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The Actinorhizal Symbiosis

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ABSTRACT

The term "actinorhiza" refers both to the filamentous bacteria *Frankia,* an actinomycete, and to the root location of nitrogen-fixing nodules. Actinorhizal plants are classified into four subclasses, eight families, and 25 genera comprising more than 220 species. Although ontogenically related to lateral roots, actinorhizal nodules are characterized by differentially expressed genes, supporting the idea of the uniqueness of this new organ. Two pathways for root infection have been described for compatible *Frankia* interactions: root hair infection or intercellular penetration. Molecular phylogeny groupings of host plants correlate with morphologic and anatomic features of actinorhizal nodules. Four clades of actinorhizal plants have been defined, whereas *Frankia* bacteria are classified into three major phylogenetic groups. Although the phylogenies of the symbionts are not fully congruent, a close relationship exists between plant and bacterial groups. A model for actinorhizal specificity is proposed that includes different levels or degrees of specificity of host-symbiont interactions, from fully compatible to incompatible. Intermediate, compatible, but delayed or limited interactions are also discussed. Actinorhizal plants undergo feedback regulation of symbiosis involving at least two different and consecutive signals that lead to a mechanism controlling root nodulation. These signals mediate the opening or closing of the window of susceptibility for infection and inhibit infection and nodule development in the growing root, independently of infection mechanism. The requirement for at least two molecular recognition steps in the development of actinorhizal symbioses is discussed.

Key words: *Frankia*; Root nodules; N_2 fixation; Actinorhiza; Plant microbe interaction

INTRODUCTION

Reduction of atmospheric N_2 to ammonia and its further assimilation into amino acids and other biomolecules enables gaseous nitrogen to be assimilated into life processes. Because all organisms need N to survive, nitrogen fixation is probably the second most important biochemical pathway after CO_2 fixation. Nevertheless, the ability to fix nitrogen is found in only one biologic kingdom, the Prokaryota (Sprent and Sprent 1990). Thus, other organisms have exploited the ability of prokaryotes to fix nitrogen by establishing various types of interactions (Werner 1992).

Cyanobacteria- and plant-microbe symbioses can be considered among the major milestones in evolution of life on Earth, bringing together the two most essential biochemical pathways—carbon fixation and nitrogen fixation. There are two main types of symbioses between nitrogen-fixing bacteria and vascular plants: one between *Rhizobium* and leguminous plants, and the other between *Frankia* and actinorhizal plants. The rhizobia-legume symbiosis involves more than 1700 plant species of the family Fabaceae (Leguminosea) distributed in three subfamilies—Mimosoideae, Ceaesalpinoideae, and Papilionoideae. The gram-negative bacterial partner

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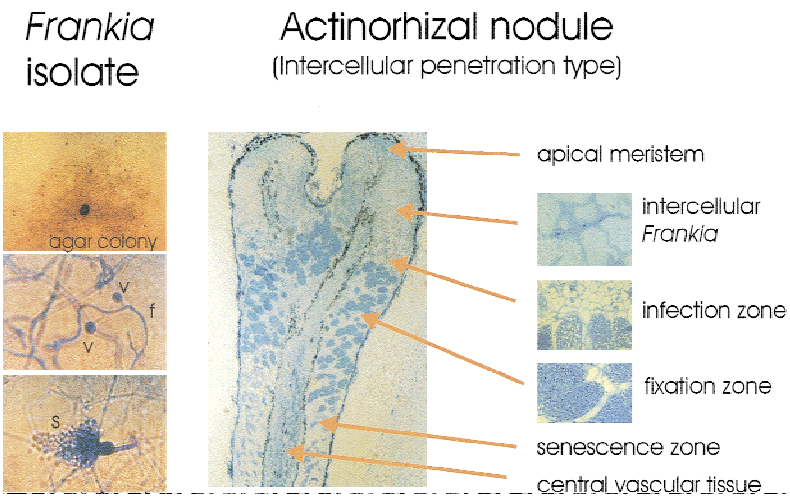

Figure 1. Morphology of *Frankia* isolates and actinorhizal nodule anatomy. Examples shown are from strain BCU110501 (Chaia 1998) and a *Discaria trinervis* 12-week-old nodule (Valverde 2000); *f.* filament, *v.* vesicle, *s.* sporangia; *Frankia* intercellular filament in nodule parenchyma; infection zone showing hyperplasic infected cells without vesicle differentiation; fixation zone showing hyperplasic infected cells with vesicles interspersed and normal-sized uninfected cells.

(*Rhizobium, Azorhizobium, Sinorhizobium, Bradyrhizobium,* and *Mesorhizobium*) is a member of the family Rhizobiaceae (Crespi and Gálvez 2000). Actinorhizal plants comprise more than 220 species symbiotically associated with the filamentous actinomycete *Frankia* (see later). *Gunnera,* which establishes a symbiosis with cyanobacteria in a specialized stem structure, represents a third type of nitrogen-fixing symbiosis. A fourth type is that which occurs between cyanobacteria and cycads. Other diverse diazotrophs, such as *Azospirillum, Herbaspirillum,* and *Acetobacter,* have been isolated and identified from the rhizosphere or from roots of many other plants, generally grasses, but are not symbiotically associated in root nodules (Dobereiner 1994).

In all cases of rhizobia-legume or *Frankia*-actinorhizal symbioses, a new plant organ is developed in which the bacteria differentiate, express the enzyme nitrogenase, and fix nitrogen into ammonia. These compounds are then assimilated and transported to the rest of the plant. Legumes and actinorhizal plants develop root nodules as a consequence of compatible plant-bacteria interactions through the switching on and off of genes in both genomes to establish a newly developed, shared structure. In some cases, so-called shoot nodules are formed (Prin and others 1992), but these aerial nodules are modified adventitious roots, having the same structure as root nodules but are located above the soil or the stems.

Actinorhizal and legume nodules can be easily distinguished at the anatomical level (see Pawlowski and Bisseling 1996). Legume nodules have a central infected tissue surrounded by nodule parenchyma and peripheral vascular bundles, whereas actinorhizal nodules are characterized by a central vascular bundle and peripheral infected tissue surrounded by cortical nodule parenchyma (Figure 1). Legume nodules have been proposed to have a shootlike anatomy, whereas actinorhizal nodules are ontogenically related to roots. Hirsch and LaRue (1997) discuss some of the hypotheses regarding the origin of nodules.

