

Actinorhizal Symbioses: Recent Advances in Plant Molecular and Genetic Transformation Studies

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ABSTRACT: Infection of actinorhizal plants roots by the actinomycete *Frankia* leads to the formation of a nitrogen-fixing root nodule (actinorhiza) consisting of multiple lobes, each of which is a modified lateral root. Actinorhiza development involves several specific steps, for example, root hair infection, prenodule formation, and initiation of lobe primordia from root pericycle. This article summarizes the latest development in the isolation and characterization of nodule-specific and -enhanced transcripts isolated from actinorhiza. The amino acid sequence derived from the nucleotide sequence of the cDNAs, in combination with localization data, showed that gene products are involved in nitrogen, carbon, and oxygen metabolism. Furthermore, some transcripts represented encoded gene products that might be part of infection and senescence mechanisms in actinorhiza. The article also reviews experiments designed to establish genetic transformation systems for actinorhizal plants. This research has led to the obtainment of transgenic plants of the *Casuarinaceae* family by using *A. rhizogenes* and *A. tumefaciens*. These new findings are discussed in view of future studies on actinorhizal symbiosis. As molecular and cellular studies on *Casuarinaceae* and *Betulaceae* are more advanced than on the other six actinorhizal plant families, we concentrate primarily on species within these two families.

KEY WORDS: nodulation, nodulin, *Alnus*, *Casuarinaceae*, nitrogen fixation.

I. INTRODUCTION

Actinorhizal plants are non-leguminous plants belonging to 8 angiosperm families and 24 genera. They are capable of forming root nodules as a result of infection by *Frankia*. The first description of an actinorhiza was made by Meyen (1829), who discovered large nodules on alder roots (*Alnus glutinosa*). Since then, actinorhizal

symbioses have been reported in numerous plants all over the world except Antarctic (for review see Baker and Schwintzer, 1990). Most of the genus, for example, *Alnus* (*Betulaceae*), *Myrica* (*Myricaceae*), *Purshia* (*Rosaceae*), are indigenous to temperate regions. The range of some genera such as *Casuarina* has been extended considerably through artificial dissemination. All of the host species are perennial dicots, woody

shrubs, or trees, with the exception of *Datisca*, which has herbaceous shoots. Also they all are able to grow on N-deficient disturbed areas because of their high N₂-fixing capacity. Actinorhizal plants can contribute as much nitrogen per hectare as most productive legumes (Torrey, 1976). In Egypt, a N₂-fixing potential of 288 kg/N/ha has been reported (Gauthier et al., 1984). Thus, these plants can grow in poor and disturbed soils and are important elements of plant communities worldwide. In addition, different actinorhizal species have the ability to grow well under a range of environmental stresses such as high salinity, heavy metal, extreme pH (Dawson, 1990). Such adaptation has drawn great interest to actinorhizal plants, particularly to several species of *Casuarinaceae*, which can be used for fuelwood production, agroforestry, and land reclamation in the tropics and subtropics (Diem and Dommergues, 1990).

The microsymbiont *Frankia* belongs to the order of filamentous bacteria, the actinomycetales. Unlike rhizobia, which were first obtained in pure culture toward the end of the 19th century, the first successful isolation of *Frankia* strain CpII by Callaham et al. was only reported in 1978. Since then, *Frankia* has been widely studied, but due to lack of genetic tools (including appropriate vectors, conjugation and transformation techniques), most aspects of *Frankia* biology, particularly regarding symbiosis, are still unknown. In pure culture, *Frankia* presents three major structures: vegetative hyphae, vesicles that are the site of nitrogen fixation, and sporangia. Vesicles are produced under conditions of combined nitrogen limitation and allow *Frankia* to fix nitrogen at atmospheric pO₂ in the free-living state (Torrey and Callaham, 1982). Vesicles wall is considered to be involved in oxygen protection (Parsons et al., 1987). Hopanoids lipids are the major compounds of the extracellular envelope of *Frankia* vesicles (Berry et al., 1993). In response to oxygen concentration,

both lipids content and concentration of the vesicle envelope varied, suggesting a role of oxygen-diffusion barrier (Kleemann et al., 1994). Although all effective *Frankia* strains isolated to date can form vesicles in pure culture, the structure of *Frankia* in nodules differs among actinorhizal host plant species. Vesicles are present in the nodules of most host plant species, where their shape and form are host-dependent and are absent in *Casuarinaceae* nodules. These observations indicate that the differentiation of *Frankia* is under some control of the host plant. This also raises the question of the localization of the nitrogenase in nodules without vesicles. The structure of *Frankia* in a free-living state and in a symbiotic state has been reviewed extensively by Newcomb and Wood (1987). Using conservation of *nif* (nitrogen fixation) gene sequence, it has been possible to isolate some *nif* genes of *Frankia* by heterologous probing (Normand et al., 1988). The characterization of *Frankia* isolates at the DNA level has revealed the existence of a large diversity (Rouvier et al., 1996). Recent developments in *Frankia* classification and diversity are reviewed elsewhere (Benson and Silvester, 1993; Mullin and Dobritsa, 1996).

Although recent phylogenetic studies suggest a single origin for the predisposition to become nodulated with the symbioses originating multiple times within the Rosid 1 clade (Soltis et al., 1995), legume and actinorhizal nodules differ in development pattern and final structure. Legume nodules originate in the root cortex and have peripheral vascular bundles and central infected cells, whereas actinorhizal nodules exhibit the same origin and structure as the lateral root, that is, they arise from cell divisions in the pericycle and possess both a central vascular bundle and cortical-infected cells. In legume nodules, the characterization of plant (nodulin) and *Rhizobium* genes involved in nodulation has progressed considerably during the last few years (for reviews see

Franssen et al., 1995; Mylona et al., 1995; Long, 1996). As in the legume-*Rhizobium* symbiosis, the establishment of the actinorhizal symbiosis is a complex multistep process; both plant and prokaryote undergo biochemical, physiological, and molecular changes during the successive steps of the nodulation. Molecular studies on actinorhizal plants have been carried out during the last 5 years in order to learn more about specific and common mechanisms of legume and actinorhizal nodule formation. Molecular tools, including nodule cDNA library, plant transformation/regeneration procedure, have been developed and have led to the cloning and characterization of actinorhizal plant genes.

The aim of this review is to focus on the following aspects of actinorhizal symbiosis: first, current state of knowledge on plant genes involved in actinorhizal nodule development and functioning, and, second, recent advances in genetic transformation of actinorhizal plants and their use in studying symbiosis.

II. PLANT GENES ASSOCIATED WITH ACTINORHIZAL NODULE DEVELOPMENT AND FUNCTIONING

By homology to legume nodulins (Legocki and Verma, 1980), actinorhizal nodulins are actinorhizal nodule-specific proteins (Mullin and Dobritsa, 1996). An actinorhizal nodulin gene will be a gene expressed in nodules but not in roots, irrespective of its expression in other organs, as it was defined for legume nodulin genes (van Kammen, 1984).

A. Host Plants and *Frankia* Signals

In legume symbiosis, signal exchanges between the symbiotic partners have been researched thoroughly, and signal molecules

have been characterized. First, flavonoids are secreted by the plant under nitrogen starvation conditions and interact with the *Rhizobium* NodD protein to induce *nod* genes expression. This interaction constitutes the first level of host specificity determination. The *nod* genes products are involved in the synthesis of lipo-chitin oligosaccharides (LCOs) signals, the Nod factors (reviewed in Dénarié et al., 1996). Nod factors, which are the major determinants of host specificity, induce plant responses such as root hair deformation and trigger the nodule developmental program (Schultze and Kondorosi, 1996; Spaink, 1996).

Lack of a genetic analysis system on *Frankia* has kept knowledge of signal exchange in actinorhizal symbioses in its infancy. Because of the similarities of early steps of infection between actinorhizal plants and legumes, it was postulated that similar signaling mechanisms might exist. Several unsuccessful attempts have been made to isolate homologues of *Rhizobium nod* genes in *Frankia* (Simonet et al., 1990; Chen et al., 1991). It has been claimed that there is genetic complementation of *nodD* mutant of *Rhizobium* with *Frankia* DNA (Chen et al., 1991). Because cloning and sequencing of the corresponding *nodD*-like gene of *Frankia* has not been reported, it is not clear whether *Frankia* contains genes equivalent to the common nodulation genes. However, several findings indicate common features in the signaling processes in *Rhizobium*-legume and *Frankia*-actinorhizal plant symbioses. Cultures of *Frankia* induce root hair branching and curling that resembles root hair deformation in legumes (Torrey, 1976; Diem et al., 1982; Berry and Torrey, 1983; Burggraaf et al., 1983). Furthermore, it has been shown that culture filtrate of *Frankia* strain Cp11 induces root hair deformation after incubation of *Frankia* culture with *A. glutinosa* root (Prin and Rougier, 1987). Recently, it was observed that flavonoid extracts from *Casuarina glauca* seeds could

induce the synthesis of low molecular mass (<5000 Da) heat-resistant deforming factors in a *Frankia* culture (J. Schwencke, personal communication).

