Actinorhizal, mycorhizal and rhizobial symbioses: how much do we know?

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In this review, we discuss the recent progress in research on symbiotic association of rhizobia, *Frankia* and fungi with plant roots. We compare infection processes of symbiotic establishment; structure, functioning and molecular biology of the symbiotic organ including the regulation of genes implicated in rhizobial, actinorhizial and arbuscular mycorhizal symbioses.

Key words: Symbiosis, nodule, mycorrhiza, symbiotic genes.

INTRODUCTION

The symbiotic association between certain plants and microorganisms plays an important role in soil fertilization, and improves their growth and mineral nutrition. Microorganisms implicated in this symbiotic interaction are from two groups: bacteria and fungi. The bacteria group is implicated on nitrogen fixation (for review see Pawlowski and Bisseling, 1996), while the fungal group is involved in the uptake of nutrients with low mobility (Diop, 1996; Gianinazzi-Pearson, 1996). Among the bacteria which establish symbiotic association with dicotyledonous plants, nitrogen fixation is exclusively carried out by rhizobia and *Frankia* in a specialized organ, the root nodule where atmospheric nitrogen is reduced to ammonium. Rhizobia and *Frankia* are soil bacteria which are unicellular gram -negative and filamentous branching gram-positive, respectively. Rhizobia exist in symbiotic association with legumes and one species member of Ulmaceae family, *Parasponia andersonii*. In contrast, *Frankia* can interact with diverse group of dicotyledonous plants which are called actinorhizal plants. More than 90% of terrestrial plants live symbiotically with arbuscular mycorhizal fungi. Plants with few or short root hairs are very mycotrophic and depend on mycorrhizae for nutrition and growth (Baylis, 1975). Observations of fossil plants from Devonian indicate that arbuscular mycorhizal fungi played an important role in plant colonization of land (Pirozinski and Malloch, 1975). Legume and actinorhizal plants can establish at the same time a symbiotic association with the arbuscular mycorrhizal fungi of the order Glomales. Recent studies conducted in Gymnostoma have shown that root nodules can also be colonized by arbuscular

mycorrhize (Duhoux et al., 2001). In this review, we summarize the current knowledge and recent progress in rhizobial, actinorhizal and fungus symbioses.

EARLY EVENTS OF INFECTION PROCESS

Plant-rhizobia interaction begins with molecular signals exchange between the two partners (for review see Sánchez et al., 1991). These Plants (legume and *Parasponia*) exude flavonoid compounds (flavones, isoflavones and flavanones) from their roots which were identified as the inducing molecules for rhizobial chemotaxis and for the expression of *Rhizobium* nodulation (*nod*) genes. In *Phaseolus vulgaris* six types of flavonoids (diadzein, coumestrol, naringenin, genistein, liquiritigenin and isoliquiritigenin) have been identified (Bolaños-Vásquez and Werner, 1997) and their relative concentrations depend on the nature of the inoculating bacteria. Flavonoids are active at very low concentration (10^{-8} M to 10^{-7} M) and stimulate *nodD* gene transcription, which subsequently induce *nod ABC* expression (Carlson et al., 1994; Mylona et al., 1995; Vijn et al., 1993). Nod ABC participate in Nod factor biosynthesis which consist of a backbone of three to five β-1,4-linked N-acetylglucosamines bearing a fatty acid on the non reducing sugar residue (Long, 1996). Nod factors exhibit various substitutions on both the reducing and non-reducing terminal sugar residues. Others rhizobial *nod* genes (*EFHLPQ*) products have also been implicated in the host-specificity. Moreover, the cytoplasmic membrane location of *nodI* and *nodJ* products suggest that they play an important role in Nod factors transport (Carlson et al., 1994). It has been shown that Nod factors induce the deformation of the root hairs at a very low concentration

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$(10^{-12}$ M) and plant genes expression (Figure 1). These morphological modifications are preceded by a depolarization of the cytoplasmic membrane, increase of intracellular free calcium, proton efflux, rearrangement of the actin filaments and increased cytoplasmic streaming (for review see Gehring et al., 1997; Mylona et al., 1995). This result suggests that Nod factor transduction signal may be calcium dependent.
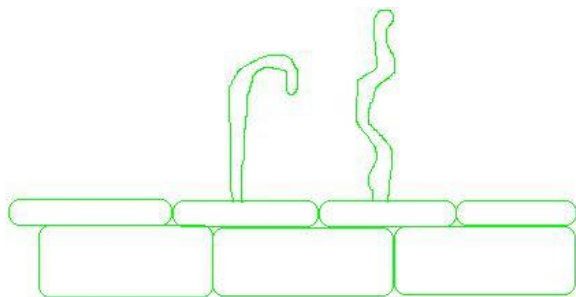
Figure 1. Deformation of the root hairs before the entry of microsymbiont into plant cells.