Both *Frankia*-actinorhizal plant and *Rhizobium*-legume symbioses have been known for many years to benefit soil fertility. The nature of these nitrogen-fixing symbioses and the microsymbionts involved were discovered at the end of 19^{th} century (Quispel 1990). The successful isolation of rhizobia from legume root nodules and the ability to manipulate rhizobia genetically in the last 25 years contrasts with the difficulties of isolating, culturing, and genetically manipulating *Frankia*. Nevertheless, as stated by Huss-Danell (1997): "while the huge amount of work on legumes has focused on only a small fraction of the thousands of symbioses with rhizobia, the comparably small research effort concerned with actinorhizas has included several host genera and has therefore given an insight into a number of morphological, physiological and biochemical variations among actinorhizal nodules."

A number of excellent reviews and book chapters on actinorhizal symbioses are available (Benson and Clawson 2000; Benson and Silvester 1993; Berry 1994; Franche and others 1998; Huss-Danell 1997; Mullin and Dobritsa 1996; Pawlowski 1997; Pawlowski and Bisseling 1996; Schwintzer and Tjepkema 1990). In this review I give a current overview of the subject and discuss a model for regulation of symbiosis between *Frankia* and actinorhizal plants.

The Bacteria

The microsymbiont of actinorhizal plants was first referred to as *Frankia* in 1888 by Brunchorst and was later classified as an actinomycete after studies by Krebber in 1932 (Quispel 1990). The genus *Frankia* is comprised of gram-positive and gram-variable actinomycetes (Lechevalier and Lechevalier 1990). The first cultured *Frankia*, isolated from *Alnus* root nodules was reported by Pommer (1959), but unfortunately the culture was lost. In 1978, the first successful isolation of *Frankia* was reported from *Comptonia peregrina* root nodules (Callaham and others 1978), beginning a new era in actinorhizal symbiosis research (Quispel 1990). At present, there are isolates reported from many, although not all, actinorhizal plant species. In some cases, isolation has not been tried, whereas in others, attempts to isolate *Frankia* from field- or greenhouse-grown nodules have either failed or have yielded nontypical *Frankia*, that is, *Frankia*-like bacteria that cannot reinfect the original host (Benson and Silvester 1993).

Frankia grows as a filamentous colony on agar plates and is usually further cultured in liquid media (Lechevalier and Lechevalier 1990). In batch static cultures, the bacteria grow as threadlike submerged colonies without aerial or floating growth, and when grown under N limitation, form three characteristic cell types: filaments, vesicles, and multilocular sporangia (Akkermans and Hirsch 1997; Benson and Silvester 1993) (Figure 1). Vegetative cells are generally poorly branched. Vesicles are the site of nitrogenase expression and nitrogen fixation (Huss-Danell and Bergman 1990; Tisa and Ensign 1987b). They exclude oxygen, thereby protecting nitrogenase (Parsons and others 1987) and exhibit a distinctive metabolism (Tisa 1998; Tisa and Ensign 1987b; Tisa and Ensign 1988). Vesicles are usually spherical in cultivated *Frankia,* whereas in nodules they often assume different shapes (spherical, elliptical, club-shaped). Vesicles can also be septate or nonseptate. The third type of differentiated structure, the multilocular sporangia, is filled with spores, which can remain for long periods in dry soil as infective particles (Tortosa and Cusato 1991). On the basis of the presence or absence of sporangia within a root nodule, *Frankia* strains have been classified as either spore$^+$ or spore$^-$ (Schwintzer 1990). Spore$^+$ strains appear to be much more infective than spore$^-$ strains (Normand and Lalonde 1982); both have been characterized at the molecular level (Simonet and others 1994).

Filaments, vesicles, and sporangia have the potential for being infective particles (Burleigh and Torrey 1990; Schultz and Benson 1989), although they must germinate and grow as new filaments to infect the root. Spores are probably a major means of *Frankia* propagation in nature. It has been shown that *Frankia* cells are distributed through air by birds (Pashke and Dawson 1993) and also accumulate in river and lake sediments (Huss-Danell and others 1997). All three cell types can be found in the symbiotic state (Newcomb and Wood 1987) (Figure 1), although there are some exceptions.

Cultivated *Frankia* cells behave as heterotrophic aerobic bacteria with doubling times of 15 h, compared with 3 h for rhizobia. Nevertheless, the growth of *Frankia in planta* seems to be unrestricted, because timing of root infection, nodule development, and host cell infection are similar to those of rhizobia-legume nodules. Thus, the difficulties of growing *Frankia* in culture or isolating *Frankia* from some plant species reflect our limited knowledge of isolation and growth requirements. Lipidlike compounds, such as dipterocarpol, from plant extracts (Quispel and others 1989) and even simple fatty acids (Selim and others 1996) have been used to get exponential growth with some isolated strains. Stirred cultures, where oxygen availability is changed, have also yielded enhanced growth for some strains. Nevertheless, the successful use of these substances or conditions is not universally applicable for all *Frankia* isolates, highlighting the diversity of this group of bacteria. We cannot discard the idea that some *Frankia* strains may be nonculturable. Fortunately, we have the molecular tools to detect and analyze *Frankia* in soil and in nodules without the need for culturing the bacteria.

Polymerase chain reaction (PCR) techniques using primers specific to 16S rDNA genes, intergenic region of 16S-23S rDNA, intergenic region of *nifD-nifK* genes, or rep-PCR primers have been applied to *Frankia* isolated from almost all actinorhizal plant genera (Benson and others 1996; Clawson and others 1998; Jamann and others 1993; Jeong and others 1999; Murry and others 1997; Nalin and others 1995; Nazarett and others 1991; Nitayajarn and others 1990; Normand and others 1996; Sellstedt and others 1992; Simonet and others 1991). Defined molecular phylogeny groupings of *Frankia* are obvious from those studies (Clawson and Benson 1999; Lumini and Bosco 1999; Mirza and others 1994; Rouviere and others 1996). Biochemical characterization of whole cell sugar pattern (St. Laurent and others 1987), fatty acid analysis (Simon and others 1989), or isozymes profiles (Gardes and Lalonde 1987), complement the picture on diversity. Now that more *Frankia* isolates are available from a number of different host plants (Carú 1993; Chaia 1998), further phenotypic characterization will help to refine *Frankia* classification to evaluate diversity for the purpose of proposing or defining *Frankia* species (Benoist and Schwenke 1990; Benson and others 1984; Bloom and others 1989b; Bloom and others 1989a; Carú and Cabello 1998; Gardes and Lalonde 1987; Tisa and others 1999).