Recent studies in plant-cyanobacteria symbioses suggest a conservation of chemical signals between legume-*Rhizobium* and plant-cyanobacteria symbioses (Bergman et al., 1996). *Gunnera* mucilages and seed rinse can induce symbiosis-specific genes of *Rhizobium*, and homologies exist between flavonoid-responding genes of *Rhizobium* and genomic sequences of some symbiotic cyanobacteria (Rasmussen et al., 1996). These results and those with actinorhizal symbioses indicate that plant signals (i.e., flavonoids) may be conserved between these three different symbioses.

Future progress in understanding signal exchanges between the two partners of actinorhizal symbiosis is undoubtedly dependent on the development of a genetic analysis system for *Frankia*. Biochemical studies also benefit from the isolation of cDNAs corresponding to plant genes expressed early in the infection process. This would make it possible to design specific molecular assays for the isolation and characterization of signaling factors. Interestingly, *Penicillium nodositatum*, a saprophytic fungus, is able to infect *Alnus glutinosa* and *Alnus incana* following an intracellular infection pathway. This invasion induces the formation of actinorhizal-like nodules, the so-called myconodules (Capellano et al., 1987; Sequerra et al., 1994). Thus, this fungus seems to be able to mimic *Frankia* signals and avoid plant defense reactions and therefore could be used as an alternative model to study signal exchanges in actinorhizal symbioses.

B. Infection and Nodule Formation

Frankia strains can infect their hosts by two mechanisms, intercellular penetration as

in *Elaeagnus*, *Hippophae*, *Ceanothus*, and *Cercocarpus* (Miller and Baker, 1985; Berry and Sunnel, 1990) and root hair infection as in *Myrica*, *Comptonia*, *Alnus*, and *Casuarina* (Berry and Sunnel, 1990). Both types of infection can be induced, in some conditions, by the same *Frankia* strain, which indicates that the symbiotic infection process is controlled by the host plant (Racette and Torrey, 1989). A more documented review of the cytological aspects of infection process and nodule formation is given by Berry and Sunell (1990).

Intracellular infection (Figure 1) starts with the induction of root hair curling by *Frankia*. Most observations show the initial infection site in the deformed apical region of a root hair (Callaham and Torrey, 1977; Berry et al., 1985). Torrey (1976) has also described penetration of hyphae at the base of a deformed root hair in *Casuarina cunninghamiana*. After penetration, *Frankia* hyphae are encapsulated by a cell wall deposit that is believed to consist of xylans, cellulose, and pectins of host origin (Lalonde and Knowles, 1975; Berg, 1990). In response to the initial root hair infection, some cell division occurs in the cortex, near the invaded root hair, and causes the formation of a swelled structure, the prenodule (Berry and Sunnel, 1990). Infection threads, consisting of lines of encapsulated hyphae, progress intracellularly toward this mitotically active zone, and then further invade some cells of the prenodules that appeared hypertrophied. These cells become filled with endophyte. As the prenodule develops, cell divisions are induced in the pericycle opposite protoxylem pole and give rise to the nodule primordium.

All of the published reports on the molecular aspects of actinorhizal symbiosis involve plants with an intracellular mode of infection, such as *Alnus* or *Casuarina*. No gene specifically expressed during the early step of infection has been isolated yet. Nevertheless, the expression of plant symbiotic

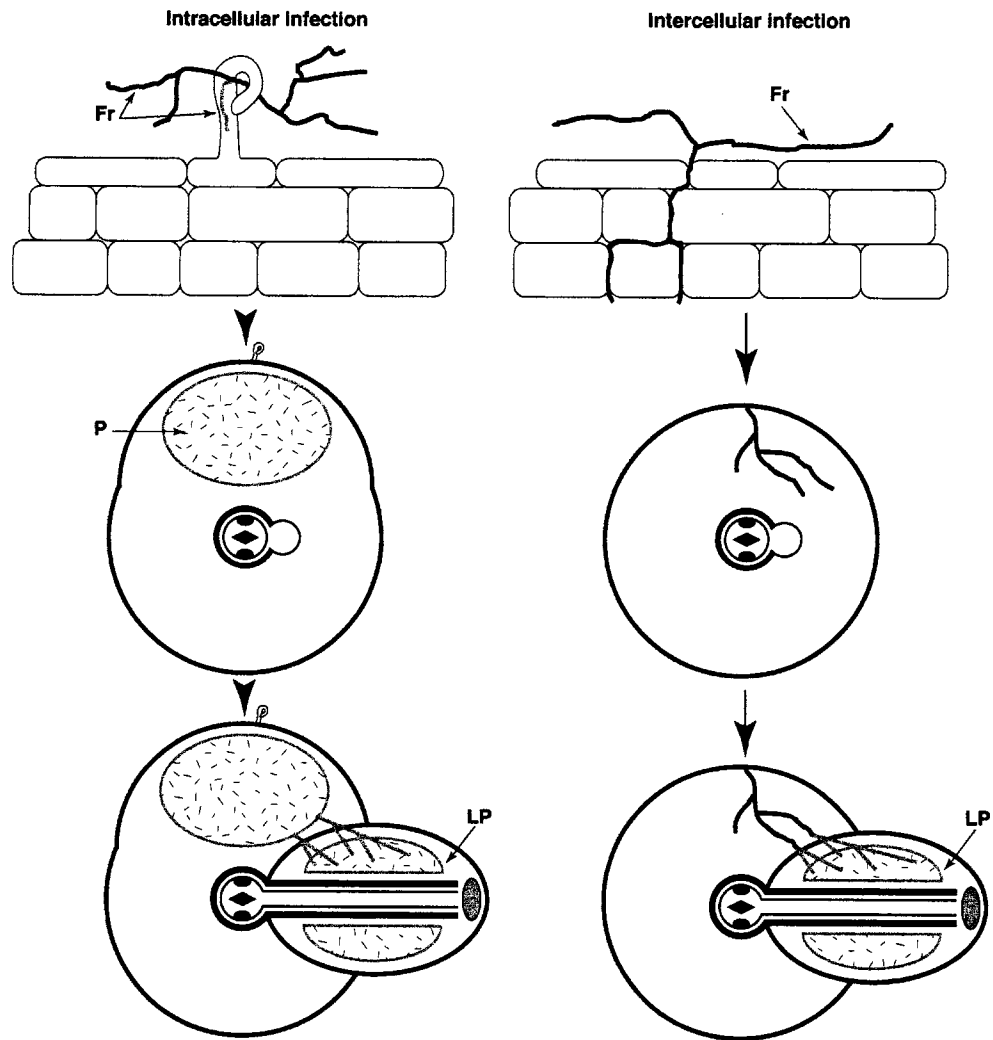


FIGURE 1. Infection and early organogenesis of a nodule lobe in actinorhizal plants. This diagram represents the two known infection pathways (intra- and intercellular infection). Dark lines represent intercellular *Frankia*, while grey lines represent intracellular hyphae. *Intracellular infection* (*Myrica*, *Comptonia*, *Alnus*, and *Casuarina*): *Frankia* hyphae (Fr) penetrate a curled root hair. Concomitantly with the progression of *Frankia*, mitotic activity occurs in cortical cells adjacent to infected root hair. Sustained cell divisions and expansion of infected cells give rise to a region called the prenodule (P) producing a small external protuberance. The nodule lobe (LP) is initiated in the pericycle opposite to protoxylem poles. *Frankia* hyphae coming from the prenodule progress through root cortex to the young nodule lobe (LP). *Intercellular infection* (*Elaeagnus*, *Ceanothus*, *Cercocarpus*): *Frankia* hyphae penetrate the middle lamella between adjacent root epidermal cells. Infection is followed by intercellular growth of the microsymbiont toward the nodule lobe primordium (LP), where intracellular colonization occurs. In intercellular infection, prenodule step has not been reported.