In actinorhizal symbiosis, substances implicated on plant-Frankia-fungi interaction have not been clearly identified. Nevertheless, factors inducing root hairs deformation (Figure 1) have been observed in *Frankia* culture surpernant (Burggraaf et al., 1983; Prin and Rougier, 1987; Selim, 1995). These substances are active at high concentration $(10^{-5}$ M) and their biosynthesis is induced by flavonoid extracts but it is not certain if they are the same as Nod factors in rhizobial symbiosis (for review see Duhoux et al., 1996).

Plants which establish symbiotic association with arbuscular mycorrhizal fungi stimulate hyphal growth by synergistic interaction between volatile compounds and exudates produced by roots (Bécard and Piché, 1989). Bécard et al. (1995) also showed that maize plants deficient in chalcone synthase activity, necessary for flavonoids biosynthesis, were similarly colonized by arbuscular mycorrhizal just like maize with chalcone synthase. However, flavonoids may act as signals for the initiation and the development of arbuscular mycorrhizal symbioses and are essentials in the regulatory system which control the fungal growth (Piché, personal communication) . Non-host status of certain plants is generally due to exudation of fungal inhibitory compounds (Bécard and Piché, 1989). Identification of mycorrhiza-resistant (myc$^-$) phenotypes among non-nodulating (nod$^-$) genotypes of different legumes demonstrates that some steps in the recognition or infection process are controlled by common plant genetic determinants. These observations also suggest that some molecular mechanism could be common to actinorhizal, rhizobial and mycorrhizal symbioses (Balaji

et al., 1994; Gianinazzi-Pearson et al., 1991). On the other hand, the existence of myc$^+$ nod$^-$ plant mutants mean that different plant genes occur in the two types of symbiosis (Duc et al., 1989).

Both rhizobia and *Frankia* can produce a conserved enzyme, the nitrogenase which catalyses the reduction of dinitrogen and which is highly sensitive to oxygen (Burris, 1991). In free-living state, *Frankia* can produce vesicles at the end of its hyphae. In this case the nitrogenase is located inside the vesicles and protected from oxygen by multilayered lipid envelope (Berry, 1994). Rhizobia are capable of fixing dinitrogen only in the symbiotic state. Vesicular arbuscular mycorrhizae are the common association which produce fungal structure (vesicles and arbuscules) in cortical root cells. These vesicular arbuscular mycorrhizae are formed by septate phycomycetous fungi belonging to four genera: *Glomus*, *Gigaspora*, *Acaulospora* and *Sclerocystis*. These fungi are obligate symbionts, and are not host-specific. But certain endophytes may form preferential association with certain host plants. This symbiotic association is found in most plant families, of arctic, temperate and tropical regions, aquatic and desert environments (for review see Powell and Bagyaraj, 1984). Biochemical and molecular studies of fungi have been limited by difficulties in obtaining pure fungal culture. However, two fungal transcripts encoding a novel class of proteins known as hydrophobins have been isolated in ectomycorrhiza and hyphae of *Pisolithus tinctorius* (for review see Martin et al., 1995). Hydrophobins could be involved in the hyphal aggregation and binding in plant roots to form ectomycorrhizal mantle (Tagu and Martin, 1996; Tagu et al., 1996).

During symbiosis functioning, bacteroids, *Frankia* and arbuscules are surrounded by membrane of plant origin called peribacteroid membrane, capsule and periarbuscular membrane respectively, which play an important role in exchange of metabolites between the symbiotic partners (Newcomb and Wood, 1987, Smith and Smith, 1990). Furthermore, rhizobia are surrounded by a peribacteroid space which does not exist in actinorhizal and mycorrhizal symbioses (Roth and Stacey, 1989).