Frankia comprises not only symbiotic bacteria but also free-living actinomycetes in the soil. Genome unit analysis, using *Frankia*-specific primers and the most probable number method, show that the population of these bacteria in the soil is higher than the infective units found in the same soil (Myrold and others 1994). Like cyanobacteria, *Frankia* fixes nitrogen in the free-living state, at least in culture; rhizobia are unable to do this. Free-living *Frankia* populations could be important under the roots of nonactinorhizal plants (Maunuksela and others 1999), but it is not known whether *Frankia* fixes nitrogen close to other plant roots in an associative way, as does *Azospirillum* with some grasses, or shows some degree of specificity for rhizosphere colonization. At present, there are no data demonstrating whether free-living *Frankia* contribute significantly to general N cycling.

Several years ago, the isolation of *Frankia* from roots of *Atriplex,* a xerophytic shrub was reported (Caucas and Oliva 1990). Although an actinorhizal symbiosis was not well supported, a beneficial effect of *Frankia* inoculation on those plants was shown (Caucas and Abril 1996). Further investigation is needed, especially because there seems to be a novel, beneficial, but nonsymbiotic rhizospheric effect on these plants. *Frankia* has been detected in soils devoid of actinorhizal plants for more than 30 years (Smolander and Sundman 1987). Thus, *Frankia* might have other benefits to soil-ecology in addition to its symbiotic ability.

Although almost all available techniques or strategies have been tried, *Frankia* has not yet been transformed, thus limiting genetic studies (Cournoyer and Normand 1992; Cournoyer and Normand 1994; Mullin and An 1990). One explanation may be the presence of extracellular DNAses (Tavares and Sellstedt 1997). *Frankia* cultures from single spores have been obtained, and they could be a source for defined *Frankia* mutants (Lumini and Bosco 1996). Some chemically induced *Frankia* mutants have been reported and partially characterized (Carú and Cabello 1998), but analyses on the symbiotic behavior of these mutants in cross-inoculation assays have not been reported.

Frankia cell wall and cell envelope composition is distinct from that of other bacteria, in particular, because of the presence of hopanoids in the multilayer envelope of the vesicle (Harriot and others 1991). This lipid envelope acts as a gas diffusion barrier to prevent high oxygen tension within vesicles, thereby permitting nitrogenase expression and activity, both in culture and in symbiotic state (Berry and others 1993; Parsons and others 1987). *Frankia* can regulate not only the thickness but also the composition of the vesicle envelope. Depending on the soil depth where the *Frankia* cells are growing, the hopanoid composition changes, suggesting a fine-tuning control for exact gas permeability to fulfill metabolic needs (Nalin and others 1998).

It is worth noting that *Frankia* is not the only microorganism that can be isolated from actinorhizal root nodules. For instance, a previously unrecognized actinomycete was isolated from root nodules of *Casuarina* trees growing in Mexico. This microorganism appears to fix nitrogen, on the basis of acetylene reduction assays, but does not develop vesicles or sporangia in culture, and moreover, it is unable to reinfect its original host (Niner and others 1996).

The Actinorhizal Plant

All the actinorhizal plants are trees or shrubs, except for the genus *Datisca,* which is herbaceous. Some species are very well adapted to flooded lands, warm arid and semiarid regions, and areas of devastation (for example, rock slides). Actinorhizal plants have numerous uses: soil restoration, fuel wood, production of wood and derivatives, agroforesty, coastal restoration, and the prevention of desertification. Several excellent reviews have discussed the application of actinorhizal plants (Benoit and Berry 1990; Dawson 1986; Diem and Dommergues 1990; Sprent and Parsons 2000). Many actinorhizal plants are also mycorrhizal (Barker and Tagu 2000). This tripartite symbiosis gives a high degree of autotrophy to these plant-microorganism associations. Thus, actinorhizal plants are natural pioneers in succession on land, and they are frequently the first species colonizing disturbed areas. Nitrogen fixation by actinorhizal plants in nature seems to be of similar magnitude as that of the legumes showing diurnal and seasonal variation with an estimated annual rate of 240—350 kg ha^{-1} y^{-1}. Actinorhizal plants are perennial, so their contribution to N cycle through litter fall and soil decomposition is ecologically relevant (Huss-Danell 1997).

The Root Nodule

Actinorhizal nodules resemble modified lateral roots, having a central vascular bundle. Because of its indeterminate structure, the development of the symbiotic association is recapitulated longitudinally in a mature nodule (Figure 1). At the nodule tip is the uninfected apical meristem from which nodule parenchyma develops. Adjoining the meristem in a basipetal direction is a region of uninfected cells followed by a region of recently infected cells without vesicle differentiation, known as the infection zone. The central nodule tissue, or fixation zone, contains two types of cells: mature infected cells with differentiated vesicles, where nitrogen fixation takes place, and uninfected cells, which are probably involved in assimilation of the fixed N and exchange of C. The distribution of infected and uninfected cells in the fixation zone differ depending on the actinorhizal plant genus. The different arrangements are attributed to differences in oxygen protection mechanisms (Laplaze and others 1999a; Silvester and others 1990). At the nodule base, the senescent zone is present. Because actinorhizal nodules are perennial, they show seasonal variations in the proportion of these zones (Chaia 1993). In Casuarinaceae, Myricaceae, and Datiscaceae families, the apical meristem develops a special structure, the nodule root, which exhibits negative geotropism. This gives further evidence of the indeterminate growth habit of these nodules (Huss-Danell 1997).

Although actinorhizal nodules share a similar anatomic origin with lateral roots, that is, initial cell divisions starting at the pericycle, they are not directly derived from lateral roots, nor do they ever develop root cap. Moreover, the distribution of lateral roots is not modified by the development of *Frankia* nodules, suggesting that the two distinct developmental pathways are independently regulated (Valverde 2000).