genes such as an infection-associated serine protease gene (Ribeiro et al., 1995), symbiotic hemoglobin of *C. glauca* (Gherbi et al., 1997) and *nifH* gene (Pawlowski et al., 1995) of *Frankia* were studied using *in situ* hybridization in *A. glutinosa* and *C. glauca*

prenodules (L. Laplaze, A. Ribeiro, D. Bogusz, and K. Pawlowski, unpublished results). The expression patterns of these genes were similar in prenodule- and nodule-infected cells. These observations suggested that nitrogen fixation may already occur in

the prenodule stage. The early works of Angulo Carmona (1974) describe the appearance of *Frankia* vesicles in infected cells of *A. glutinosa* prenodules that suggests the existence of nitrogen fixation activity. Thus, the prenodule might already be considered a symbiotic stage, raising the question of its origin and its function in the nodulation process. Considering recent phylogenetic data that suggest a single evolutionary origin for the predisposition of legumes and actinorhizal plants to become nodulated (Soltis et al., 1995), prenodule may be a leftover coming from a common ancestor. In this case, the absence of a prenodule stage during actinorhizal intercellular infection would be a loss-of-function. It is interesting that prenodule formation also occurs during the infection process of the non-legume *Parasponia rigida* by *Rhizobium* (Lancelle and Torrey, 1984a,b). In the case of legumes, the nodule originates from cortical cells divisions; however, unlike the actinorhizal prenodule, differentiation leads to new organ formation. The inability of the prenodule to generate the formation of an organized nodule from dividing cortical cells might be the consequence of the nodule primordia inhibiting prenodule development as the result of early pericycle cell divisions. Another hypothesis would be that the prenodule is a “leftover” from pathogenic interaction, with the induction of the expression of plant defense genes limiting bacterial infection and thus preventing the symbiotic association from expanding. The characterization of genes specifically induced during prenodule induction and ontogeny should provide information about the origin of this unique structure.

Intercellular infection (Figure 1) was first described in *E. augustifolia* by Miller and Baker (1985). Hyphae penetrate between two adjacent rhizoderm cells and progress apoplastically through cortical cells within an electron-dense matrix secreted into the intercellular spaces (Miller and Baker, 1985;

Racette and Torrey, 1989). Unlike the intracellular mode of infection, no prenodule is formed in the root cortex during nodule formation. Interestingly, in *Ceanothus*, during the intercellular infection, limited cortical cell divisions occur, but the corresponding cells are not infected (Liu and Berry, 1991). Also, *Frankia* vesicles are observed in the intercellular spaces during intercellular infection, suggesting that, as for intracellular infection, nitrogen fixation might take place before the nodule lobe formation (Miller and Baker, 1985; Racette and Torrey, 1989).

In both intracellular and intercellular modes of infection, nodule development starts with the induction of mitotic activity in pericycle cells (Figure 1) as a fashion of lateral roots, opposite or at an angle of 45 degrees from the xylem pole in tetrarch and diarch root, respectively (Torrey, 1976). The number of root primordia initiated were shown to vary according to plant family: usually one or two in *Casuarina* (Torrey, 1976; Duhoux, unpublished), up to 14 in *Comptonia* (Callaham and Torrey, 1977). In *C. cunninghamiana* (Torrey, 1976), the root primordia traverse the cortical tissue in the midst of infected prenodule cells, whereas in *A. glutinosa* (Angulo Carmona, 1974) and in *C. glauca* (our laboratory) the root primordia develop outside the infected tissue. The meristematic cells of the primordia always remain free of *Frankia* by an unknown mechanism. It has been suggested that tannin-filled cell layers that surround the primordia may prevent infection (Torrey, 1976). While the nodule primordium develops, *Frankia* hyphae infect young cortical cells and start invading the nodule cortex acropetally.

C. Nodule Functioning

The development of the pericycle-born nodule primordium gives rise to an indeterminate actinorhizal nodule lobe that re-

sembles a modified lateral root without a root cap. New lobes arise continuously to form a coralloid nodule. In each lobe there is a central vascular bundle, and *Frankia* is restricted to the cortical cells (Figure 2). In some cases (e.g., *Casuarina*), an agravitropic nodule root is created at the apex of the nodule (Figure 2). It has large air spaces and is supposed to facilitate oxygen diffusion to the nodule (Silvester et al., 1990). Nodule physiology has been studied extensively (see review in Huss-Danell, 1990).

To identify plant genes involved in the symbiosis, *Alnus glutinosa* and *Casuarina glauca* nodule cDNA libraries have been constructed, and several cDNA clones have been isolated by differential screening (Goetting-Minesky and Mullin, 1994; Ribeiro et al., 1995, Gherbi et al., 1997). As molecular data are available only for those two species, we focus on them in the following paragraphs.

Due to the presence of a meristem at the apex of actinorhizal nodule lobes, a devel-

opmental gradient exists. Different zones (Figure 2) have been characterized using both morphological (Angulo Carmona, 1974) and gene expression studies (Ribeiro et al., 1995; Gherbi et al., 1997). They represent differentiation stages.

The **meristem** at the apex is designated as **zone 1**. Because of the differences between lateral roots and nodules, for example, the lack of root cap in the nodule lobe, one can expect nodule-specific genes determining these differences to be expressed in this zone. However, no meristem-associated actinorhizal nodulin gene has been isolated to date.

In the **infection zone (zone 2)**, adjacent to the apical meristem, hyphae infect some of the new cells derived from meristem activity that subsequently enlarge. Unlike *Rhizobium* in legume, *Frankia* is not released from the infection thread into the cytoplasm of invaded cells; hyphae remain encapsulated by plant cell-derived material as they grow and differentiate (Lalonde and

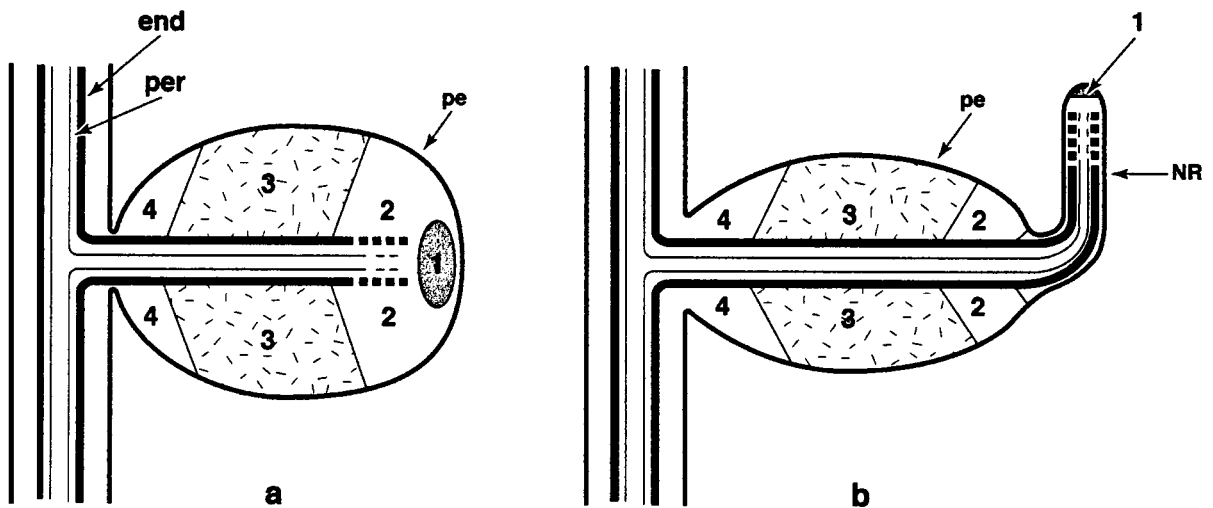


FIGURE 2. Structure of actinorhizal nodule lobes. Nodule consists of discrete or densely packed lobes. Each nodule lobe is a modified lateral root without root cap, including central vascular tissue, cortical parenchyma infected with *Frankia* and a superficial periderm (pe). A zonation of the cortex with four different zones can be defined (Ribeiro et al., 1995; Gherbi et al., 1997). (1) meristem, (2) infection zone, (3) fixation zone, (4) senescence zone. (end) endoderm; (per) pericycle. a, *Alnus* type lobe. b, *Myrica, Casuarina* type lobe. These lobes exhibit a nodule root (NR) at the apex of nodule lobe. Nodule roots are devoid of *Frankia* hyphae.

