Kloska S, Trevaskis B, Freund S, Altmann T and Udvardi M K 2002a Novel aspects of symbiotic nitrogen fixation uncovered by transcript profiling with cDNA arrays. Mol. Plant-Microbe Interact. 15, 411–420.

Colebatch G, Trevaskis B and Udvardi M 2002b Symbiotic nitrogen fixation research in the postgenomics era. New Phytol. 153, 37–42.

Costa J-L and Lindblad P 2002 Cyanobacteria in symbiosis with cycads. *In* Cyanobacteria in Symbiosis, Eds. A N Rai, B Bergman and U Rasmussen. pp. 195–202. Kluwer Academic Publishers, Dordrecht.

Christensen T, Dennis E S, Peacock J W, Landsmann J and Marcker K A 1991 Hemoglobin genes in non-legumes: cloning and characterization of a *Casuarina glauca* hemoglobin gene. Plant Mol. Biol. 16, 339–344.

Clawson M L and Benson D R 1999. Natural diversity of *Frankia* strains in actinorhizal root nodules from promiscuous hosts in the family Myricaceae. Appl. Environ. Microbiol. 65, 4521–4527.

Cohen M F and Meeks J C 1997 A hormogonium regulating locus, *hrmUA*, of the cyanobacterium *Nostoc punctiforme* strain ATCC 29133 and its response to an extract of a symbiotic plant partner *Anthoceros punctatus*. Mol. Plant-Microbe Interact. 10, 280–289.

Cullimore J V and Bennett M J 1992 Nitrogen assimilation in the legume root nodule: current status of the molecular biology of the plant enzymes. Can. J. Microbiol. 38, 461–466.

Cvitanich C, Pallisgaard N, Nielsen K A, Hansen A C, Larsen K, Pihakaski-Maunsbach K, Marcker K A and Jensen E O 2000 CPP1, a DNA-binding protein involved in the expression of a soybean leghemoglobin c3 gene. Proc. Natl. Acad. Sci., USA 97, 8163–8168.

Day D A, Poole P S, Tyerman S D and Rosendahl L 2001 Ammonia and amino acid transport across symbiotic membranes in nitrogen-fixing legume nodules. Cell. Mol. Life Sci. 58, 61–71.

Day A A and Udvardi M K 1993 Metabolite exchange across symbiosome membranes. Symbiosis 14, 175–189.

Denison R F 1992 Mathematical modeling of oxygen diffusion and respiration in legume root nodules. Plant Physiol. 98, 901–907.

Denison R F 2000 Legume sanctions and the evolution of symbiotic cooperation by rhizobia. Am. Nat. 156, 567–576.

Denison R F and Okano Y 2003 Leghaemoglobin oxygenation gradients in alfalfa and yellow sweetclover nodules. J. Exp. Bot. 54, 1085–1091.

Diem H G and Dommergues Y R 1990. Current and potential uses and management of Casuarinaceae in the tropics and subtropics. *In* The Biology of *Frankia* and Actinorhizal Plants. Eds. C R Schwintzer and J D Tjepkema. pp. 317–342. Academic Press, New York.

Dommergues Y R 1995 Nitrogen fixation by trees in relation to soil nitrogen economy. Fertil. Res. 42, 215–230.

Doyle J J 1998 Phylogenetic perspectives on nodulation: evolving views of plants and symbiotic bacteria. Trends Plant Sci. 3, 473–478.

Franche C, Laplaze L, Duhoux E and Bogusz D 1998 Actinorhizal symbioses: Recent advances in plant molecular and genetic transformation Studies. Crit. Rev. Plant Sci. 17, 1–28.

Galibert F, Finan T M, Long S R, Puhler A, Abola P, Ampe F, Barloy-Hubler F, Barnett M J, Becker A, Boistard P, Bothe G, Boutry M, Bowser L, Buhrmester J, Cadieu E, Capela D, Chain P, Cowie A, Davis R W, Dreano S, Federspiel N A, Fisher R F, Gloux S, Godrie T, Goffeau A, Golding B, Gouzy J, Gurjal M, Hernandez-Lucas I, Hong A, Huizar L, Hyman R W, Jones T, Kahn D, Kahn M L, Kalman S, Keating D H, Kiss E, Komp C, Lalaure V, Masuy D, Palm C, Peck M C, Pohl T M, Portetelle D, Purnelle B, Ramsperger U, Surzycki R, Thebault P, Vandenbol M, Vorholter F J, Weidner S, Wells D H, Wong K, Yeh K C and Batut J 2001 The composite genome of the legume symbiont *Sinorhizobium meliloti*. Science 293, 668–672.

Gallon J R 1992 Tansley Review No. 44: Reconciling the incompatible: N_2 fixation and O_2. New Phytol. 122, 571–609.

Gallon J R 2001 N_2 fixation in phototrophs: Adaptation to a specialized way of life. Plant Soil 230, 39–48.

Gantar M and Elhai J 1999 Colonization of wheat para-nodules by the N_2-fixing cyanobacterium *Nostoc* sp. strain 2S9B. New Phytol. 141, 373–379.

Gauthier D, Jaffre T and Prin Y 2000 Abundance of *Frankia* from *Gymnostoma* spp. in the rhizosphere of *Alphitonia neocaledonica*, a non-nodulated Rhamnaceae endemic to New Caledonia. Eur. J. Soil Biol. 36, 169–175.

Gherbi H, Duhoux E, Franche C, Pawlowski K, Berry A M and Bogusz D 1997 Cloning of a full-length symbiotic hemoglobin cDNA and *in situ* localization of the corresponding mRNA in *Casuarina glauca* root nodule. Physiol. Plant. 99, 608–616.