Many differentially expressed genes have been detected in actinorhizal nodules, supporting the idea of the uniqueness of this organ (Goetting-Minesky and Mullin 1994; Guan and others 1997). Recently, the first early nodulin gene from an actinorhizal plant has been cloned from *Datisca glomerata.* This gene shares homology with an early nodulin gene from a legume (Okubara and others 2000). It is expressed very early; 4 weeks after inoculation, the transcripts were detected in nodule meristem. Other genes that are expressed early in nodule development are a subtilisin-like protease in *Alnus glutinosa* (Ribeiro and others 1995) and glycine-rich and histidine-rich proteins in both *Alnus glutinosa* and *Casuarina glauca* (Pawlowski and others 1997). Other nodule-specific or nodule-enhanced genes are related to N and C metabolism (Guan and others 1996; Kim and An 1999; Laplaze and others 1999b; Okubara and others 1999; Ribeiro and others 1996; van Ghelue and others 1996). Hemoglobin is an example of a late actinorhizin (protein expressed specifically in actinorhizal nodules) detected in *Casuarina* and *Myrica* nodules (Christensen and others 1991; Gherbi and others 1997; Séguin and Lalonde 1993). Some of the genes expressed in actinorhizal nodules are novel, whereas others are homologs of legume nodulins. Homologs for the legume early nodulin gene *ENOD40*, which is expressed in other plant-microbe interactions including VA-symbiosis (van Rhijn and others 1997), have been found in actinorhizal plants (K. Pawlowski 2000). It will be interesting to determine whether *NIN,* a plant gene important for nodule initiation in legumes (Schauser and others 1999), is also expressed in actinorhizal plants during nodule development. The accumulation of more data regarding the similarities and differences in gene expression and other features will be useful for a critical analysis of evolutionary relationships between different nitrogen-fixing plants.

Infection and Nodulation

It is not known whether the growing filaments of *Frankia* search for infectible sites on the root surface, whether the root tip chemotactically attracts *Frankia,* or whether the two meet just by chance. *Frankia* is a nonmotile bacteria, but a few days after inoculation, there was an accumulation of bacteria cells at certain points on the growing root of *Discaria trinervis* seedlings inoculated with a dense suspension of homogenized *Frankia* BCUI110501. Later, root nodules developed at the points where the clouds of *Frankia* filaments were observed (Valverde and Wall, unpublished). These localized accumulations of *Frankia* filaments resembled the swimming clouds of rhizobia attracted to defined points on clover roots (Gulash and others 1984). The “clouds” may indicate the location of susceptible sites for infection as discrete points of chemotaxis at the root surface. *Frankia* cells should be able to attach to root surface for infection, and *Frankia* cells have been shown to discriminate different sugar-specific lectins (Chaboud and Lalonde 1982). Nonetheless, there is no information on the role of lectins in *Frankia* infection or attachment to the root surface.

Once in the rhizoplane, there are two pathways for host root infection and subsequent nodule development by compatible *Frankia:* intracellular or intercellular. Root hair infection, the intracellular infection pathway, is characteristic of the so-called more primitive actinorhizal plants. It begins with root hair deformation, then the *Frankia* filaments become entwined by the deformed root hair. An infection thread is formed by the invagination of root hair cell wall (Berry and Sunell 1990) around the *Frankia* filaments. As the infection thread grows toward the inner root cortex, host cells ahead of the infection thread alter their cytoskeletal components, forming preinfection thread structures, known as cell bridges (Berg 1999b), which were originally described for rhizobial infection in pea (van Brussel and others 1992). Preinfection thread formation suggests the existence of a signal transduction pathway for nodule development following *Frankia* root infection. Immediately after root infection, a new center of cell division is induced in the outer cortex, in a way similar to the initiation of determinate legume nodules. *Frankia* infects this new tissue by means of the infection thread. Subsequently, vesicles differentiate from the filaments and express nitrogenase (Laplaze and others 2000). This transient structure is called the prenodule and is typical of the intracellular infection pathway. Then, cell divisions are induced in the cells of the pericycle, and the true nodule primordium emerges and grows toward the root surface, incorporating the prenodule tissue and merging with it.

The intercellular infection pathway is initiated when *Frankia* filaments enter the root tissue through intercellular spaces. The filaments cross the epidermis and invade the first cell layers of the root parenchyma (Liu and Berry 1991; Miller and Baker 1985; Valverde and Wall 1999a). This ingression implies that the middle lamella is degraded, starting at the epidermal cell junction (Sprent and de Faria 1989). In response to *Frankia* invasion, the plant deposits extracellular electron-dense material at the infected site or nearby intercellular areas (Liu and Berry 1991; Valverde and Wall, 1999a). No root hair deformation is associated with this infection pathway, although the invasive *Frankia* can cause root hair deformation on a host that undergoes intracellular infection. Concurrently, cell division and nodule organogenesis begin at the pericycle. The nodule primordium emerges from the activated pericycle and endodermis analogous to a lateral root. Although the nodule primordia grow outwards, *Frankia* proceeds to invade inwards. When these two meet, an infection thread containing invasive *Frankia* filament, sheathed in plant cell wall material invaginates the nodule cells. Infection threads are recognized by their ontogeny and morphology as being the cell-invasive structures, in both the intercellular or intracellular infection pathways (Berg 1999a). Cellulose, hemicellulose, and pectin have been immunologically detected in the extracellular matrix of the infection thread sheath, suggesting that it is of plant origin (Berg 1990; Berg 1999a). Within the infected cell, and independently of the infection pathway, the vegetative filament proliferates by branching from the infection thread. N_2-fixing symbiotic vesicles differentiate from the tips of those filaments.

Is the activation of the pericycle a consequence of a signal transduction pathway triggered during root infection or is it the consequence of an independent signaling process? Root hair deformation was proposed as a necessary step for nodules to develop, although by itself it is not sufficient for nodulation (van Ghelue and others 1997). We have recently found that it is possible to induce nodule organogenesis without root hair deformation in cultivated *Alnus acuminata* roots by inoculation with *Frankia* HFPArI3 (Enrico and Wall unpublished). This suggests that there are independent signaling pathways for these two steps in nodulation.

Phylogenetic Studies on Actinorhizal Plants and *Frankia*

A study of the phylogeny of seed plants, based on *rbcL* gene sequences, revealed that all nitrogen-fixing and nodulated plants, cluster in the Rosid I lineage of the angiosperms. This result suggests that the predisposition to develop nitrogen-fixing nodules of any type arose only once during the evolution of the angiosperm (Doyle 1998). Nodulated plants within the Rosid I clade can be grouped into four major lineages: three of them include actinorhizal plants (Soltis and others 1995). One includes the Hamamelid families, Myricaceae, Betulaceae, and Casuarinaceae, whereas a second includes the Rosid families Elaeagnaceae, Rhamnaceae, and Rosaceae, as well as the *Bradyrhizobium*-infected *Parasponia* (Ulmaceae). Coriariaceae and Datiscaceae define the third line of actinorhizal plants. The fourth line of nodulated plants includes the rhizobia-infected legumes of the Fabaceae.