Knowles, 1975). This zone corresponds to the first stage of interaction; therefore, genes involved in the formation of the interface between the two symbionts are expected to be expressed there. *ag12*, an actinorhizal nodulin gene coding for a serine protease of the subtilisin family, is highly expressed in the infected cells of zone 2 (Ribeiro et al., 1995). The presence of a putative signal peptide in the sequence of the deduced protein, Ag12, suggests an extracellular location of the protein. As the matrix surrounding *Frankia* is an extracellular compartment, and subtilisin-like proteases are involved in protein processing, it has been proposed that Ag12 may participate in the processing of proteins of the cell-wall-derived-matrix surrounding the bacteria (Ribeiro et al., 1995). Due to the proliferation of *Frankia* in the infected cells of zone 2, a lot of matrix is created; this explains the high expression level of *ag12*. The *C. glauca* homologue of *ag12*, *cg12*, has been isolated recently, and its expression is similar to that of *ag12* (L. Laplaze, A. Ribeiro, D. Bogusz, and K. Pawlowski, unpublished data). Moreover, the protein deduced from the *cg12* sequence also possesses the signal peptide, thus contributing to the idea of an extracellular localization of this actinorhizal nodulin.

The plant must provide energy and a carbon source for the proliferating endosymbiont early in the interaction. The main form of photosynthates transport from aerial parts is sucrose. In nodulated legumes, sucrose is the main transport form of carbohydrate from leaves to nodules (Reibach and Streeter, 1983). Nodule-enhanced sucrose synthase and enolase genes were isolated from *A. glutinosa* and were shown by *in situ* hybridization to be expressed in the infected cells of the infection zone (Van Ghelue et al., 1996). Cytoplasmic sucrose synthase is responsible for sucrose degradation, whereas enolase catalyzes the phosphoenolpyruvate (PEP) formation. As energy is generated by

glycolytic processes, and as C4 dicarboxylic acids are supposed to supply carbon to the endosymbiont (Akkermans et al., 1981), sucrose has to be degraded to PEP, which is phosphorylated by PEP carboxylase (PEPC). Co-localization of sucrose synthase and enolase transcripts together with immunolocalization of PEPC in infected cortical cells of *A. glutinosa* nodule (Perrot-Rechenmann et al., 1981) are in agreement with this model. Moreover, a nodule-enhanced *A. glutinosa* gene, *agthi1* whose product is involved in the biosynthesis of thiamine, a cofactor of enzymes involved in glycolysis and the Calvin cycle is also expressed in the infected cells of the infection zone (Ribeiro et al., 1996). Thus, these results reflect the high metabolic activity of infected cells of zone 2.

Two other actinorhizal nodulin genes, *ag164* and *agNt84*, are expressed in infected cells of zone 2 (Pawlowski et al., 1997). The deduced polypeptide sequences have a putative extracellular localization peptide and an arrangement of glycine and histidine residues that suggests that they can bind metal ions. Ag164 and AgNt84 could be involved in transport/storage of metal for biosynthesis of metalloenzymes or metalloproteins involved in symbiosis such as cytochromes, oxidases, or nitrogenase. Alternatively, they might be structural proteins involved in the infection as the corresponding genes expression can only be detected in zone 2.

The **fixation zone (zone 3)** is composed of infected and uninfected cells. Within this zone, infected host cells are hypertrophied. *Frankia* hyphae and vesicles are present, *Frankia nif* genes, coding for the nitrogenase complex, are expressed (Pawlowski et al., 1995), and the protein nitrogenase is detected (Huss-Dannell and Bergman, 1990). Therefore, in this zone, active nitrogen fixation takes place. Uninfected cells are smaller than infected cells and, in *Alnus*, contain amyloplasts and phenolic compounds (Berry and Sunnel, 1990). The product of nitrogen

fixation, ammonium, is exported by the bacteria to the plant cell cytoplasm, where it is assimilated via the GS/GOGAT pathway in both legume and actinorhizal symbioses and is then metabolized to synthesize nitrogen transport compounds (Schubert, 1986). Genes involved in nitrogen metabolism: a nodule-enhanced glutamine synthetase gene and an actinorhizal nodulin gene corresponding to acetylornithine transaminase (AOTA) from *A. glutinosa* have also been isolated (Guan et al., 1996). AOTA is an enzyme involved in the biosynthesis of citrulline, the nitrogen transport form in *Alnus*. These two genes are expressed in infected cells of the fixation zone (Guan et al., 1996). Thus, ammonium assimilation and synthesis of citrulline might occur in these cells.

Here again, the plant has to provide energy and a carbon source for the endosymbiont and acceptor molecules for fixed nitrogen. The nodule-enhanced *sucrose synthase*, *enolase*, and *agthi1* are highly expressed in the infected cells of the fixation zone (Ribeiro et al., 1996; van Ghelue et al., 1996). The absence of detection of sucrose synthase mRNA in uninfected cells of *A. glutinosa* contrasts with the presence of starch accumulation observed in these cells (Wheeler and Lawrie, 1976). Van Ghelue et al. (1996) suggested that apoplastic invertase instead of sucrose synthase might be responsible for starch biosynthesis.

As mentioned previously, the nitrogenase function requires iron and molybdenum. Therefore, for nitrogen fixation to occur, the plant must provide these two metal ions. A cDNA corresponding to a nodule-enhanced gene of metallothionein of *C. glauca*, *cgMT1*, is expressed in infected cells of the fixation zone (our laboratory). Metallothioneins are small proteins involved in various biological processes such as metal homeostasis and cell differentiation. As the expression of this metallothionein gene in *C. glauca* nodules seems to be correlated

with *nifH* expression, CgMT1 could play a role in providing metal ions for the biosynthesis of the iron-molybdenum cofactor of the nitrogenase complex (our laboratory).

In actinorhizal nodules, oxygen is supplied to the symbionts in the infected cells for respiration, but, at the same time, nitrogenase of the symbiont is destroyed by traces of oxygen. Within the nodules of actinorhizal plants, several mechanisms are present to limit oxygen access to the nitrogenase complex. As mentioned before, in most of actinorhizal symbiosis *Frankia* is able to form vesicles where nitrogenase biosynthesis occurs. Parsons et al. (1987) have shown that the thickened glycolipid vesicle membranes provide an adaptative barrier to the penetration of oxygen. Furthermore, the high respiration rate within those vesicles further reduced oxygen concentration (Vikman, 1992). When vesicles are absent, as in *Casuarina*, the lignification of infected cell walls may prevent oxygen diffusion (Berg and McDowell, 1988). Besides those mechanisms limiting oxygen access to symbiotic tissue, hemoglobin has been found in several actinorhizal plants (Suharjo and Tjepkema, 1995). The role of Hb in symbiosis is to facilitate the supply of oxygen to the bacterial respiration chain and, at the same time, preserve the activity of the oxygen-intolerant nitrogenase enzyme complex (Appleby, 1984). Fleming et al. (1987) purified an Hb from *C. glauca* nodule that was shown to present a low oxygen affinity compared with that of legume nodule hemoglobins (Gibson et al., 1989). This suggests that symbiotic nitrogen fixation operates at a higher free oxygen concentration than in legumes even in the absence of vesicles in *C. glauca* nodule. *In situ* hybridization to longitudinal sections of *C. glauca* nodule lobes established that *hb* mRNAs were concentrated in the mature infected cells of the cortical tissues where the induction of *nifH* of *Frankia* occurred (Gherbi et al., 1997). Slight hybrid-

ization was also noticed in young infected cells of zone 2. Thus, as in legume nodules, *Casuarina hb* genes are expressed before *nif* genes (Yang et al., 1991). This is consistent with the role of Hb in reducing oxygen tension, thereby allowing the induction of *nif* genes (Soupène et al., 1995). The occurrence of Hb is in other nodules where *Frankia* form vesicles varies; a very low amount is detected in *A. glutinosa* (Tjepkema, 1982), whereas a high concentration occurs in nodules of *Myrica gale* (Pathirana and Tjepkema, 1995). The presence of Hbs of different molecular weights in *Casuarina* roots and nodules suggested that these proteins were encoded by separate genes (Appleby et al., 1988). The two sets of *Casuarina* genes have been isolated and sequenced (Jacobsen-Lyon et al., 1995). Sequence analyses reveal that *Casuarina* Hb isolated from nodules is closely related to symbiotic leghemoglobin from legumes, whereas the *hb* gene expressed in nonsymbiotic tissue shows extensive homology to the *Trema* nonsymbiotic *hb* gene (Bogusz et al., 1988) and to the *hb* gene found in *Parasponia* (Appleby et al., 1983; Christensen et al., 1991). The promoters corresponding to symbiotic and non-symbiotic *hb* genes were analyzed in transgenic *Lotus corniculatus*. These promoters retained the symbiotic and nonsymbiotic pattern of expression observed in *Casuarina* (Jacobsen-Lyon et al., 1995). Deletion analysis showed that the two motifs (cis-acting elements), which are present in most nodulin promoters (Sandal et al., 1987), are also essential for expression of symbiotic *Casuarina hb* (Jacobsen-Lyon et al., 1995). Besides *Casuarina*, an *hb* gene expressed in nonsymbiotic tissue was also reported recently in barley (Taylor et al., 1994) and soybean (Anderson et al., 1996). The function of this nonsymbiotic Hb is still unknown. Appleby et al. (1988) suggested that Hb could act as a sensor of oxygen tension in fast-growing tissue.