INFECTION PROCESS

A. Intracellular infection

Rhizobia induce a localized hydrolysis of the cellular wall of the deformed root hairs (Kijne, 1992; Van Spronsen et al., 1994). Fungi can also hydrolyse lignin and cellulose during the infection process (Harley and Smith, 1983). The invagination of plasmalemma is similar between endomycorrhozas, *Rhizobium* and *Frankia* (Figure 2A) forming a new structure, the infected thread. In legume plants, before the infected thread cross the cortical cells,

some modifications such as movement of nucleus to the centre, as well as microtubules and cytoplasm rearrangement to form phragmoplast like-structure were operated (Pawlowski, 1997). These modifications are followed by mitotic reactivation of the target cortical cells. The infected thread invades the nodule primordium and its release resemble on endocytosis process in Mimosoideae and Papilionideae (Bassett et al., 1977). In nodules of the non-legume *Parasponia* and of some tropical legumes, bacteria do not differentiate into bacteroids. The persistent infected thread is enclosed by cytoplasmic membrane and fibrillar materials (Pawlowski, 1997).
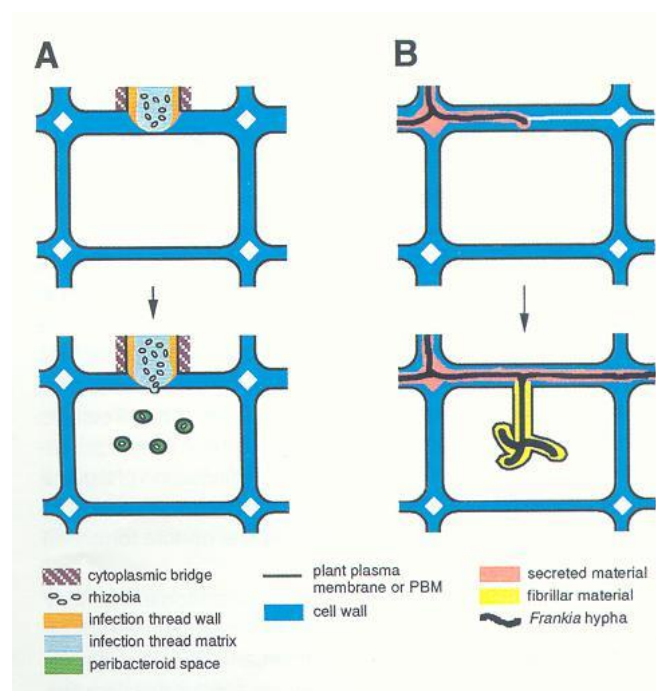
Figure 2. Different pathway of endosymbiont entry into plant cells (Pawlowski and Bisseling, 1996).
A: Intracellular penetration like as in legume nodule primordium.
B: Intercellular penetration in actinorhizal nodule primordium.

In actinorhizal symbiosis, intracellular infection has been described for *Myrica, Comptonia, Alnus* and *Casuarina* genera (Berry and Sunell, 1990). *Frankia* penetrate into the deformed root hairs localized on the bottom of lateral root where it induces cell wall hydrolysis. After penetration, it is enclosed by a plant-derived membrane composed of celluloses, pectines and hemicelluloses. This process is also associated with nuclear migration in the cell centre, cytoplasmic movement and appearance of phragmoplast-like structures. *Frankia* hyphae colonize the dividing cortical cells which form the prenodule. The prenodule does not develop into a nodule.

Intracellular hyphae can display signs of deterioration characterized by cytoplasm degeneration and wall collapse (Kinden and Brown, 1975). The host cell cytoplasm undergoes important modifications with increasing metabolic and golgi activities, highly polyploidy nucleus, greatly enlarged nucleolus and increased endoplasmic reticulum (Safir, 1987).

B. Intercellular infection

Intercellular infection has been described only in tropical legumes such as *Sesbania rostrata* and *Aeschynomene* (Alazard and Duhoux, 1990). In actinorhizal plants intercellular infection has been recorded in *Elaeagnus* (Miller and Baker, 1985) and is not associated by prenodule formation except in *Ceanothus* where some mitotic activity was found without cellular expansion or infection (Liu and Berry, 1991). In this case *Frankia* and *Rhizobium* penetrate by different ways. The first proceed in the middle lamella between adjacent epidermal cells and the second into the gaps in the epidermis (for review see Pawlowski and Bisseling, 1996) (Figure 2B). *Frankia* move through the cortex and become intracellulary. In some legumes symbiosis, infection threads are formed after intercellular penetration (Ndoye et al., 1994) or rhizobia enter the cells of the primordium directly via invagination of the plant plasma membrane (Alazard and Duhoux, 1990).