Morphologic and anatomic features of actinorhizal nodules correlate with a more detailed analysis of *rbcL* grouping (Swensen and Mullin 1997). To date, four clades of actinorhizal plants have been defined. One of the above-mentioned groups of Rosid families is divided into two subclades: one including Elaeagnaceae and Rhamnaceae, and the second defined by the Rosaceae (Figure 2). Fossil records and the geographical distribution of actinorhizal species give extra support to these groupings (Benson and Clawson 2000).

Phylogenetic studies on *Frankia* have focused mainly on 16S RNA gene sequences (Benson and others 1996; Jeong and others 1999; Normand and others 1996; Ritchie and Myrold 1999b). Similar results have been obtained with *nifD* gene sequences (Normand and others 1992), and recently confirmed using *recA* and *glnII* sequences (Cournoyer and

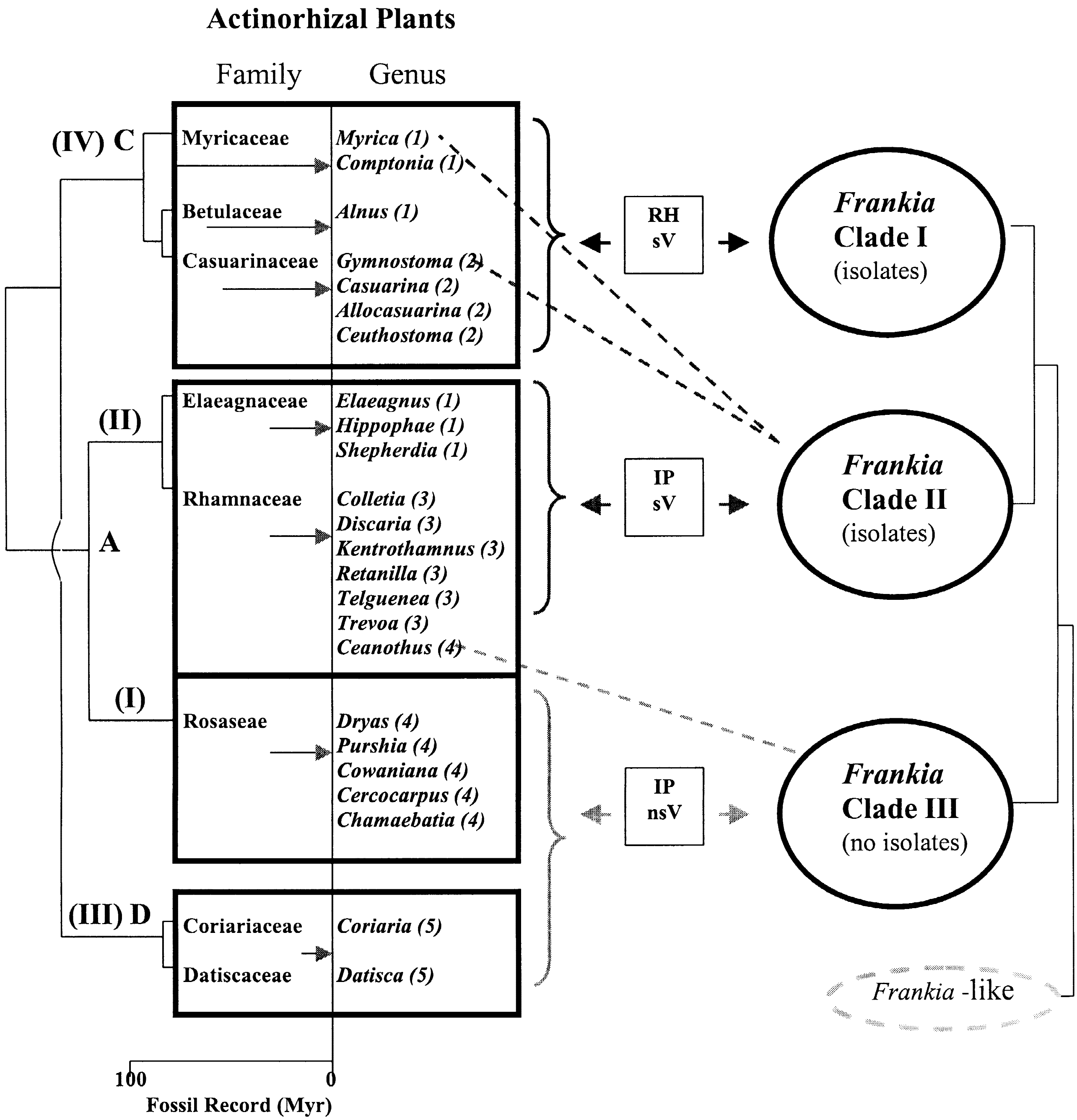

Figure 2. Phylogenetic grouping of actinorhizal plants and *Frankia.* Number between brackets of plant genus indicates native geographical distribution *(1)* to most continents, *(2)* to Australia and western Pacific, *(3)* to South America and southern New Zealand, *(4)* western North America, *(5)* disjunct distribution in northern and southern temperate zones. *RH,* root hair infection; *IP,* intercellular penetration; *sV,* septated vesicles in nodule; *ns* V, nonseptated vesicles in nodule. Based on Benson and Clawson (2000); Jeong and others (1999); Huss-Danell (1997). Groups I–IV proposed by Soltis and others (1995); Clades A–D proposed by Swensen and Mullin (1977).

Lavire 1999). In all these studies, there has been difficulty with isolating *Frankia* from root nodules. This problem has been partially overcome by direct amplification of total nodule DNA, using specifically designed primers for *Frankia.* Consensus phylogenetic trees generated from 16S rDNA sequences consistently yield three major groups of *Frankia,* and a fourth "*Frankia*–like" clade of Nod$^-$/Fix$^-$ actinomycetes (Figure 2). Subgroups can be found, although these are not statistically well supported.

There are many well-known isolates included in groups I and II, whereas no one isolate has been obtained from group III, which is defined only on the basis of analysis of nodule-extracted DNA. Physiologically, at least, the absence of a septum in vesicles of nodules of host plants infected with group III *Frankia* agrees with the proposed division.

Although the phylogenies of the microsymbiont are not congruent with the four host clades, a close relationship exists between the plant and bacterial groups. Further analysis shows that the plant clades diverged earlier than the *Frankia* clades, suggesting that the *Frankia*-actinorhizal symbiosis evolved independently, at least three or four times, rather than co-evolving from an ancestral symbioses (Benson and Clawson 2000; Jeong and others 1999; Swensen 1996). Nevertheless, once the symbiosis was established, the plants or *Frankia* were retained within certain taxonomic groups, with limited lateral transfer and probable coevolution from that point onwards (Simonet and others 1998).