In older nodule lobes, there is a **senescence zone (zone 4)** where host cytoplasm and endophyte degeneration is observed (Newcomb and Wood, 1987), *nifH* expression is switched off, and nitrogenase activity is lost in legume and actinorhizal nodules (Vikman et al., 1990; Swaraj et al., 1993). A nodule-specific cysteine proteinase AgNOD-CP1 cDNA has been isolated from *A. glutinosa* and might be involved in senescence (Goetting-Minesky and Mullin, 1994; Pawlowski, 1997). This protease may act extracellularly, because it presents a peptide signal for extracellular localization (Goetting-Minesky and Mullin, 1994) and may play a role in endosymbiont degradation (Pawlowski, 1997). A cysteine protease activity was found in aging legume nodules and was assumed to play a role in the recovery of the nitrogenous compounds from the senescing tissues (Pladys and Vance, 1993). *ag13*, a nodule-enhanced gene from *A. glutinosa*, is expressed in infected cells of the senescence zone (Guan et al., 1997). The protein deduced from the *ag13* sequence is rich in glutamic acid and proline; it has a putative signal peptide that suggests an extracellular function, probably in the compartment surrounding the endophyte. While proline-rich legume nodulins are involved in the infection process (ENOD2, Franssen et al., 1987; ENOD5 and ENOD12, Scheres et al., 1990; MtPRP4, Wilson et al., 1994), Ag13 is involved in late events of nodule functioning (Guan et al., 1997) probably as a defense-related protein. It has been proposed that Ag13 is a member of a new family of acidic extracellular proteins (Guan et al., 1997).

Nodule functioning is associated with a high exchange rate between the fixing nodule and the rest of the plant. The **pericycle** acts as an interface between nodule cortical cells and vascular tissues. Interestingly, in *A. glutinosa* the pericycle of the nodule is formed of several layers of small cells with dense cytoplasm and is rich in mitochondria

(Burgess and Peterson, 1987). Moreover, several actinorhizal nodulins or nodule-enhanced genes are expressed in nodule pericycle. Genes associated with carbon metabolism such as sucrose synthase, enolase (van Ghelue et al., 1996), *agthil* (Ribeiro et al., 1996), and with nitrogen metabolism, such as glutamine synthetase (Guan et al., 1996), are highly expressed in pericycle cells, thereby reinforcing the idea that this tissue has a high metabolic activity and a function in nutrient exchanges. It should be noted that *AOTA* is not expressed in *A. glutinosa* pericycle cells, which suggests that the synthesis of the nitrogen-transport molecule, citrulline, only occurs in the infected cells of the fixation zone (Guan et al., 1996). Furthermore, because the *GS* gene is expressed in pericycle cells, these cells must contain free ammonium. This means that the degradation of primary assimilation products should take place and be immediately followed by reassimilation (Guan et al., 1996). In *C. glauca* nodules, the presence of *cgMTI* transcripts in the pericycle suggests that the pericycle is also involved in metal exchanges (our laboratory). Finally, *ag13* transcripts are detected in the multilayered pericycle of *A. glutinosa* (Guan et al., 1997). As *Ag13* is thought to act as a defense protein, the expression of the corresponding gene in the pericycle may be involved in limiting the endosymbiont invasion.

III. TRANSGENIC ACTINORHIZAL PLANTS: A TOOL FOR THE MOLECULAR STUDY OF THE SYMBIOSIS

Recent advances in tissue culture, DNA technology, and gene transfer techniques have opened new avenues in transformation of higher plants in general and of actinorhizal plants in particular. The most convenient method for introducing foreign genes into

dicotyledonous plants is based on the *Agrobacterium tumefaciens*-Ti plasmid or *Agrobacterium rhizogenes*-Ri plasmid systems. For the plants that do not respond well to *Agrobacterium*, alternative direct DNA transfer methods have been developed (see Potrykus, 1991; Christou, 1993; Vasil, 1994; Songstad et al., 1995). This article reviews the different techniques of genetic transformation that have been applied to actinorhizal plants, the use of these transgenic plants for molecular study of the symbiosis, and genetic engineering of actinorhiza.

A. Transformation Systems for Actinorhizal Plants

1. Direct Gene Transfer

A great variety of direct gene transfer techniques have been developed in recent years, for example, PEG-mediated DNA uptake, electroporation of protoplasts and tissues, fusion of protoplasts with DNA-containing liposomes, ultrasonication, pollen transformation (Potrykus, 1991; Fisk and Dandekar, 1993; Songstad et al., 1995). To our knowledge, two of these techniques have been applied to actinorhizal plants.

a. Electroporation

Electroporation employs electrical impulses of high field strength for a short period; this process enables transient openings of the plasmalemma and the diffusion of macromolecules such as nucleic acids (Fromm et al., 1985). This technique was first applied to plant protoplasts (Fromm et al., 1985), and, more recently, to intact tissue (Dekeyser et al., 1990; D'Halluin et al., 1992; Songstad et al., 1993).

Transient expression of the β -glucuronidase reporter gene placed under the con-

trol of the cauliflower mosaic virus 35S promoter was obtained following electroporation of protoplasts derived from a cell suspension of *Alnus incana* (Séguin and Lalonde, 1988). The reporter gene activity was quantified by fluorometric analysis, and the conditions of electroporation were optimized by modifying the electroporation voltage and the DNA concentration of the plasmid vector. The addition of PEG in the electroporation medium enhanced the β -glucuronidase activity in the protoplasts three- to sixfold. The growth of stably transformed tissues of *Alnus incana* was not obtained by the authors. The major drawback of the protoplast-electroporation technique is linked to the difficulty in regenerating protoplasts into complete plants (Potrykus, 1991). Electroporation of intact tissues has never been reported for actinorhizal plants.

b. Particle Bombardment

Microprojectile bombardment appears as an attractive technique for direct gene transfer into actinorhizal plants as it does not require protoplasts. The plasmid DNA containing the transgenes is coated onto microparticles that are accelerated to high velocity by a particle gun apparatus and delivered into intact organs or cultured tissues (Klein et al., 1987). Any organ of the plant can be subjected to particle bombardment, and transient expression systems can be easily developed (Sanford, 1990; Christou, 1992; Klein et al., 1992). This technology is particularly valuable in evaluating gene constructs in a plant that has never been transformed.

Before developing a genetic transformation procedure for *Casuarinaceae* in our laboratory, we determine whether the cauliflower mosaic virus 35S promoter could drive the expression of the β -glucuronidase reporter gene in *Casuarina glauca* and *Allocasuarina verticillata*. The plasmid pUC18 carrying the 35S promoter (Benfey and Chua, 1990), the

uidA (GUS) gene (Jefferson et al., 1987), and the NOS 3' end (Bevan, 1984) was coated onto tungsten microparticles that were accelerated to high velocities using a gun powder device (Le et al., 1996). Different plant targets were used, including cotyledons, epicotyls, and hypocotyls excised from 1-month-old *Casuarina* and *Allocasuarina* seedlings. Bombardments of cotyledons led to the most efficient and reproducible data of transient expression. The intensity and number of the blue spots resulting from the expression of the reporter gene in *Casuarinaceae* cells was similar to the one observed in bombarded *Nicotiana tabaccum* leaves, indicating that the 35S promoter was a useful sequence for expressing transgenes in actinorhizal trees (C. Franche and A. Lappartient, unpublished data). The same approach can be used on any actinorhizal plant, thereby providing a quick means to determine the activity of gene constructs in the recipient cells.

The ultimate objective of any gene transfer method is to regenerate transgenic plants from transformed cells. Stably transformed lines using particle bombardment have been reported in various plants in the literature (for review see Christou, 1992), but there is no publication on a transgenic actinorhizal plant obtained after particle bombardment. The low conversion frequency of transient-to-stable transformation events appears to be a major drawback of this technique (Sanford, 1990; Christou, 1993). However, it is reasonable to expect that this technology does hold promise and will progress considerably when more information becomes available on the biology of plant cells capable of stable DNA uptake and regeneration.