Intercellular infection is also observed in mycorrhizal symbiosis (Safir, 1987). The hyphae form H- or Y-connection, which provoke intercellular spaces expansion. The infecting hyphal branches originating from the appressorium grow through the intercellular spaces and colonize the outer root cortical cells. Simultaneously, some hyphae spread out in all directions and bear auxiliary cells and terminally or intercallary spores. Auxiliary cells are ones of the main distinct characteristics of the arbuscular mycorrhizal fungi belonged to the familly of Gigasporinae. Sporulation of most arbuscular mycorhizal fungi begins 3 days after contact and spores can be produced far from the rooting zone (Diop, 1995).

STRUCTURE AND FUNCTIONING OF NODULES AND MYCORRHIZA

In legume and actinorhizal plants, the establishment of nitrogen fixation is associated with nodule formation. In contrast, fungal symbiosis differentiation of mycorrhize takes place before phosphorus assimilation. Two types of legume nodules are known: determinate nodule and indeterminate nodule. These two types of nodules are differentiated on the cortical tissue root. Determinate nodules are frequently found in tropical legumes in which the apical meristem stops its activity early in development (for rewiev see Pawlowski and Bisseling, 1996). Determinate nodules are differentiated in the outer cortex (Figure 3C). However, indeterminate nodule exhibited an
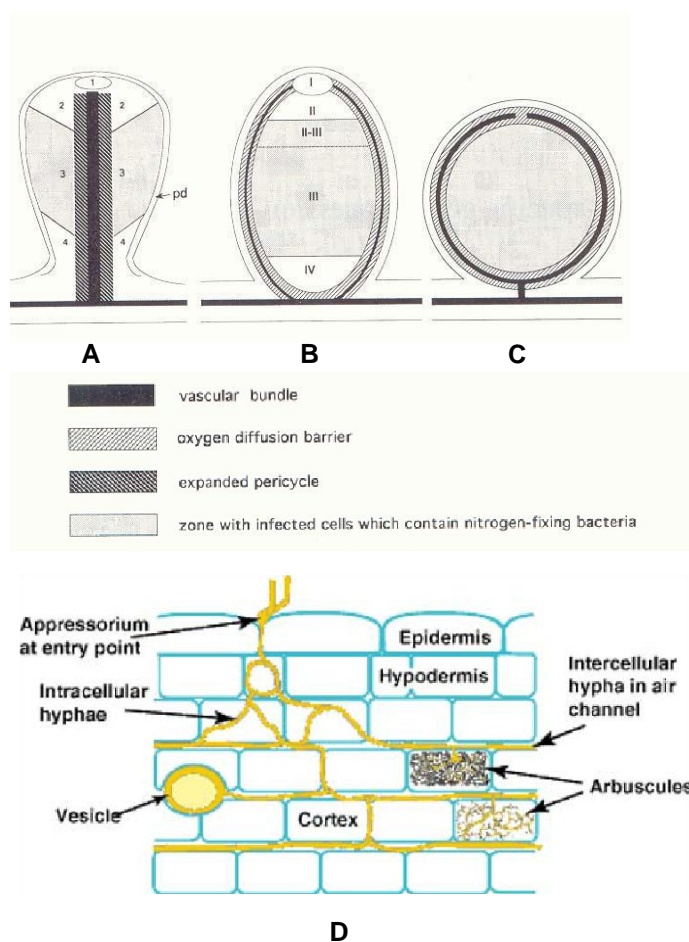
Figure 3. Nodule and mycorrhiza structure. A: Actinorhizal nodule (pd: Periderm); B: Indeterminate legume nodule; C: Determinate legume nodule; D: Mycorrhiza structure.

apical meristem, which is continuously activated, and the new differentiated cells are infected by rhizobia (Figure 3B). The only nonlegume infected by *Rhizobium*, *Parasponia* (Ulmaceae) shows nodule ontogeny and tissue organization similar to the actinorhizal nodules and root. Actinorhizal nodules have an indeterminate growth pattern (Figure 3A). The *Rhizobium*-induced nodule can be spherical, cylindrical or collar-shaped according to the patterns of plant cell division and growth of cortical cells (Newcomb, 1981). Occasionally, legume nodules are multiple-lobed cylindrical-shaped in *Pisum sativum* (Syoño et al., 1976). Actinorhizal nodules generally consist of numerous conical- shaped lobes (for review see Newcomb and Wood, 1987) and may or may not exhibit nodular roots. The *Alnus* type nodule lack roots, whereas the *Casuarina* type shows nodular roots. Nodular roots present a negative geotropism and play an important role in facilitating gas exchange between the nodule and atmosphere. Legumes and actinorhizal nodules or lateral roots are predominantly formed opposite to the protoxylem poles. This result suggests that a mitotic factor can reactivate only the protoxylem cells (for review