These analyses as a whole reinforce a model for host preferences of *Frankia* strains or for symbiotic specificity in actinorhizal symbioses.

Symbiotic Recognition and Signals in Actinorhizal Symbiosis

Studies of cross-inoculation groups and compatibility groups or host ranges of *Frankia* have been done since the 1980s, with a limited number of available *Frankia* isolates (Baker 1987; Torrey 1990). The availability of more isolates from diverse species in the Rhamnaceae open up the possibility of extending those studies, but pure isolates from *Ceanothus, Datisca,* and *Coriaria,* all of them recognized as *Frankia* Clade III from 16S rDNA sequence analysis, are still lacking. However, in consideration of all the present experimental evidence, we can propose a model for symbiotic specificity in the actinorhizal symbioses that takes into account host ranges and degrees of specificity. The model must also explain for the exceptions to the rule. Incompatibility should be expected as one extreme of the model, whereas on the other, we should find full symbiotic compatibility. This implies that infection and nodulation after root inoculation exhibit optimal timing. In between these extremes, compatible but delayed or limited interactions can be found (Wall and others 2000).

The main level of specificity can be defined at the actinorhizal plant clade-*Frankia* clade interaction. For example, plants of the Hammamelidae clade are nodulated by *Frankia* from clade I; plants of the Elaeagnaceae-Rhamnaceae clade are nodulated by *Frankia* from clade II; and Rosaceae plants are infected by *Frankia* from the less characterized clade III (no isolates are available), as are plants from the clade defined by Coriariaceae and Datiscaceae (Figure 2). This specificity should be expressed at the molecular level by different families of signal molecules, or alternatively, by different families of chemical substitutions on a common backbone molecule. A second level of specificity can be found within a cross-inoculation group. Additional degrees of compatibility might be expressed here as different nodulation timing of different *Frankia* strains on different compatible host plants, the best being between a *Frankia* isolate and its original host. At the molecular level, this should be expressed as minor chemical modification of a compatible signal making it more suitable for slightly different host receptors. Figure 3 summarize this model for specificity and recognition in actinorhizal symbioses.

Remarkable exceptions to this model are *Myrica* and *Gymnostoma,* which behave as promiscuous host plants, being nodulated by *Frankia* from Clade I or II (Navarro and others 1997). These hosts could be considered the most primitive plants in that they recognize a common basic feature in the structure of the putative recognition molecule, such as its backbone. By contrast, other members of the clades recognize a specific chemical substitution or modification of a basic structure. *Casuarina* and *Allocasuarina* form a very narrow cross-inoculation group within the Hammamelidae clade, probably because of very specific modification of the recognition signal. Another important exception to the rule is *Ceanothus,* which is generally nodulated by *Frankia* of clade III (Ritchie and Myrold 1999a). The explanation might be found in the similar geographic distribution of *Ceanothus* with other Rosaceae (Ritchie and Myrold 1999b; Silvester 1977). Finally, the strains able to cross boundaries between incompatible groups, such as *Alnus* and *Elaeagnus* (Bosco and others 1994; Miller and Baker 1986), may be capable of synthesizing more than one recognition signal, as occurs with the broad host strain *Rhizobium* sp. NGR234 (Pueppke and Broughton 1999). Also, the possibility of coinfection or physiologic complementation for infection and nodulation in field conditions may explain the finding of boundary crossings between different groups of *Frankia* and host plants based on analysis of DNA extracted from field-collected nodules (Ramirez Saad and others 1998). Nonetheless the idea of lateral gene transfer between *Frankia* strains should not be discarded, although little information is available (Harriot and others 1995; Hirsch and others 1995).

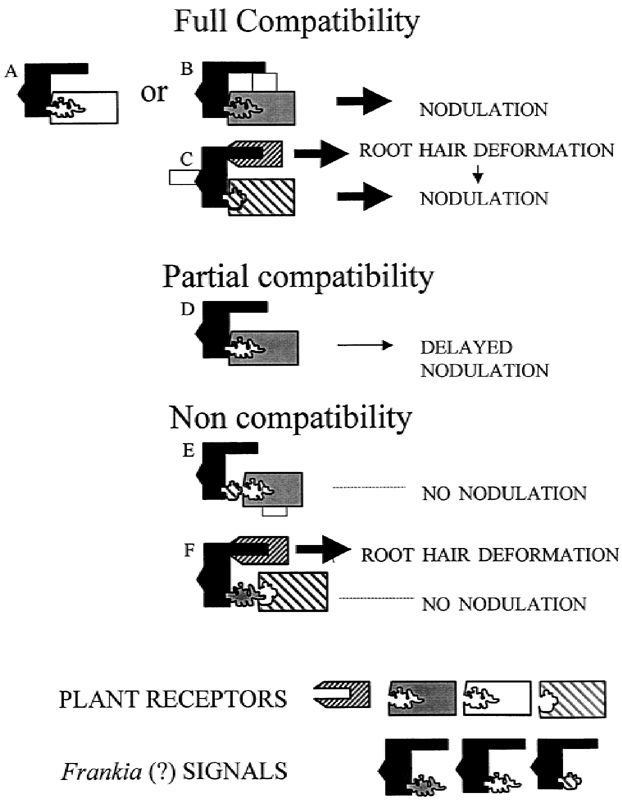

Figure 3. Model for the degree of symbiotic specificity between *Frankia* and actinorhizal plants. Bacterial signals carry information for root hair deformation and for host recognition to induce nodule development once the signal transduction pathway is triggered. Plant receptors exist for root hair deformation factor and symbiont recognition. Root hair deformation is necessary but not sufficient for nodulation to occur in intracellular infected actinorhizal plants, such as *Alnus incana,* but is not necessary for intercellular infected plants such as *Discaria trinervis.* Three possibilities can be described at this later recognition step: (A–C) full recognition between *Frankia* strain and actinorhizal species, with normal or optimal nodulation rates, for example, *Frankia* BCU110501-*Discaria trinervis* **(A),** *Frankia* BCU110601-*Discaria articulata* **(B)** or *Frankia* ArI3-*Alnus incana* **(C),** D) partial recognition, for example, within compatible clades of *Frankia* and actinorhizal plants, but between *Frankia* strains and plant species with delayed nodulation rates, for example, *Frankia* BCU110501-*Discaria articulata* **(D)** or BCU110501-*Eleagnus angustifolia,* **(E–F)** noncompatible pairs, for example, *Frankia* BCU110501-*Alnus incana* **(E),** *Frankia* ArI3-*Discaria trinervis* **(F).** For more details see the text and Wall and others (2000).