2. Transfer Based on *Agrobacterium rhizogenes*

The soil bacterium *Agrobacterium rhizogenes* is responsible for the hairy root

disease in dicotyledonous plants; this disease results from the transfer of a portion of DNA from a large bacterial plasmid (the root-inducing or Ri plasmid) into the plant genome (for reviews see Zambryski et al., 1989; Tepfer and Casse-Delbart, 1987). In legumes, this vector has proven to be very valuable in obtaining transgenic plants and in developing quick procedures for the study of symbiotic gene expression in roots and nodules (for review see Stougaard, 1995).

a. Susceptibility of Actinorhizal Plants to Wild-Type A. rhizogenes Strains

The host-range of *A. rhizogenes* has been reviewed in detail (De Cleene and De Ley, 1981; Porter, 1991), and several actinorhizal plants have been reported to be sensitive to these agrobacteria. *Alnus glutinosa*, *Alnus acuminata*, and *Elaeagnus angustifolia* were found susceptible to four strains of *A. rhizogenes* (Savka et al., 1992): two agropine-type strains, A4 (Moore et al., 1979) and 1855 (Cardarelli et al., 1985), one mannopine-type strain, 8196 (Koplow et al., 1984), and one cucumopine-type strain, K599 (Savka et al., 1992). When hypocotyls from 1-month-old seedlings of *A. acuminata* and *A. glutinosa* were inoculated with any of the four strains, root primordia developed within 14 d at the inoculation sites; these roots exhibited non-geotropic growth and were characterized by abundant lateral roots. Evidence for genetic transformation was evaluated by specific opine synthesis and by examination of the typical phenotypes associated with hairy roots. For *E. angustifolia*, transformation experiments were performed on cotyledons excised from 2-week-old seedlings. Fourteen days after inoculation with the strain 8196, globular non-friable callus grew on the wounded cotyledons; from these structures nodule-like root tissues emerged, but they failed to proliferate on hormone-free nutrient medium. When the inoculation was

performed using strain the K599, a similar response was observed on the wounded sites of *E. angustifolia*, but the nodule-like root structures proliferated without any supply of growth regulators. These structures were called pseudoactinorhizae and Savka et al. (1992) hypothesized that the *A. rhizogenes* strain K599 might produce a molecule that could act as a signal for nodule-like root structure differentiation.

In our laboratory, three wild-type strains of *A. rhizogenes* were used in preliminary inoculation experiments on the tropical actinorhizal tree *A. verticillata* (Phelep et al., 1991; Franche et al., 1994): an agropine strain, A4 (Moore et al., 1979), a cucumopine strain, 2659 (Davioud et al., 1988), and a mannopine strain, 8196 (Koplow et al., 1984). To generate hairy roots, 2 month-old aseptic seedlings of *A. verticillata* were inoculated with these strains by wounding the hypocotyls with a needle dipped in the agrobacterial culture. Seven to 10 d after inoculation, about 50% of the inoculated hypocotyls developed roots showing a typical hairy root phenotype (high growth rate, extensive lateral branching, and lack of geotropism) due to the expression of the oncogenes of the T-DNA from *A. rhizogenes*. Opines were detected in 100% and 42%, respectively, of the roots induced by 2659 and A4 (Phelep et al., 1991).

Although results reported in the literature suggest that *A. rhizogenes*-induced hairy roots are difficult to establish in woody species (Tepfer, 1990), actinorhizal trees tested so far exhibit a good sensitivity to a wide range of *A. rhizogenes* strains.

b. Fast A. rhizogenes-Based Transformation System for the Study of Gene Expression in Roots and Nodules of Actinorhizal Plants

To facilitate molecular analysis of symbiotic genes in legumes, several rapid procedures for production of transgenic root nod-

ules have been established on *Lotus corniculatus* (Hansen et al., 1989), *Trifolium repens* (Jorgensen et al., 1988; Diaz et al., 1989), and *Vicia hirsuta* (Quandt et al., 1993). The procedures rely on direct nodulation by *Rhizobium* of transgenic roots induced by *A. rhizogenes*. Using the “composite plant” approach, the expression of a chimeric gene can be studied within approximately 2 months. A similar approach has been developed on *Casuarina glauca* in our laboratory (Diouf et al., 1995).

Young seedlings of *C. glauca* were wounded on the hypocotyl and inoculated with an overnight culture of A4RS (Jouanin et al., 1986) containing, in the binary vector BIN19 (Bevan, 1984), the intron- β -glucuronidase gene (GUSINT) under the control of the 35S promoter (Vancanneyt et al., 1990). After 1 week, a small callus was visible at the inoculation site, and 7 d later highly branched quick-growing roots developed directly out of the callus. The normal root system was then removed at the stem basis, while the composite plant was decontaminated by incubation in a liquid medium with cefotaxim. The plants were then cultivated in glass tubes containing a nutrient medium prior to inoculation by *Frankia*. Transfer of the TR- and TL-DNA from A4RS was studied by PCR analysis using primers specific for *rolA* and agropine genes. The analysis of 20 putatively transformed roots demonstrated the amplification of the two groups of pRi genes. The β -glucuronidase reporter gene activity was detected in 50% of *C. glauca* hairy roots, indicating a co-transfer of the genes carried by the vector BIN19-GUSINT.

Six weeks after their transfer in glass tubes, the composite plants were starved in nitrogen and inoculated by the *Frankia* strain Thr (Girgis et al., 1990). Two to five nodules generally appeared 1 month after inoculation by the actinomycete (Diouf et al., 1995). Nodulation by *Frankia* of transgenic hairy roots was found to be slightly reduced:

40% of the transformed roots induced on *C. glauca* by *A. rhizogenes* A4RS could be nodulated by the strain Thr, whereas 100% of the non-transformed control plants developed nodules. Similar results have been reported by Beach and Gresshoff (1988) when *A. rhizogenes* transformed roots of three forage legumes (*Trifolium pratense*, *Macroptilium atropurpureum*, and *Medicago sativa*) were inoculated by *Rhizobium*. Up to 60% of the plants failed to nodulate, and when nodules did appear they were far fewer per given length of hairy root than in non-transformed control roots. The authors suggested that the inhibition of nodulation was linked to the expression of Ri T-DNA genes rather than to persisting agrobacteria in the plant tissues. For *Lotus corniculatus* and *Vicia hirsuta* nodulation of the composite plants and the wild-type control plants was found to be similar (Hansen et al., 1989; Quandt et al., 1993).

Using “composite plant” system developed on *C. glauca*, the analysis of nodulated transgenic hairy roots can be made within 4 months. This system should facilitate in the future the molecular studies of the symbiotic process in the actinorhizal plants.

c. Regeneration of Transgenic Actinorhizal Plants Following *A. rhizogenes* Genetic Transformation

For plant transformation, the use of *A. rhizogenes* provides features that might be more advantageous than the more commonly used *A. tumefaciens*: as the transgenic hairy roots can be easily recognized, separated, and cultivated *in vitro*, a number of plants can regenerate spontaneously from these root cultures or following hormone addition (Tepfer, 1990). Therefore, despite the hairy root phenotype of the regenerated plants, *A. rhizogenes* is a valuable genetic transformation strategy for recalcitrant species. Although hairy roots can be induced on

several actinorhizal plants, the regeneration of transgenic plants from *A. rhizogenes*-induced roots has been reported for only one species, *A. verticillata* (Phelep et al., 1991).

Young seedlings of *A. verticillata* were inoculated on the hypocotyls with the wild-type strains A4 and 2659, and the transgenic roots were excised and cultivated on a nutrient medium. Shoot regeneration from A4-transformed roots was obtained by adding exogenous phytohormones and buds developed in 2 months on some of the transformed roots. Shoot regeneration occurred spontaneously on 90% of the *Agrobacterium rhizogenes* 2659 transformed roots after 3 months of culture on a hormone-free medium (Phelep et al., 1991). After an IBA treatment, all the putatively transformed shoots were rooted. The phenotype of the transgenic *A. verticillata* plants was different from that of the control plants: the root system was more developed, plagiotropic, and branched than the normal non-transformed regenerants, and the aerial system exhibited reduced apical dominance with highly branched shoots. T-DNA transfer into putatively transgenic *A. verticillata* trees was demonstrated by opine synthesis and Southern blot analysis (Phelep et al., 1991). After 2 months of inoculation by the strain of *Frankia* DEC, 20% of the plants transferred into a glasshouse developed nitrogen-fixing nodules (Franche et al., 1994).