Grobbelaar N, Hattingh W and Marshall J 19876 The occurrence of coralloid roots on the South African species of the Cycadales and their ability to fix nitrogen symbiotically. S. Afr. J. Bot. 52, 467–471.

Grobbelaar N, Scott W E, Hattingh W and Marshall J 1987 The identification of the coralloid root endophytes of the southern African cycads and the ability of the isolates to fix dinitrogen. S. Afr. J. Bot. 53, 111–118.

Grove T S, O'Connell A M and Malajczuk N 1980 Effects of fire on the growth, nutrient content and rate of nitrogen fixation on the cycad *Macrozamia riedlei*. Aust. J. Botany 28, 271–281.

Gualtieri G and Bisseling T 2000 The evolution of nodulation. Plant Mol. Biol. 42, 181–194.

Guan C H, Ribeiro A, Akkermans A D L, Jing Y X, van Kammen, Bisseling T and Pawlowski K 1996 Nitrogen metabolism in actinorhizal nodules of *Alnus glutinosa:* Expression of glutamine synthetase and acetylornithine transaminase. Plant Mol. Biol. 32, 1177–1184.

Guinel F C and Geil R D 2002 A model for the development of the rhizobial and arbuscular mycorrhizal symbioses in legume and its use to understand the roles of ethylene in the establishment of these two symbioses. Can. J. Bot. 80, 695–720.

Hafeez F, Akkermans A D L and Chaudhary A H 1984 Observations on the ultrastructure of *Frankia* sp. in root nodules of *Datisca cannabina* L. Plant Soil 79, 383–402.

Halliday J and Pate J S 1976 Symbiotic nitrogen fixation by coralloid roots of the cycad *Macrozamia riedlei*: physiological characteristics and ecological significance. Aust. J. Plant Physiol. 3, 349–358.

Haselkorn R, Schlictman D, Jones K and Buikema W J 1997 Heterocyst differentiation and nitrogen fixation in cyanobacteria. *In* Biological Nitrogen Fixation for the 21st Century. Eds. C Elmerich, A Kondorosi and W E Newton. pp. 93–96. Kluwer, Dordrecht.

Heidstra R, Nilsen G, Martinez-Abarca F, van Kammen A and Bisseling T 1997 Nod factor-induced expression of leghemoglobin to study the mechanism of NH_4NO_3 inhibition on root hair deformation. Mol. Plant-Microbe Interact. 10, 215–220.

Hirsch A M, Fang Y, Asad S and Kapulnik Y 1997 The role of phytohormones in plant–microbe symbioses. Plant Soil 194, 171–184.

Hughes M, Donnelly C, Crozier A and Wheeler C T 1999 Effects of the exposure of roots of *Alnus glutinosa* to light on flavonoids and nodulation. Can. J. Bot. 77, 1311–1315.

Hunt S and Layzell D B 1993 Gas exchange of legume nodules and the regulation of nitrogenase activity. Annu. Rev. Plant Physiol. Plant Mol. Biol. 44, 483–511.

Hunt P W, Klok E J, Trevaskis B, Watts R A, Ellis M H, Peacock W J and Dennis E S 2002 Increased level of hemoglobin 1 enhances survival of hypoxic stress and promotes early growth in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci., USA 99, 17197–17202.

Hunt P W, Watts R A, Trevaskis B, Llewelyn D J, Burnell J, Dennis E S and Peacock W J 2001 Expression and evolution of functionally distinct haemoglobin genes in plants. Plant Mol. Biol. 47, 677–692.

Huss-Danell K 1990 The physiology of actinorhizal nodules. *In* The Biology of *Frankia* and Actinorhizal Plants. Eds. C R Schwintzer and J D Tjepkema. pp. 129–156. Academic Press, New York.

Huss-Danell K 1997 Tansley Review No. 93. Actinorhizal symbioses and their N_2 fixation. New Phytol. 136, 375–405.

Huss-Danell K and Bergman B 1990 Nitrogenase in *Frankia* from root nodules of *Alnus incana* (L.) Moench: immunolocalization of the Fe- and MoFe-proteins during vesicle differentiation. New Phytol. 116, 443–455.

Jacobson-Lyon K, Jensen E O, Jorgensen J E, Marcker K A, Peacock W J and Dennis E S 1995 Symbiotic and nonsymbiotic hemoglobulin genes of *Casuarina glauca*. Plant Cell 7, 213–223.

James E K, Iannetta P P M, Naisbitt T, Goi S R, Sutherland J M, Sprent J I, Minchin F R and Brewin N J 1994 A survey of N_2-fixing nodules in the Leguminosae with particular reference to intercellular glycoprotein in the control of oxygen diffusion. Proc. Royal Soc. Edinburgh 102B, 429–432.

James E K, Iannetta P P M, Deeks L, Sprent J I and Minchin F R 2000 Detopping causes production of intercellular space occlusions in both the cortex and infected region of soybean nodules. Plant Cell Environ. 23, 377–386.

Johansson C and Bergman B 1992 Early events during the establishment of the *Gunnera/Nostoc* symbiosis. Planta 188, 403–413.

John T R, Wiggington J, Bock J V, Klemt R and Johnson J D 2003 An insertion sequence unique to *Frankia* strain ArI5. Plant Soil 254, 107–113.

Kaneko T, Nakamura Y, Sato S, Asamizu E, Kato T, Sasamoto S, Watanabe A, Idesawa K, Ishikawa A, Kawashima K, Kimura T, Kishida Y, Kiyokawa C, Kohara M, Matsumoto M, Matsuno A, Mochizuki Y, Nakayama S, Nakazaki N, Shimpo S, Sugimoto M, Takeuchi C, Yamada M and Tabata S 2000 Complete genome structure of the nitrogen-fixing symbiotic bacterium *Mesorhizobium loti*. DNA Res. 7, 331–338.