The mechanisms for recognition are probably similar in either root hair–infected or intercellular-infected actinorhizal plants, but differences in the chemistry of the molecular signals, or different locations of the plant receptor, could explain symbiotic recognition and specificity. Initiation and development of actinorhizal root nodules take just a few days in intercellular-infected plants such as *Shepherdia* (Elaeagnaceae) (Racette and Torrey 1989) and *Discaria* (Rhamnaceae) (Valverde and Wall 1999a). On the other hand, root hair–infected actinorhizal plants such as *Alnus japonica* (Burgesss and Petersson 1987) and *Comptonia peregrina* (Callaham and Torrey 1977) are reported to require weeks to reach a similar stage of development. This difference suggests that signal transduction follows a different route, perhaps more direct or faster in intercellular-infected plants than in root hair–infected plants. One of the first signals involved is likely to be a root hair deformation factor that is produced in *Frankia* pure cultures (van Ghelue and others 1997). The factor appears to be structurally different from the *Rhizobium* Nod factor (Cérémonie and others 1999). However, root hair deformation cannot by itself explain symbiotic specificity, although it appears to be a necessary, but not sufficient, early step for root hair infection (Chaia and others 1998; van Ghelue and others 1997; Wall and Huss-Danell 1997). Some recent reports strongly suggest the participation of flavonoid substances in the nodulation process (Benoit and Berry 1997; Hughes and others 1999), but their role as a specific signal for symbiotic recognition is not yet determined as it is for the *Rhizobium*-legume symbiosis (Bladergroen and Spaink 1998).

Thus, some sort of recognition step must occur at the beginning of the interaction. Signal transduction consequently leads to prenodule induction in the root cortex or directly to cell division induction at the pericycle and nodule primordia development, but as yet we do not know the nature of the signals. Undoubtedly, *Frankia* mutagenesis or success in actinorhizal plant transformation (Berg and others 1992; Diouf and others 1995; Franche and others 1997; Franche and others 1998; Savka and others 1992) will open a lot of possibilities to test these hypotheses. Meanwhile physiologic complementation experiments with incompatible actinorhizal plants have been performed to test the model.

Regulation of Symbiosis

Plant control of symbiosis could be simply regulation of the proportion of symbiotic tissue in the plant, as a general developmental control of plant growth. This action can be achieved either by controlling new infections or by controlling the development of existing nodules. It should be noted that the microorganism behaves, to some extent, as a parasite inside the host root until it begins to reduce atmospheric nitrogen (Werner 1992). This kind of host control over the nodulation and nitrogen fixation processes has been intensively studied in legumes (Caetano-Anollés and Gresshoff 1991) and actinorhizal plants (Dobritsa and Novik 1992; Wall and Huss-Danell 1997; Valverde and Wall 1999b; Valverde and others 2000). Environmental factors (light, water, nitrogen and phosphate availability, soil pH, pO_2, pCO_2), as well as bacterial factors (physiologic state, concentration, nitrogen-fixing efficiency), are also known to modulate nodule development, growth, and function in actinorhizal plants (see review by Huss-Danell 1997).

The number of effective (nitrogen-fixing) root nodules a plant produces under both field and laboratory conditions is regulated; in either root hair–infected plants such as *Alnus incana* (Wall and Huss-Danell 1997) or in intercellular-infected plants such as *Discaria trinervis* (Chaia 1997; Valverde and Wall 1999b). Analysis of the pattern of nodule formation along the tap root indicates a transient window of susceptibility for nodulation (Burgraaff and others 1983; Chaia 1997; Valverde and Wall 1999b; Wall and Huss-Danell 1997). Formation of nodules occurs mainly in a region close to the position of the tap root tip at the moment of inoculation; the result is clustered nodules. The inoculum dose and the culture age of *Frankia* affects nodule distribution, but not the degree of nodulation (Valverde and Wall 1999b).

Experimental results of delayed re-inoculation on tap root or split-root systems and the effect of removal of mature nodules support a two-step model for the regulation of nodulation (Figure 4). At least two different and consecutive signals, S1 and S2, lead to the appearance of at least one inhibitor molecule *"I"* that controls root nodulation by (1) opening or closing the window of susceptibility for infection, (2) arresting nodule primordia at stages before nodule host cell invasion by *Frankia* and vascular bundle differentiation, and (3) inhibiting nodule development in the growing root (Valverde and Wall 1999b; Wall and Huss-Danell 1997). These features occur independently of the infection pathway.

The first step occurs soon after infection by *Frankia* and induction of root cell division and suppresses further infection. This inhibition acts locally at the growing root tip and becomes systemic throughout the root system. The nature of the S1 signal is still unknown in actinorhizal plants. The second step mediated by the S2 signal is also unknown, but it involves mature, N_2-fixing nodules (Valverde and Wall 1999b; Wall and Huss-Danell 1997). It appears to be related to a threshold value for N concentration in plant tissue, which is reached either by root absorption of soluble N (nitrate or ammonium), or through N_2 fixation, or by a combination of both (Valverde 2000). This feedback regulation of nodulation and N_2 fixation, which is related to the N internal concentration of certain plant tissues, was proposed for legumes (Parsons and others 1993) and later also for actinorhizal plants (Baker and others 1997; Valverde 2000). Undoubtedly, the nodules are the source for long-term inhibition of nodule development in the root system because only nodule removal allows the development of arrested nodules (Valverde and Wall 1999b; Wall and Huss-Danell 1997).

N inhibition of nodulation, either by nodule number or nodule biomass (Arnone and others 1994; Kohls and Baker 1989; Thomas and Berry 1989) and N inhibition of N_2 fixation (Baker and Parsons 1997) have been reported for many actinorhizal genera and seem to be similar to that known for legumes (MacConnell and Bond 1957). Detailed studies with split root systems have demonstrated that N inhibition is both localized and systemic. Recent studies on different nitrogen-fixing plants suggest that N inhibition depends on the external N/P ratio that is sensed by the plant. Thus, inhibition by high N can be counteracted if P levels are high. This P effect has been shown in root hair–infected plants (Wall and others 1998; Yang 1995; Yang and others 1997) and in intercellular-infected plants (Valverde 2000). The positive effect of P on nodulation, and its interaction with N inhibition, are systemic physiologic features. Nodulation analysis, on root or plant dry matter basis, supports the hypothesis of a direct positive effect of P on nodulation that can be distinguished from a nonspecific effect of general plant growth promotion by P (Israel 1993; Reddel and others 1997; Valverde 2000). Taken together, this information supports a model for a homeostatic regulation of symbiosis that involves more than one controlling factor or signal (Figure 4).