Due to the poor efficiency of nodulation and to the altered phenotype of the transgenic *A. verticillata* plants, it is very likely that this strategy of genetic transformation based on *A. rhizogenes* will not be of great interest for basic studies on actinorhizal symbiosis.

3. Transfer Based on *Agrobacterium tumefaciens*

A. tumefaciens is the causative agent of crown gall, a disease of dicotyledonous

plants characterized by a tumor growth. Using this natural gene transfer system, a wide range of plants, many of which are of economical importance, have been transformed genetically (for review see Hooykaas and Schilperoort, 1992; Lindsey, 1992). Although legumes are usually recalcitrant to transformation and regeneration, several of them have been transformed successfully by *A. tumefaciens* (Jordan and Hobbs, 1994), and the transgenic plants have been used for the study of symbiotic genes (e.g., Pichon et al., 1992; Chabaud et al., 1996).

a. Susceptibility of Actinorhizal Plants to Wild-Type A. tumefaciens Plants

The host-range of *A. tumefaciens* is broad; De Cleene and De Ley (1976) have shown that over 600 dicotyledonous hosts, representing more than 300 genera, are susceptible to crown gall infection. The susceptibility of actinorhizal plants to *Agrobacterium tumefaciens* was first established by Hoerner in 1945; tumors developed on inoculated *Alnus rubra* in approximately 1 year. Mackay et al. (1988) reported the genetic transformation of *Alnus glutinosa* and *Alnus incana* by two wild-type *A. tumefaciens* strains: the octopine-type Ach5 (Chilton et al., 1985) and the nopaline-type C58 (Van Larebeke et al., 1974). *In vitro* propagated shoots were inoculated either by decapitation or by superficially wounding the stem. Tumors were visible within 7 to 14 d after inoculation. Genetic transformation was demonstrated by tumor growth in the absence of phytohormones, the production of opines, and Southern blot analysis.

Two different oncogenic strains of *A. tumefaciens* were used on *C. glauca* in our laboratory: the nopaline strain 82139 (Brasileiro et al., 1991) and Antib 12 (A. Petit, Institut des Sciences Végétales, Gif

sur Yvette, France). One to 2 month-old seedlings of *C. glauca* were wounded on the hypocotyls, epicotyls, or cotyledons with a needle previously soaked with a fresh colony of the wild-type *Agrobacterium* strains; 82139–inoculated *C. glauca* developed tumors within 4 weeks on 58% of the hypocotyls. *C. glauca* appeared slightly more sensitive to inoculation by the strain Antib 12. Twelve days after inoculation, tumors developed on 78% of the hypocotyls and on 57% of the epicotyls of the plantlets; tiny tumors appeared on 8% of the cotyledons, but did not develop any further. The phenotype of the tumors induced by the two wild-type *A. tumefaciens* strains was undifferentiated. Some of the tumors were excised and grown for several months on a nutrient medium without any growth regulator.

b. Regeneration of Transgenic Actinorhizal Plants

In order to successfully regenerate transgenic plants using the natural *A. tumefaciens* gene transfer system, several parameters have to be fulfilled: (1) the virulence of the *Agrobacterium* strain has to permit the transfer of the T-DNA into the wounded plant cells; (2) the transformed cells have to be efficiently selected among the population of non-transformed cells; (3) the transformed cells have to be regenerated into plants. The strategy used in most studies of plant transformation usually consists of establishing culture conditions in which plants can be regenerated from callus, and co-cultivating the explant with the chosen disarmed strain of *A. tumefaciens* that contains the desired DNA construct. This approach was used in our laboratory first on *C. glauca* and then on *A. verticillata*.

Gene transfer from *A. tumefaciens* to wounded plant cells requires a set of genes localized on the Ti plasmid *vir* region

(for review see Hooykaas and Schilperoort, 1992; Zambryski, 1992; Hooykaas and Beijersbergen, 1994; Sheng and Citovsky, 1996). A number of physiological and environmental factors such as the presence of plant phenolic compounds (acetosyringone), sugars, pH, temperature, and osmoprotectant compounds affect the induction of virulence genes and consequently the efficiency of T-DNA transfer. Therefore, conditions for gene transfer by a disarmed strain of *Agrobacterium tumefaciens* were optimized on the actinorhizal tree *Casuarina glauca* (Le et al., 1996). The highest number of transgenic calli were obtained when *C. glauca* epicotyls were excised from 45-d-old seedlings of *C. glauca*, co-cultivated for 3 d with the strain C58C1(GV2260; pBIN19GUSINT) in the presence of 25 μ M acetosyringone at a pH of 5.6 and subsequently grown in the presence of 50 mg/l kanamycin and 250 mg/l cefotaxim. Within 2 months of culture on a nutrient medium containing growth regulators and antibiotics, 26% of the transformed epicotyls had developed one to three calli that grew in the presence of kanamycin, and 95% of these calli expressed the β -glucuronidase reporter gene activity. The presence of the transgenes was confirmed further by PCR and Southern blot analyses (Le et al., 1996). Although we have obtained numerous transgenic calli, differentiation of buds was observed on only 10% of them, and it was not possible to regenerate transgenic plants. Recently, in our laboratory adventitious organogenesis has been optimized for *C. glauca* and some transgenic plants have been obtained (C. Franche and A. Smouni, unpublished data).

The most efficient and reproducible *A. tumefaciens*-based transformation procedure was developed on *A. verticillata* (Franche et al., 1997). The major difference with *C. glauca* is the high potential of *A. verticillata* for regeneration from callus induced on mature zygotic embryos (Duhoux

et al., 1996). Therefore, the embryos of *A. verticillata* were used as a target for T-DNA transfer by the strain C58C1(GV2260; pBIN19GUSINT). Within approximately 6 months, transgenic plants were regenerated on 70% of the kanamycin-resistant calli. The phenotype and the nodulation efficiency by *Frankia* was found similar in the transgenic *A. verticillata* and in the non-transformed control plants, and the transgenic nodules were shown to fix nitrogen (Franche et al., 1997).

The transformation procedure developed on *A. verticillata* has several major advantages: the kanamycin selection is efficient and there are very few escapes; a single medium is required for both bud differentiation and shoot elongation; it is possible to regenerate numerous trees from a single callus and within a period of time that is rather short considering that *Casuarina* is a tree.

B. Transgenic Actinorhizal Plants for Molecular Analysis of Symbiosis

In legumes, transgenic plants have become a major tool for a better understanding of the symbiotic interaction with *Rhizobium* (de Bruijn et al., 1990). Promoter analysis has led to the identification of cis-acting elements involved in plant gene regulation during nodule formation and functioning. An example of this is provided by the analysis of the promoters of nonlegume and legume hemoglobin genes in the transgenic legume *Lotus corniculatus*. Internal deletions demonstrated the essential role of regulatory elements for organ specificity and for the expression level of these hemoglobin genes (Stougaard et al., 1987; Jacobsen-Lyon et al., 1995; Andersson et al., 1997). Moreover, legume and nonlegume *hb* gene promoter analysis has generated information on the evolution of *hb* genes and nitrogen fixing symbiotic association among the An-

giosperms (Bogusz et al., 1990; Andersson et al., 1997). Furthermore, transgenic legumes have helped to refine the knowledge of spatial and temporal expression of nodulin genes such as *Enod12* (Journet et al., 1994; Chabaud et al., 1996). The availability of transgenic actinorhizal plants open the way to similar studies.

To investigate whether regulatory mechanisms of hemoglobin gene expression were maintained between *Rhizobium*-nodulated plants and actinorhizal plants, we introduced the promoter region from the soybean c_3 (*lbc_3*) (Stougaard et al., 1987) and the *Parasponia* (Bogusz et al., 1990) hemoglobin genes into *A. verticillata*. *Parasponia andersonii* is a member of the *Ulmaceae* family, a non-legume that lives in symbiotic association with *Rhizobium*. In transgenic *Lotus corniculatus*, the soybean *lbc3* promoter directs the expression in *Rhizobium*-infected cells (She et al., 1993). In transgenic nodules of *C. glauca* and *A. verticillata*, the soybean *hb* promoter was found expressed in *Frankia*-infected cells, thus indicating a conservation of the cell-specific expression in the actinorhizal plants (unpublished, our laboratory). A similar pattern of expression was observed in transgenic *C. glauca* and *A. verticillata* plants with the *Parasponia hb* promoter, the reporter gene activity being restricted to *Frankia*-infected cells. In the corresponding transgenic *Lotus corniculatus*, low levels of GUS staining were reported in bacteroid-containing cells. The fact that the more intense blue was located in the uninfected cells suggested poor recognition of the symbiotic functions of the *Parasponia* promoter in legumes (Andersson et al., 1997).