Kaneko T, Nakamura Y, Sato S, Minamisawa K, Uchiumi T, Sasamoto S, Watanabe A, Idesawa K, Iriguchi M, Kawashima K, Kohara M, Matsumoto M, Shimpo S, Tsuruoka H, Wada T, Yamada M, Tabata S 2002 Complete genomic sequence of nitrogen-fixing symbiotic bacterium *Bradyrhizobium japonicum* USDA110. DNA Res. 9, 189–197.

Kapranov P, Routt S M, Bankaitis V A, de Bruijn F J and Szczyglowski K 2001 Nodule-specific regulation of phosphatidylinositol transfer protein expression in *Lotus japonicus*. Plant Cell 13, 1369–1382.

Karr D B, Oehrle N W and Emerich D W 2003 Recovery of nitrogenase from aerobically isolated soybean nodule bacteroids. Plant Soil 257, 27–33.

Kelner D J, Vessey J K and Entz M H 1997 The nitrogen dynamics of one, two and three year stands of alfalfa in a cropping system. Agric. Ecosyst. Environ. 64, 1–10.

Klint J, Ran R, Rasmussen, U and Bergman B 2003 Proteomics of the symbiotic cyanobacterium *Nostoc punctiforme*: identification of developmentally regulated proteins in hormogonia. Mol. Plant-Microbe Interact. (submitted)

Knight C D and Adams D G 1996 A method for studying chemotaxis in nitrogen-fixing cyano-bacterium-plant symbioses. Physiol. Mol. Plant Pathol. 49, 73–77.

Knowlton S, Berry A and Torrey J G 1980 Evidence that associated soil bacteria may influence root hair infection of actinorhizal plants by *Frankia*. Can. J. Microbiol. 26, 971–977.

Kuzma M M and Layzell D B 1994 Acclimation of soybean nodules to changes in temperature. Plant Physiol. 106, 263–270.

Lancelle S A and Torrey J G 1984 Early development of *Rhizobium*-induced root nodules of *Parasponia rigida*. I. Infection and early nodule initiation. Protoplasma 123, 26–37.

Lancelle S A and Torrey J G 1985 Early development of *Rhizobium*-induced root nodules of *Parasponia rigida*. II. Nodule morphogenesis and symbiotic development. Can. J. Bot. 63, 25–35.

Lavire C and Cournoyer B 2003 Progress on the genetics of the N_2-fixing actinorhizal symbiont *Frankia*. Plant Soil 254, 125–137.

Lavire C, Louis D, Perriere G, Briolay J, Normand P and Cournoyer B 2001 Analysis of pFQ31, a 8551-bp cryptic plasmid from the symbiotic nitrogen-fixing actinomycete *Frankia*. FEMS Microbiol. Lett. 197, 111–116.

Layzell D B 1990 N_2 fixation, NO_3^- reduction and NH_4^+ assimilation. *In* Plant Physiology, Biochemistry and Molecular Biology. Eds. D T Dennis and D H Turpin. pp. 389–406. Longman Group, Essex.

Lee H, Sung S B, Kim H B and An C S 2001 Sequence analysis and expression patterns of two *nifA* genes from *Frankia* EuIK1. Austral. J. Plant Physiol. 28, 939–949.

Lhuissier F G P, De Ruijter N C A, Sieberer B J, Esseling J J, Emons A M C 2001 Time course of cell biological events evoked in legume root hairs by *Rhizobium* Nod factors: State of the art. Ann. Bot. 87, 289–302.

Li Y Z, Parsons R, Day D A and Bergersen F J 2002 Reassessment of major products of N_2 fixation by bacteroids from soybean root nodules. Microbiology-SGM 148, 1959–1966.

Lindblad P 1992 Ornithine cycle in *Nostoc* PCC 73102. Occurrence and localization of ornithine carbamoyl transferase and the effect of external carbon and ornithine on nitrogenase activity and citrulline synthesis. Protoplasma 152, 87–95.

Lindblad P, Atkins C A and Pate J S 1991 N_2 fixation by freshly isolated *Nostoc* from coralloid roots of the cycad *Macrozamia riedlei* (Fisch. ex Gaud.) Gardn. Plant Physiol. 95, 753–759.

Lindblad P and Bergman B 1986 Glutamine synthetases: activity and localization in cyanobacteria of the cycads *Cycas revoluta* and *Zamia skinneri*. Planta 169, 1–7.

Lindblad P and Bergman B 1990 The cycad-cyanobacterial symbiosis. *In* Handbook of Symbiotic Cyanobacteria. Ed. A N Rai. pp. 137–159. CRC Press, Boca Raton.

Lindbald P, Bergman B, Hofsten A, Hällblom L and Nylund J-E 1985a The cyanobacterium-*Zamia* symbiosis: an ultrastructural study. New Phytol. 101, 707–716.

Lindblad P, Hällblom L and Lindblad P 1985b The cyanobacterium-*Zamia* symbiosis: C_2H_2 reduction and heterocyst frequency. Symbiosis 1, 19–28.

Liu Q and Berry A M 1991 The infection process and nodule initiation in the *Frankia-Ceanothus* root nodule symbiosis: A structural and histochemical study. Protoplasma 163, 82–92.

Lodwig E and Poole P 2003 Metabolism of *Rhizobium* bacteroids. Crit. Rev. Plant Sci. 22, 37–78.