A difference appears between regulation of nodulation in root hair–infected plants such as *Alnus* (Wall and Huss-Danell 1997) and in intercellular-infected plants such as *Discaria* (Valverde and Wall 1999b). If the N feedback mechanism operating in nodulated plants temporarily disappears, that is, by growing nodulated roots under Ar atmosphere without N_2 and *Frankia* cells are provided to the growing root, the plant will increase the proportion of symbiotic tissue either by new infections and nodulation or by developing already existing nodules. Whereas *Alnus* regained its susceptibility for new infections (Wall and Huss-Danell unpublished; Wall and others 1998) *Discaria* did not form new

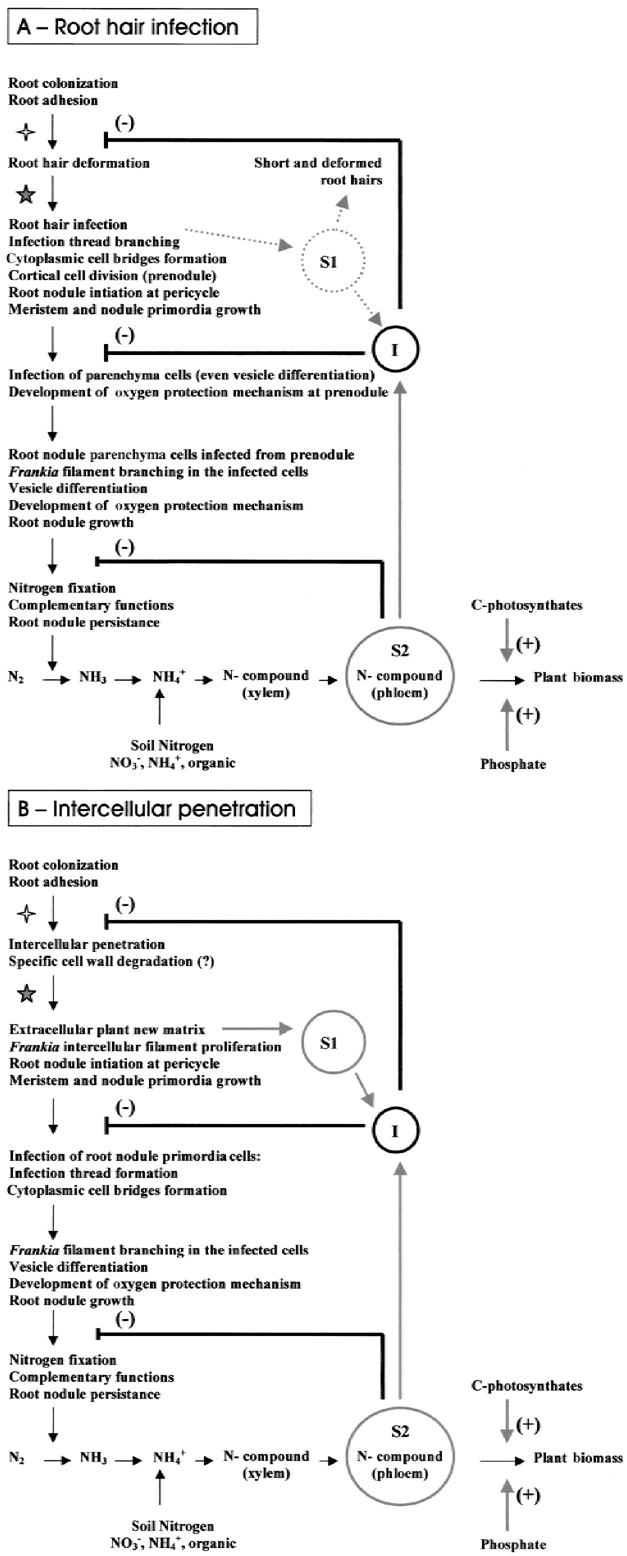

Figure 4. Model for regulation of nodulation in actinorhizal symbioses. Dotted line means transient pathway. *Four-pointed star;* first molecular signal involving the root hair deformation factor. *Five-pointed star;* second symbiotic interaction and molecular recognition step involving flavonoids. (–) Inhibition. (+) Activation. See text for further explanation.

nodules, but rather increased the biomass of existing nodules (Valverde 2000). In the model shown in Figure 4, the N-feed back inhibition of nodulation is mediated by S2. It seems that neither S2 nor S1 are present in intact, inactive root nodules of *Alnus* to further inhibit infection and nodule development, but S1 should be present in intact, inactive *Discaria* nodules to inhibit new infections. Only if nodules are removed do new infections take place in the growing root tip in both species. If S1 is produced after the initial infection of host roots, this state would still exist in *Discaria* nodules as at the beginning of the interaction. By contrast, in *Alnus* nodules this would not occur. The intercellular *Frankia* filaments (Figure 1), interacting with meristematic nodule cells in *Discaria* nodule, resemble the intercellular *Frankia* filaments found in early infection (Valverde and Wall 1999a) and could be the source for S1 signal production in the nodule. By contrast, deformation of emerging root hairs that is related to early infection steps, in root hair–infected plants as *Alnus,* is a transient plant response that does not occur in already nodulated plants (Wall and Huss-Danell 1997). This observation is in agreement with a transient expression of S1 related to early infection, which is not present in mature nodules. The differences in regulatory mechanisms reinforce the idea of independent evolutionary origin of at least these two types of actinorhizal symbioses.

PROSPECTS

Actinorhizal plants could be useful tools to develop a sustainable economy. If there is interest in extending the ability to establish a nitrogen-fixing symbiosis to a plant species with economic potential, efforts should concentrate first on close relatives of well-known actinorhizal plants. We know very little about the complex interactions between actinorhizal plants with *Frankia* and mycorrhizal fungi (Gardner 1986) or other microorganisms (Knowlton and others 1980). Some reports show a beneficial and synergistic effect of multi symbioses (Mark and others 1999), whereas experiments showed no improvement in plant growth compared with controls (Ekblad and Huss-Danell 1995; Russo 1989). Another beneficial role of the symbiotic state, not related to N nutrition is that there may be greater pathogenic resistance induced after *Frankia* nodulation (Baker and others 1980; Wolters 1998). More basic studies on the complex interactions between *Frankia* and the actinorhizal plants will help us achieve a better understanding not only of symbiosis but also of plant growth regulation.

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