see Pawlowski and Bisseling, 1996). This factor induces the susceptibility of cells to phytohormones and uridine. These nodules show a periderm on their surface, a parenchyma, a vascular bundle and an infected zone. In the case of legume nodules, the vascular system is peripherical and divides the nodule parenchyma into two parts. The infected cells are localized in the middle of the nodule unlike in the actinorhizal and *Parasponia* nodules where they are peripherical and the vascular bundle is situated in the central cylinder. The infected zone of actinorhizal nodules contains two cell populations: infected and uninfected cells which have unknown special function. The uninfected cells are the seat of starch storage and genes implicated in this biosynthesis have not been not cloned. In contrast, the infected zone of legume and non-legume nodules contains only one cell population. Indeterminate legume nodules and actinorhizal nodules structure may be divided in four zones: the zone I is formed by the meristem, the zone II or prefixation zone contains cells that become infected, the fixation zone or zone III, and zone IV or senescence zone where plant cytoplasm and bacteria are degraded. In legume nodules, the bacterial nitrogen fixation starts in the interzone II-III (Yang et al., 1991). This interzone has not been described for actinorhizal nodules.

Arbuscular mycorrhizal structures are normally not visible to the naked eye. Roots must be cleared and stained to see typical infection. Arbuscular mycorrhizal fungi do not fix atmospheric nitrogen. However, they exude enzymes such as nitrate reductase and glutamine synthetase for nitrate and ammonium uptake (Strullu et al., 1991). Fungal hyphae extend far from the rooting zone and absorb mineral nutrients of low mobility, mainly phosphorus but also cooper and zinc (Kothari et al., 1990; Tarfdar and Marschner, 1994). Arbuscular mycorhizal fungi can produce phosphatases for utilization of organic phosphorus. In addition to their role in improving host's mineral nutrition, arbuscular mycorhizal fungi ensure a protection against certain soil-borne pathogens (Diop, 1996). Arbuscular mycorrhizal fungi influence microbial populations and improve soil structure by secretion of mucilaginous coumponds (Strullu et al., 1991). Vesicles are often lipid-filled and act as storage reserve for the fungus. These vesicles are initiated after the formation of arbuscules but live longer after senescence of arbuscles (Figure 3D). Vesicles and some spores found within the roots can be intercellular or intercallary. The arbuscule is the privileged site for fungus/plant metabolite exchange while the vesicle and spore are reproductive and survival organs (Strullu et al., 1991).

MOLECULAR BIOLOGY OF NODULES AND MYCORRHIZA

In the case of legume-*Rhizobium* symbiosis, proteins specifically expressed during nodules formation and

functioning have been described (for review see Nap and Bisseling, 1990). These proteins are called nodulins and classified in two groups according the time of their appearance: the early nodulins (ENOD) which are identified during the infection process and nodule formation, and the late nodulins which are expressed into the functioning nodules. Several of these nodulin genes have been isolated and characterized in legume-*Rhizobium* interaction, while only a few has been identified in actinorhizal plants and fungal symbioses.

Studies of early nodulin genes during nodule formation have provided some useful tools to evaluate the mode of action of bacterial Nod factors. A well studied early nodulin gene is *ENOD12* (Scheres et al., 1990a), and it is induced in root epidermis by Nod factors. The application of reverse transcriptase polymerase reaction (RT-PCR) and transgenic techniques suggest that *ENOD12A* is expressed adjacent to the meristematic zone (Bauer et al., 1997) of *Medicago sativum* nodules and roots and plays a role in the differentiation processes. Another nodulin gene, *ENOD5* (Scheres et al., 1990b), is also expressed during infection process and nodule development. *ENOD5, ENOD12*, MtN8, and *MtN12* encode proline-rich proteins (Gamas et al., 1996; Mylona et al., 1995). These aminoacids are extensin components which participate on the infected thread wall structure. Expression of the early nodulin gene *ENOD40* is localized in pericycle zone of root and can be induced by exogenous application of Nod factors (for review see Pawlowski and Bisseling, 1996). In *P. sativum* nodules, *ENOD7* gene is expressed in the proximal part of the prefixation zone II (Kozik et al., 1996). In legume nodules, *ENOD2*, *MtPRP* and nodule-specific lectin gene are expressed in parenchyma and these products provide an O_2 diffusion barrier in order to preserve nitrogenase activity (for review see Pawlowski, 1997). A cluster of three *ENOD8* is present in *Medicago trunculata* but one of them is probably a pseudogene for its lack of a 5' exon. The expression of the other two genes is located only in the nodule (Dickstein et al., 2002).