Lopez M F, Fontaine M S and Torrey J G 1984 Levels of trehalose and glycogen in *Frankia* sp. HFPArI3 (Actinomycetales) [Nitrogen-fixing bacteria]. Can. J. Microbiol. 30, 746–752.

Lopez M F, Young P and Torrey J G 1986 A comparison of carbon source utilization for growth and nitrogenase activity in two *Frankia* isolates. Can. J. Microbiol. 32, 353–358.

Madsen E B, Madsen L H, Radutoiu S, Olbryt M, Rakwalska M, Szczyglowski K, Sato S, Kaneko T, Tabata S, Sandal N and Stougaard J 2003 A receptor kinase gene of the LysM type is involved in legume perception of rhizobial signals. Nature 425, 637–640.

Marie C, Deakin W J, Viprey V, Kopcinska J, Golinowski W, Krishnan H B, Perret X and Broughton W J 2003 Characterization of Nops, nodulation outer proteins, secreted via the type III secretion system of NGR234. Mol. Plant-Microbe Interact. 16, 743–751.

Mariotti A, Sougoufara B and Dommergues Y R 1992. Estimation of nitrogen fixation using the natural abundance method in a plantation of *Casuarina equisetifolia* (Forst). Soil Biol. Biochem. 24, 647–653.

Martel A, Jansson E, Garcia-Reina G and Lindblad P 1993 Ornithine cycle in *Nostoc* 73102. Arginase, OCT and arginine deaminase, and the effects of addition of external srginine, ornithine and citrulline. Arch. Microbiol. 59, 506–511.

Martinez-Romero E 2003 Diversity of *Rhizobium-Phaseolus vulgaris* symbiosis: Overview and perspectives. Plant Soil 252, 11–23.

Marvel D J, Torrey J G and Ausubel F M 1987 *Rhizobium* symbiotic genes required for nodulation of legume and nonlegume hosts. Proc. Natl. Acad. Sci., USA 84, 1319–1323.

Mattsson U, Johansson L, Sandström G and Sellstedt A 2001 *Frankia* KB5 possesses a hydrogenase immunologically related to membrane-bound [NiFe]-hydrogenases. Curr. Microbiol. 42, 438–441.

Mattsson U and Sellstedt A 2002 Nickel affects activity more than expression of hydrogenase protein in *Frankia*. Curr. Microbiol. 44, 88–93.

McClure P R, Coker III G T and Schubert K R 1983 Carbon dioxide fixation in roots and nodules of *Alnus glutinosa*. Plant Physiol. 71, 652–657.

McEwan N R, Green D C and Wheeler C T 1992 Utilisation of the root hair curling reaction in *Alnus glutinosa* for the assay of nodulation signal compounds. Acta Oecologica 13, 509–510.

Meeks J C, Campbell E, Hagen K, Hanson T, Hitzeman N and Wong F 1999 Developmental alternatives of symbiotic *Nostoc punctiforme* in response to its symbiotic partner *Anthoceros punctatus*. *In* The Phototrophic Prokaryotes, Eds. G A Peschek, W Löffelhardt and G Schmetterer. pp. 665–678, Kluwer Academic Publisher, New York.

Meesters T M 1987 Localization of nitrogenase in vesicles of *Frankia* sp. Cc1.17 by immunogoldlabelling on ultrathin cryosections. Arch. Microbiol. 146, 327–331

Miklashevichs E, Rohrig H, Schell J and Schmidt J 2001 Perception and signal transduction of rhizobial NOD factors. Crit. Rev. Plant Sci. 20, 373–394.

Miller I M and Baker D D 1985 The initiation, development and structure of root nodules in *Elaeagnus angustifolia* L. (Elaeagnaceae). Protoplasma 128, 107–119.

Miller I M and Baker D D 1986 Nodulation of actinorhizal plants by *Frankia* strains capable of both root hair infection and intercellular penetration. Protoplasma 131, 82–91.

Mohapatra S S and Gresshoff P M 1984 Carbon-nitrogen requirements for the expression of nitrogenase activity in cultured *Parasponia-Rhizobium* strain ANU 289 [Nitrogen-fixing bacteria]. Arch. Microbiol. 137, 58–62.

Newcomb W and Pankhurst C E 1982 Fine structure of actinorhizal nodules of *Coriaria arborea* (Coriariaceae). New Zealand J. Bot. 20, 93–103.

Nilsson M, Bhattacharya J, Rai A N and Bergman B 2002 Colonization of roots of rice (*Oryza sativa*) by symbiotic *Nostoc* strains. New Phytol. 156, 517–525.

Obukowicz M, Schaller M and Kennedy G S 1981 Ultrastructure and phenolic histochemistry of the *Cycas revoluta-Anabaena* symbiosis. New Phytol. 87, 751–759.

Oh C J, Kim H B and An C S 2003 Molecular cloning and complementation analysis of nifV gene from *Frankia* EuIK1 strain. Mol. Cells 15, 27–33.

Oke V and Long S R 1999 Bacteroid formation in the *Rhizobium*-legume symbiosis. Curr. Opin. Microbiol. 2, 641–646.

Okubara P A, Fujishige N A, Hirsch A M and Berry A M 2000 *Dg93*, a nodule-abundant mRNA of *Datisca glomerata* with homology to a soybean early nodulin. Plant Physiol. 122, 1073–1079.

Ow M C, Gantar M and Elhai J 1999 Reconstitution of a cycad-cyanobacterial association. Symbiosis 27, 125–134.

Pandya S, Iyer P, Gaitonde V, Parekh T and Desai A 1999 Chemotaxis of rhizobium SP.S2 towards *Cajanus cajan* root exudate and its major components. Curr. Microbiol. 38, 205–209.