The availability of transgenic actinorhiza can also offer the possibility of modifying the expression of symbiotic genes and allowing their biological function to be examined. In legumes, the antisense strategy was used to study the regulation of ureide biosynthesis and the development of the

peribacteroid membrane in transgenic root nodule (Cheon et al., 1993; Lee et al., 1993).

The cauliflower mosaic virus 35S (CaMV) promoter has been the most widely used sequence for the expression of foreign genes into plants and has proven to be very effective in dicots and in some monocots (Benfey and Chua, 1990). A detailed knowledge of the spatial and temporal expression of this constitutive promoter is necessary before introducing new valuable traits into actinorhizal trees or driving antisense genes putatively involved in the symbiotic process.

As mentioned previously, the possibility of expressing the β -glucuronidase gene under the control of the 35S promoter was first demonstrated by the analysis of electroporated protoplasts from *Alnus incana* (Séguin and Lalonde, 1988). In our laboratory, we conducted a detailed analysis of the level and specificity of expression of the β -glucuronidase gene driven by the 35S promoter in transgenic *A. verticillata* regenerated after transformation by the *A. tumefaciens* strain C58C1(GV2260; BIN19GUSINT) (Franche et al., 1997). Some 150 plants regenerated from 23 calli resulting from independent transformation events were analyzed. Blue-colored cells were observed in both the stems and the roots of transgenic *A. verticillata* plants. A constitutive *uidA* expression was observed in shoots, whereas in roots, reporter gene activity was found to be stronger in the vascular tissue and the meristematic region. The root tips stained more intensely than any other region of the roots. In transgenic nodules, a heterogeneous pattern of blue staining was also obtained. The data suggest that the CaMV 35S is not expressed in the nodule periderm and endoderm, and that its level of expression is higher in the cells that are not infected by *Frankia*. Nevertheless, any interpretation of differences in staining should take into account a number of factors such as

cell size, degree of vacuolation, substrate accessibility, and level of transcription (Jefferson et al., 1987). It is interesting to note that the pattern of staining obtained in roots and nodules of *A. verticillata* transformed by *A. tumefaciens* was very similar to the one obtained in hairy roots and nodules of composite *C. glauca* plants obtained by *A. rhizogenes* (Diouf et al., 1995). In the control non-transformed plants, β -glucuronidase expression was never detectable in stems, roots, or nodules, thus indicating that *A. verticillata* had no or very low background of GUS activity.

The region -343 to -90 of the CaMV 35S promoter has been shown to act as an enhancer sequence in higher plants (Odell et al., 1988). The enhancement of heterologous gene expression in plants can be obtained by duplication of this region (Kay et al., 1987; Timmermans et al., 1990; Mitsura et al., 1996). A gene construct carrying the *uidA* gene driven by the enhanced 35S promoter was introduced into *A. verticillata*. Qualitative and quantitative data obtained in our laboratory indicate a better expression of this promoter, especially in roots.

From these data it appears that the 35S promoter is a valuable sequence to drive the constitutive expression of a foreign gene in *Allocasuarina*, especially in shoots. For a more uniform expression in roots, the enhanced 35S promoter is required.

C. Transgenic Plants and Genetic Engineering of Actinorhiza

Actinorhizal plants include a number of woody dicotyledonous species that play a major ecological and economical role in temperate and tropical areas (Wheeler and Miller, 1990; Diem and Dommergues, 1990). The possibility of genetically engineering some of these species has great potential considering the constraints in classic genetic improve-

ment in forestry, such as the large size of the mature plants and the long sexual generation time. Although genetic engineering in trees is still in its infancy, several studies developed on the model tree poplar have clearly established the potential of this approach for the introduction of novel genetic traits such as resistance to herbicides, insects, pathogenic fungi, or for the modification of wood properties (for reviews see Charest and Michel, 1991; Schuerman and Dandekar, 1991; Jouanin et al., 1993; Sederoff, 1995). Similar strategies can now be applied to actinorhizal trees such as *Casuarina* and will develop further with the progress of *in vitro* culture systems and gene transfer techniques.

IV. CONCLUDING REMARKS

Because of their economic importance, strong emphasis has been placed on research into the functioning of legume symbioses. Despite the significant ecological role of actinorhizal plants, it was only in 1978 that the first *Frankia* strain was isolated and in the last few years that molecular approaches have been developed on the plant side. Recent works, presented in this article, report on the characterization of transcripts corresponding to actinorhizal nodulin and nodule-enhanced genes in *A. glutinosa* and *C. glauca*.

Among the cDNAs clones that have been characterized from *C. glauca* and *A. glutinosa* root nodules, some represent genes expressed in the infection process (*ag12*) and genes involved in oxygen (*hb*), carbon (sucrose synthase, enolase), and nitrogen metabolism (*GS*, *AOTA*). A few conclusions can be drawn from these molecular studies. Despite their possible phylogenetic relationship (Soltis et al., 1995), no homolog of legume early nodulin genes such as *ENOD12* or *ENOD2* have been isolated from actinorhiza, which suggests that different

genetic programs are induced during the infection processes of both symbioses. Actinorhizal nodules have a root-like anatomy, whereas the legume nodule structure resembles the stem, thus genes involved in nodule development might have been recruited from the stem for legumes and from the lateral root development for actinorhizal plants. In the future, it would be interesting to determine if genes specifically induced in lateral root development are also involved in the formation of nodules. It is also possible that actinorhizal nodulin genes through gene duplication have arisen from genes implicated in lateral roots development. This evolutionary strategy is thought to have occurred for the symbiotic *hb* gene (Appleby et al., 1988). An analysis of the cDNA library representing genes expressed in actinorhizal nodules showed that most of the genes expressed in nodules are also expressed in roots, thus reinforcing the idea that actinorhiza are modified lateral roots.

The diverse nature of actinorhizal plants is correlated to a diversity in nodule anatomy and physiological mechanisms. Molecular phylogenetic studies suggest that nodulation has evolved independently in actinorhizal plant families (Swensen, 1996). According to *rbcL*-based phylogeny, *C. glauca* and *A. glutinosa* belong to the same clade (clade IV), thus molecular studies on plants of different clades should indicate to what extent mechanisms are maintained throughout the four identified actinorhizal clades (Swensen, 1996).

Because of the wide diversity of actinorhizal plants, we addressed the question of developing an actinorhizal plant model for molecular biology and genetic research. *Alnus* and *Casuarinaceae* have been chosen as model families for the study of actinorhizal development at the molecular level because of their importance in agroforestry and the availability of numerous physiological and morphogenetic studies. Recent developments

on genetic transformation of *Casuarinaceae*, presented here, show that transgenic *C. glauca* and *A. verticillata* constitute valuable tools to investigate the specific function and the regulation of the expression of actinorhizal symbiotic genes. To be useful, a system for generating transgenic plants must be reproducible and allow the regeneration of large number of plants in a short period of time. The *Agrobacterium tumefaciens* transformation system developed on *A. verticillata* meets these requirements; it can be used on a routine basis, allows the regeneration of transgenic plants within 6 months, and is simple. Furthermore, the composite plants obtained after transformation by *A. rhizogenes* can be used to prescreen the expression of gene constructs, while fully transformed plants are regenerated after transformation by *A. tumefaciens*. Thus, it is very likely that *Casuarina* trees will appear in the future as a model tree for the study of actinorhizal symbioses. The study of heterologous genes expression, such as legume nodulins or developmental genes from the model plant *Arabidopsis thaliana*, should also provide information on the evolution of nodulation and nodule-specific genes. *Datisca*, the only herbaceous genus nodulated with *Frankia*, could be an alternative. Because of its short life cycle and its low DNA content, it might be a suitable model for genetic studies (Pawlowski and Bisseling, 1996). Population genetics using RAPD profiles have already been reported in *Datisca glomerata* species (Fritsch and Rieseberg, 1992), thus this species might be a good candidate.

Finally, by comparing legume and non-legume symbiotic processes, it might be possible to identify key steps that are common and unique to these associations (Pawlowski and Bisseling, 1996). This should be useful in defining strategies that lead to the extension of symbiotic nitrogen fixation to economically important non-nodulated plants.

ACKNOWLEDGMENTS

The authors would like to acknowledge Drs. K. Pawlowski (Institut für Biochemie der Pflanze, Untere Karspüle 2 37073 Göttingen, Germany) and E. Dennis (CSIRO, Division of Plant Industry, Canberra, Australia) who gave us preprints.

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