In actinorhizal plants, nodules are derivatives of lateral root primordia and proteins analogous to legume nodulins may not exist. Goetting-Minesky and Mullin (1994) have shown that a nodule-specific transcripts of host plant origin is present in total RNA of *Alnus glutinosa*. The deduced aminoacid sequence of one full-length cDNA shows similarity with cystein proteinase which plays a role in defense response to *Frankia* invasion. Differential screening of an *A. glutinosa* nodule cDNA library revealed that a subtilin-like protease gene expression is enhanced during early stages of nodule development in the infected cells before the onset of nitrogen fixation (Ribeiro et al., 1995; Laplaze et al., 2000) . Nodule-specific clones, *agNt84* and *ag164* which encodes glycine and histidine-rich proteins have also been isolated from *A. glutinosa* cDNA libraries constructed from root nodules mRNA. *In situ* hybridization shows that

this gene is expressed only in infected cells of the prefixing zone of the young nodules (Pawlowski et al., 1997). The encoded proteins share sequence similarity with nodulin- 24 (Katinakis and Verma, 1985) a protein localized in peribacteroid membrane. In *Casuarina glauca-Frankia* symbiosis, chalcone syntase transcripts were isolated from nodule cDNA library. Chalcone synthases are present in nodules, roots and aerial organs. This enzyme could contribute to the establishment of the symbiosis and nodule development (Gherbi, 1996).

In arbuscular mycorhizal fungal symbiosis, little is known about the biochemistry and molecular biology of mycorrhiza formation. The discovery of a transmembrane alpha-helix protein in mycorrhiza suggests that this protein could be implicated in metabolites transport between the two partners (Fester et al., 2002).

Late nodulins are induced shortly before nitrogen fixation and include mainly enzyme implicated in nitrogen assimilation, carbon metabolism and nitrogenase oxygen protection, as well as proteins located in the peribacteroid membrane (Delauney and Verma, 1988).

REGULATION OF SYMBIOTIC GENES

In legume plants a system using transgenic *Lotus corniculatus* has been developed for studing nodulin genes regulation (Petit et al., 1987; Hansen et al., 1989). Analysis of leghaemoglobin gene expression in transgenic *L. corniculatus* has led to the identification of a so-called organ-specific cis acting element (Bogusz et al., 1990; Ramlov et al., 1993; Szabados et al., 1990), also called the nodule-infected cell-specific elements (Szczyglowski et al., 1994) . The organ-specific cis acting element is also found in the promoters of symbiotic haemoglobin genes of *C. glauca* (Jacobsen-Lyon et al., 1995) and *P. andersonii* (Bogusz et al., 1990), in *Nodulin-45* gene promoter of *Lupinus angustifolius* (Macknight et al., 1995) and in *Nodulin-30* gene of *P. vulgaris* (Carsolio et al., 1994) . These results suggest that similar trans-acting factor may be conserved between legumes, non-legumes and actinorhizal plants.

ENDO12A and *ENOD12B* promoter deletion have also shown that the 200bp immediately usptream of the transcription start site determine nodule specific and nod factor induced gene expression in *P. sativum* (Vijn et al., 1995). Furthermore, promoter analysis of a cytosolic soybean glutamine synthetase gene *GS15* showed that regulatory elements necessary for ammonia stimulation in nodules are located between –3.5 and –1.3 kbp (Marsolier et al., 1993).

CONCLUSION AND FUTURE PROSPECTS

In this review, symbiosis establishment and functioning in rhizobial, actinorhizal and mycorrhizal symbioses was

described. These three symbioses show high similarities in several processes. Leghemoglobin, for example, is expressed in rhizobial, actinorhizal and mycorrhizal symbioses. It appears that nodule and mycorrhiza formation are under the control of specific genes plant-host which could be specially induced by bacterial or fungus products. We conclude that mycorrhiza induces a discreet modification in plant roots, actinorhizal and *Parasponia* nodule structure is very similar to roots, and legume nodule is a new organ. These morphological aspects will be further clarified by molecular studies for understanding these symbioses evolution.

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