Parker M A, Lafay B, Burdon J J and van Berkum P 2002 Conflicting phylogeographic patterns in rRNA and nifD indicate regionally restricted gene transfer in *Bradyrhizobium*. Microbiology-SGM 148, 2557–2565.

Parsons R, Silvester W B, Harris S, Gruijters W T M and Bullivant S 1987 *Frankia* vesicles provide inducible and absolute oxygen protection for nitrogenase. Plant Physiol. 83, 728–731.

Parsons R and Sunley R J 2001 Nitrogen nutrition and the role of root-shoot nitrogen signalling particularly in symbiotic systems. J. Exp. Bot. 52, 435–443.

Pate J S, Lindblad P, and Atkins C A 1988 Pathways of assimilation and transfer of the fixed nitrogen in coralloid roots of cycad-*Nostoc* symbioses. Planta 176, 461–471.

Pate J S 1989 Synthesis, transport, and utilization of products of symbiotic nitrogen fixation. Recent Adv. Phytochem. 23, 65–115.

Pathirana S M and Tjepkema J D 1995 Purification of hemoglobin from the actinorhizal root nodules of *Myrica gale* L. Plant Physiol. 107, 827–831.

Pawlowski K, Swensen S, Guan C H, Hadri A E, Berry A M and Bisseling T 2003 Distinct patterns of symbiosis-related gene expression in actinorhizal nodules from different plant families. Mol. Plant-Microbe Interact. 16, 796–807.

Pawlowski K, Twigg P, Dobritsa S, Guan C and Mullin B C 1997 A nodule-specific gene family from *Alnus glutinosa* encodes glycine- and histidine-rich proteins expressed in the early stages of actinorhizal nodule development. Mol. Plant-Microbe Interact. 10, 656–664.

Peoples M B, Giller K E, Herridge D F and Vessey J K 2002 Limitations to biological nitrogen fixation as a renewable source of nitrogen for agriculture. *In* Nitrogen Fixation – Global Perspect-

ives. Eds. T M Finan, M R O'Brian, D B Layzell, J K Vessey and W Newton. pp. 356–360. CABI Publishing, New York.

Phillips D A, Wery J, Joseph C M, Jones A D and Teuber L R 1995 Release of flavonoids and betaines from seeds of seven *Medicago* species. Crop Sci. 35, 805–808.

Plazinski J, Croft L, Taylor R, Zheng Q, Rolfe B G and Gunning B E S 1991 Indigenous plasmids in *Anabaena azollae*: their taxonomic distribution and existense of regions of homology of symbiotic genes of *Rhizobium*. Can. J. Microbiol. 37, 171–181.

Poza-Carrion C, Fernandez-Valiente E, Pinas F F and Leganes F 2001 Acclimation of photosynthetic pigments and photosynthesis of the cyanobacterium *Nostoc* sp strain UAM 206 to combined fluctuations of irradiance, pH, and inorganic carbon availability J. Plant Physiol. 158, 1455–1461.

Price G D, Mohapatra S S and Gresshoff P M 1984 Structure of nodules formed by *Rhizobium* strain ANU289 in the nonlegume *Parasponia* and the legume Siratro (*Macroptilium atropurpureum*). Bot. Gaz. 145, 444–451.

Price N P J 1999 Carbohydrate determinants of *Rhizobium*-legume symbioses. Carbohydr. Res. 317, 1–9.

Qian J H, Kwon S W and Parker M A 2003 rRNA and *nifD* phylogeny of *Bradyrhizobium* from sites across the Pacific Basin. FEMS Microbiol. Lett. 219, 159–165.

Racette S and Torrey J G 1989 Root nodule initiation in *Gymnostoma* (Casuarinaceae) and *Shepherdia* (Elaeagnaceae) induced by *Frankia* strain HFPGpI1. Can. J. Bot. 67, 2873–2879.

Radutoiu S, Madsen L H, Madsen E B, Felle H H, Umehara Y, Gronlund M, Sato S, Nakamura Y, Tabata S, Sandal N and Stougaard J 2003 Plant recognition of symbiotic bacteria requires two LysM receptor-like kinases. Nature 425, 585–592.

Rai A N, Bergman B and Rasmussen U 2002 Cyanobacteria in Symbiosis. Kluwer Academic Publishers, Dordrecht. 368 pp.

Rai A N, Söderbäck E and Bergman B 2000 Tansley Review No. 116: Cyanobacterium-plant symbioses. New Phytol. 147, 449–481.

Ramirez-Saad H, Janse J D and Akkermans A D L 1998 Root nodules of *Ceanothus caeruleus* contain both the N_2-fixing *Frankia* endophyte and a phylogenetically related Nod-/Fix- actinomycete. Can. J. Microbiol. 44, 140–148.

Rasmussen U, Johansson C, Renglin A, Pettersson C and Bergman B 1996 A molecular characterization of the *Gunnera-Nostoc* symbiosis: Comparison with *Rhizobium*- and *Agrobacterium*-plant interactions. New Phytol. 133, 391–398.

Rasmussen U and Nilsson M 2002 Cyanobacterial diversity and specificity in plant symbioses. *In* Cyanobacteria in Symbiosis, Eds. A N Rai, B Bergman and U Rasmussen. pp. 313–328. Kluwer Academic Publishers, Dordrecht.

Reinhold-Hurek B and Hurek T 1997 Interaction between diazotrophs and grasses. *In* Biological Fixation of Nitrogen for Ecology and Sustainable Agriculture. Eds. A Legocki, H Bothe and A Puhler. pp. 317–324. Springer-Verlag, Berlin.

Ribbe M, Gadkari D and Meyer O 1997 N_2 fixation by *Streptomyces thermoautotrophicus* involves a molybdenum-dinitrogenase and a manganese-superoxide oxidoreductase that couple N_2 reduction to the oxidation of superoxide produced from O_2 by a molybdenum-CO dehydrogenase. J. Biol. Chem. 272, 26627–26633.

Rippka R, Deruelles J, Waterbury J B, Herdman M and Stainer R Y 1979 Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J. Gen. Microbiol. 111, 1–61.

Robinson J B and Bauer W D 1993 Relationships between C_4 dicarboxylic acid transport and chemotaxis in *Rhizobium meliloti*. J. Bacteriol. 175, 2284–2291.

Rodriguez-Barrueco C and de Castro F B 1973 Cytokinin-induced pseudonodules on *Alnus glutinosa*. Physiol. Plant. 29, 227–280.

Roelofsen W and Akkermann A D L 1979 Uptake and evolution of H_2 and reduction of C_2H_2 by root nodules and homogenates of *Alnus glutinosa* L. Plant Soil 110, 167–176.

Santi C, Svistoonoff S, Constans L, Auguy F, Duhoux E, Bogusz D and Franche C 2003a Choosing a reporter for gene expression studies in transgenic actinorhizal plants of the Casuarinaceae family. Plant Soil 254, 229–237.

Santi C, von Groll U, Ribeiro A, Chiurazzi M, Auguy F, Bogusz D, Franche C and Pawlowski K 2003b Comparison of nodule induction in legume and actinorhizal symbioses: The induction of actinorhizal nodules does not involve ENOD40. Mol. Plant-Microbe Interact. 16, 808–816

Schneider D, Wink M, Sporer F and Lounibos P 2002 Cycads: Their evolution, toxins, herbivores and insect pollinators. Naturwissenschaften 89, 281–294.

Schopf J W, Kudryavtsev A B, Agresti D G, Wdowlak T J and Czaja A D 2002 Laser-Raman imagery of Earth's earliest fossils. Nature 416, 73–76.

Schubert K R 1986 Products of biological nitrogen fixation in higher plants: synthesis, transport, and metabolism. Annu. Rev. Plant Physiol. 37, 539–574.

Schultze M and Kondorosi A 1998 Regulation of symbiotic root nodule development. Annu. Rev. Genet. 32, 33–57.

Schwencke J and Caru M 2001 Advances in actinorhizal symbiosis: Host plant-*Frankia* interactions, biology, and applications in arid land reclamation. A Review. Arid Land Res. Man. 15, 285–327.

Schwintzer C R, Berry A M and Disney L D 1982 Seasonal patterns of root nodule growth, endophyte morphology, nitrogenase activity and shoot development in *Myrica gale*. Can. J. Bot. 60, 746–757.

Scott K F, Saad M, Price G D, Gresshoff P M, Kane H and Chua K Y 1987 Conserved nodulation genes are obligatory for nonlegume nodulation. *In* Molecular Genetics and Plant-Microbe Interactions. Eds. D-P S Verma and N Brisson. Kluwer Academic Publishers, Dordrecht.

Sellstedt A and Atkins C A 1991 Composition of amino compounds transported in xylem of *Casuarina* sp. J. Exp. Bot. 42, 1493–1497.

Sergeeva E, Liaimer A and Bergman B 2002 Evidence for production of the phytohormone indole-3-acetic acid by cyanobacteria. Planta 215, 229–238.

Serraj R, Sinclair T R and Purcell L C 1999 Symbiotic N_2 fixation response to drought. J. Exp. Bot. 50, 143–155.

Silvente S, Camas A and Lara M 2003 Heterogeneity of sucrose synthase genes in bean (*Phaseolus vulgaris* L.): Evidence for a nodule-enhanced sucrose synthase gene. J. Exp. Bot. 54, 749–755.

Silvester W B and Harris S L 1989 Nodule structure and nitrogenase activity of *Coriaria arborea* in response to varying oxygen partial pressure. Plant Soil 118, 97–110.

Silvester W B, Harris S L, Tjepkema J D 1990 Oxygen regulation and hemoglobin. *In* The Biology of *Frankia* and Actinorhizal Plants. Eds. C R Schwintzer and J D Tjepkema. pp. 157–176. Academic Press, New York.

Silvester W B, Langenstein B and Berg R H 1999 Do mitochondria provide the oxygen diffusion barrier in root nodules of *Coriaria* and *Datisca*? Can. J. Bot. 77, 1358–1366.

Silvester W B, Parsons R and Watt P W 1996 Direct measurement of release and assimilation of ammonia in the *Gunnera-Nostoc* symbiosis. New Phytol. 132, 617–625.

Silvester W B, Whitbeck J, Silvester J K and Torrey J G 1988. Growth, nodule morphology, and nitrogenase activity of Myrica

gale with roots grown at various oxygen levels. Can. J. Bot. 66, 1762–1771.

Simms E L and Taylor D L 2002 Partner choice in nitrogen-fixation mutualisms of legumes and rhizobia. Integ. Comp. Biol. 42, 369–380.

Smith C A, Skvirsky R C and Hirsch A M 1986 Histochemical evidence for the presence of a suberinlike compound in *Rhizobium*-induced nodules of the nonlegume *Parasponia rigida*. Can. J. Bot. 64, 1474–1483.

Soedarjo M and Borthakur D 1998 Mimosine, a toxin produced by the tree-legume *Leucaena* provides a nodulation competition advantage to mimosine-degrading *Rhizobium* strains. Soil Biol. Biochem. 30, 1605–1613.

Soltis D E, Soltis P S, Morgan D R, Swensen S M, Mullin B C, Dowd J M and Martin P G 1995 Chloroplast gene sequence data suggest a single origin of the predisposition for symbiotic nitrogen fixation in angiosperms. Proc. Natl. Acad. Sci., USA 92, 2647–2651.

Soltis P S, Soltis D E, Zanis M J and Kim S 2000 Basal lineages of angiosperms: relationships and implications for floral evolution. Int. J. Plant Sci. 161, S97–S107.

Smolander A, Ronkko R, Nurmiaho-Lassila E L and Haahtela K 1990 Growth of *Frankia* in the rhizosphere of *Betula pendula*, a nonhost tree species. Can. J. Microbiol. 36, 649–656.

Sprent J I and Raven J A 1992 Evolution of nitrogen-fixing root nodules symbioses. *In* Biological Nitrogen Fixation: Achievements and Objectives. Eds. G Stacey, R H Burris and H J Evans. pp. 461–196. Chapman and Hall, New York.

Sprent J I and Scott R 1979 The nitrogen economy of *Myrica gale* and its possible significance for the afforestation of peat soils. *In* Symbiotic Nitrogen Fixation in the Management of Temperate Forests. Eds. J C Gordon, C T Wheeler and A Perry. pp. 234–242. Forest Research Laboratory, State University, Corvallis.

Stevens G A Jr and Berry A M 1988 Cytokinin secretion by *Frankia* sp. HFPArI3 in defined medium. Plant Physiol. 87, 15–16.

Suganuma N, Nakamura Y, Yamamoto M, Ohta T, Koiwa H, Akao S and Kawaguchi M 2003 The *Lotus japonicus Sen1* gene controls rhizobial differentiation into nitrogen-fixing bacteroids in nodules. Mol. Gen. Genomics 269, 312–320.

Suominen L, Roos C, Lortet G, Paulin L and Lindstrom K 2001 Identification and structure of the *Rhizobium galegae* common nodulation genes: Evidence for horizontal gene transfer. Mol. Biol. Evol. 18. 907–916.

Svistoonoff S, Laplaze L, Auguy F, Santi C, Fontanillas E, Duhoux E, Franche C and Bogusz D 2003 Expression pattern of *ara12**, an *Arabidopsis* homologue of the nodule-specific actinorhizal subtilases *cg12/ag12*. Plant Soil 254, 239–244.

Swensen S M 1996 The evolution of actinorhizal symbioses – evidence for multiple origins of the symbiotic association. Amer. J. Bot. 83, 1503–1512.

Szczyglowski K, Kapranov P, Hamburger D and de Bruijn F J 1998 The *Lotus japonicus* LjNOD70 nodulin gene encodes a protein with similarities to transporters. Plant Mol. Biol. 37, 651–661.

Tamagnini P, Axelsson R, Lindberg P, Oxelfelt F, Wunschiers R and Lindblad P 2002 Hydrogenases and hydrogen metabolism of cyanobacteria. Microbiol. Mol. Biol. Rev. 66, 1–20.

Tjepkema J D and Cartica R J 1982 Diffusion limitation of oxygen uptake and nitrogenase activity in the root nodules of *Parasponia rigida* Merr. and Perry. Plant Physiol. 69, 728–733.

Torrey J G and Callaham D 1979 Early nodule development in *Myrica gale*. Soil actinomycete causing nodulation. Bot. Gaz. 140, S10–S14.

Treutlein J and Wink M 2002 Molecular phylogeny of cycads inferred from *rbcL* sequences. Naturwissenschaften 89, 221–225.

Trevaskis B, Colebatch G, Desbrosses G, Wandrey M, Wienkoop S, Saalbach G and Udvardi M 2002 Differentiation of plant cells during symbiotic nitrogen fixation. Comp. Func. Gen. 3, 151–157.

Trinick M J 1973 Symbiosis between *Rhizobium* and the non-legume, *Trema aspera*. Nature (Lond.) 244, 459–460.

Trinick M J 1979 Structure of nitrogen-fixing nodules formed by *Rhizobium* on roots of *Parasponia andersonii* Planch. Can. J. Microbiol. 25, 565–578.

Trinick M J 1981 The effective *Rhizobium* symbiosis with the non-legume *Parasponia andersonii. In* Current Perspectives in Nitrogen Fixation. Eds. A H Gibson and W E Newton. pp. 480. Elsevier/North-Holland Biomedical Press, Amsterdam.

Trinick M J and Galbraith J 1980 The Rhizobium requirements of the non-legume *Parasponia andersonii* in relationship to the cross-inoculation group concept of legumes nitrogen fixation. New Phytol. 86, 17–26.

Tyerman S D, Whitehead L F and Day D A 1995 A channel-like transporter for NH_4^+ on the symbiotic interface of N_2-fixing plants. Nature 378, 629–632.

Udvardi M K and Day D A 1990 Ammonia (^{14}C-methylamine) transport across the bacteroid and peribacteroid membranes of soybean root nodules. Plant Physiol. 94, 71–76.

Udvardi M K, Lister D L and Day D A 1992 Isolation and characterization of a ntrC and mutant of *Bradyrhizobium* (*Parasponia*) sp. ANU289. J. Gen. Microbiol. 138, 1019–1025.

Valverde C and Wall L G 1999 Time course of nodule development in the *Discaria trinervis* (Rhamnaceae)-*Frankia* symbiosis. New Phytol. 141, 345–354.

Valverde C and Wall L G 2003 Ammonium assimilation in root nodules of actinorhizal *Discaria trinervis*. Regulation of enzyme activities and protein levels by the availability of macronutrients (N, P and C). Plant Soil 254, 139–153.

Van Ghelue M, Løvaas E, Ringø E and Solheim B 1997 Early interactions between *Alnus glutinosa* (L.) Gaertn. and *Frankia* strain ArI3. Production and specificity of root hair deformation factor(s). Physiol. Plant. 99, 579–587.

Van Ghelue M, Ribeiro A, Solheim B, Akkermans A D L, Bisseling T and Pawlowski K 1996 Sucrose synthase and enolase expression in actinorhizal nodules of *Alnus glutinosa*: Comparison with legume nodules. Mol. Gen. Genet. 250, 437–446.

Van Straten J, Akkermans A D L and Roelofsen W 1977 Nitrogenase activity of endophyte suspensions derived from root nodules of *Alnus, Hippophae, Shepherdia* and *Myrica* spp. Nature 266, 257–258.

Vessey J K 2003 Plant growth promoting rhizobacteria as biofertilizers. Plant Soil 255, 571–586.

Vessey J K and Pan B 2003 Living a grounded life: Growth and nitrogenase activity of *Gluconacetobacter diazotrophicus* on solid media in response to culture conditions. Symbiosis 35, 181–197.

Vessey J K, Walsh K B and Layzell D B 1988 Oxygen limitation of N_2 fixation in stem-girdled and nitrate-treated soybean. Physiol. Plant. 73, 113–121.

Vikman P-A 1992 The symbiotic vesicle is a major site for respiration in *Frankia* from Alnus incana root nodules. Can. J. Microbiol. 38, 779–784.

Vikman P-A and Huss-Danell K 1987a Purity of *Frankia* preparations from root nodules of *Alnus incana*. Physiol. Plant. 71, 489–494.

Vikman P-A and Huss-Danell K 1987b Capacity for hexose respiration in symbiotic *Frankia* from *Alnus incana*. Physiol. Plant. 70, 349–354.

Vikman P-A and Huss-Danell K 1991 Respiration of malate and glutamate in symbiotic *Frankia* prepared from *Alnus incana*. J. Exp. Bot. 42, 221–228.

Vikman P-A and Vessey J K 1993 Ontogenetic changes in root nodule subpopulations of common bean (*Phaeolus vulgaris* L.). III. Nodule formation, growth and degradation. J. Exp. Bot. 44, 579–586.

Viterbo A, Matveyev A, Rasmussen U and Bergman B 1999 Characterization of a nodM/glmS homologous gene in the symbiotic cyanobacterium *Nostoc* PCC 9229. Symbiosis 26, 237–246.

Walsh K B, Vessey J K and Layzell D B 1987 Carbohydrate supply and N_2 fixation in soybean. The effect of varied daylength and stem girdling. Plant Physiol. 85, 137–144.

Wang C M, Ekman M and Bergman B 2004 Expression of cyanobacterial genes and proteins involved in heterocyst differentiation and dinitrogen fixation along a plant symbiosis development profile Mol. Plant-Microbe Interact. (in press).

Waters J K, Hughes B L, Purcell L C, Gerhardt K O, Mawhinney T P and Emerich D W 1998 Alanine, not ammonia, is excreted from N_2-fixing soybean nodule bacteroids. Proc. Natl. Acad. Sci., USA 95, 12038–12042.

Webster G, Davey M R and Cocking E C 1995a *Parasponia* with rhizobia: a neglected non-legume nitrogen-fixing symbiosis. AgBiotech News Info. 7, 119–124.

Webster G, Poulton P R, Cocking E C and Davey M R 1995b The nodulation of micro-propagated plants of *Parasponia andersonii* by tropical legume rhizobia. J. Exp. Bot. 46, 1131–1137.

Weisz P R and Sinclair T R 1987 Regulation of soybean nitrogen fixation in response to rhizosphere oxygen. I. Role of nodule respiration. Plant Physiol. 84, 900–905.

Wheeler C T, Watts S H, Hillman J R 1983 Changes in carbohydrates and nitrogenase compounds in the root nodules of *Alnus glutinosa* in relation to dormancy. New Phytol 95, 209–218.

Wittmann W, Bergersen F J and Kennedy G S 1965 The coralloid roots of *Macrozamia communis* L. Johnson. Aust. J. Biol. Sci. 18, 1129–1134.

Wong F C Y and Meeks J C 1994 Establishment of a functional symbiosis between the cyanobacterium *Nostoc punctiforme* and the bryophyte *Anthoceros punctatus* requires genes involved in nitrogen control and initiation of heterocyst differentiation. Microbiology-SGM 148, 315–323.

Wycoff K L, Hunt S, Gonzales M B, VandenBosch K A, Layzell D B and Hirsch A M 1998 Effects of oxygen on nodule physiology and expression of nodulins in alfalfa. Plant Physiol. 117, 385–395.

Zehr J P, Jenkins B D, Short S M and Steward G F 2003 Nitrogenase gene diversity and microbial community structure: a cross-system comparison. Environ. Microbiol. 5, 539–554.

Zeng S and Tjepkema J D 1994 The wall of the infected cell may be the major diffusion barrier in nodules of *Myrica gale* L. Soil Biol Biochem. 26, 633–639.

Zepp K, Hahn D and Zeyer J 1997 Evaluation of a 23S rRNA insertion as target for the analysis of uncultured *Frankia* populations in root nodules of alders by whole cell hybridization. Syst. Appl. Microbiol. 20, 124–132.

Zheng W, Song T, Bao X, Bergman B and Rasmussen U 2002 High cyanobacterial diversity in coralloid roots of cycads revealed by PCR fingerprinting. FEMS Microbiol. Ecol. 40, 215–222.

Zhulin I B, Bespalov V A, Johnson M S and Taylor B L 1996 Oxygen taxis and proton motive force in *Azospirillum brasilense*. J. Bacteriol. 178, 5199–5204.

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