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Review

Actinorhizal nitrogen fixing nodules: infection process, molecular biology and genomics

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Actinorhizal hosts are non-leguminous perennial plants belonging to 8 angiosperm families. They are capable of forming root nodules as a result of infection by a nitrogen-fixing actinomycete called *Frankia*. Actinorhizal nodules consist of multiple lobes, each of which represents a modified lateral root with infected cells in the expanded cortex. This article summarizes the most recent knowledge about this original symbiotic process. The infection process is described both at cytological and molecular levels. The use of transgenic *Casuarinaceae* for studying in actinorhizal nodules the regulation of several symbiotic promoters from legumes is also discussed. With progress in plant genome sequencing, comparative genomics in legumes and actinorhizal plants should contribute to the understanding of the evolutionary history of nitrogen-fixing symbioses.

Key words: Nitrogen-fixation, actinorhizal nodules, *Frankia*, *Casuarina*, symbiotic gene.

INTRODUCTION

Actinorhizal root nodules result from the interaction between a nitrogen-fixing actinomycete called *Frankia* and roots of dicotyledonous plants belonging to 8 plant families and 25 genera (Benson and Silvester, 1993). Actinorhizal plants share common features; with the exception of *Datisca,* which has herbaceous shoots, they are perennial dicots and include woody shrubs and trees such as *Alnus* (alder), *Elaeagnus* (autumn olive), *Hippophae* (sea buckthorn) and *Casuarina* (beef wood). Most actinorhizal plants are capable of high rates of nitrogen fixation comparable to those found in legumes (Torrey, 1976). In Egypt, a nitrogen-fixing potential of 288 kg N ha^{-1} has been reported for *Casuarina* (Diem and Dommergues, 1990). As a consequence, these plants are able to grow in poor and disturbed soils and are important elements in plant communities worldwide. In addition, some actinorhizal species can grow well under a range of environmental stresses such as high salinity, heavy metal, extreme pH (Dawson, 1990). This facility for adaptation has drawn great interest to actinorhizal plants,

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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 basic knowledge of the symbiotic association between *Frankia* and actinorhizal plants is still poorly understood, although it offers striking differences with the *Rhizobium*-legume symbiosis (Pawlowski and Bisseling, 1996; Franche et al., 1998b; Wall, 2000). *Frankia* is a filamentous, branching, Gram-positive actinomycete, whereas Rhizobia are Gram-negative unicellular bacteria. *Frankia* can interact with a diverse group of dicotyledonous plants; whereas, Rhizobia only enter symbiosis with plants from the legume family and with one non-legume: *Parasponia*. In actinorhizal plants, the formation of the nodule primordia takes place in the root pericycle and the nodule consists of multiple lobes, each representing a modified lateral root without a root cap and with infected cells present in the cortex. In indeterminate nodules formed on roots of temperate legumes, the nodule primordium starts in the root inner cortex and determinate nodule primordia are formed in the root outer cortex of tropical and subtropical legumes. Legume root nodules represent stemlike structures with peripheral vascular bundles and infected cells in the central tissue, whereas actinorhizal nodules conserve the structure of a lateral root with a central vascular bundle and peripheral infected cortical tissue (Pawlowski and Bisseling, 1996; Bogusz et al., 1996).

The molecular understanding of regulatory events in actinorhizal nodulation is mainly limited by the microsymbiont *Frankia*; this actinomycete is characterized by slow growth rate, high G+C DNA content and the lack of a genetic transformation system (Benson and Silvester, 1993; Simonet et al., 1990). So far, investigations to detect any DNA sequences homologous to the *nod* genes in the *Frankia* genome have failed (Cérémonie et al., 1998). However, in the past decade, some progress has been made in the knowledge of the plant genes that are expressed at different stages of actinorhizal nodule differentiation. Differential screening of nodule cDNA libraries with root and nodule cDNA has resulted in the isolation of a number of nodule-specific or nodule-enhanced plant genes in several actinorhizal plants including *Alnus, Datisca, Eleagnus* and *Casuarina* (for reviews see Pawlowski and Bisseling, 1996; Franche et al., 1998b; Wall, 2000).

Our group has concentrated on the molecular study of the plant genes involved in the interaction between *Frankia* and *Casuarina glauca*, a tropical tree from the *Casuarinaceae* family (Laplaze et al., 2000a). *Casuarinaceae* are primarily native to the Southern hemisphere, mostly to Australia, where they occur in tropical, subtropical, and temperate coastal regions as well as in arid regions; they include about 90 species of shrubs and trees belonging to four genera, *Allocasuarina, Casuarina, Ceuthostoma* and *Gymnostoma*. All members of *Casuarinaceae* are characterized by highly reduced leaves and photosynthetic deciduous branchlets (National Research Council, 1984). They are pioneer species, able to colonize severely disturbed sites, and are thought to contribute to the rehabilitation in these sites by stabilizing the soil and building up its nitrogen content (Diem and Dommergues, 1990).

The aim of this review is to report on the most recent advances in the molecular biology of the symbiotic interaction between the actinorhizal tree *Casuarina* and the actinomycete *Frankia*.

CYTOLOGICAL STUDY OF THE INFECTION PROCESS

The morphological steps of actinorhizal nodule development have been described in several excellent reviews (Newcomb and Wood, 1987; Berry and Sunnel, 1990; Wall, 2000). Depending upon the host plants, two modes of infection of actinorhizal plants by *Frankia* have been described: intercellular and intracellular. Intracellular infection (e.g. of *Casuarina*) starts with root hair curling induced by an unknown *Frankia* signal (Figure 1). Signal exchange between *Frankia* and the host plant has been investigated by several laboratories (Prin and Rougier, 1987; van Ghelue et al., 1997; Cérémonie et al., 1999). However, the active plant and *Frankia* molecules have not yet been identified. *Frankia* penetrates the curled root hairs and infection proceeds intracellularly in the root cortex. At the same time, limited cells divisions occur in the cortex, leading to the formation of a small external protuberance called the prenodule (Figure 1). Most of prenodule cells are infected with *Frankia*. But, while cortical cells divisions lead to the formation of a nodule primordium in legumes, actinorhizal prenodules do not evolve in nodules. Concomitant with prenodule development, mitotic activity occurs in pericycle cells opposite to a protoxylem pole, giving rise to an actinorhizal lobe primordium (Figure 1). The mature actinorhizal nodule consists of multiple lobes, each of which is a modified lateral root. In each lobe there is a central vascular bundle, and *Frankia* is restricted to the cortical cells. It is interesting to note that prenodule formation also occurs during the infection process of the non-legume *Parasponia* by *Rhizobium* (Lancelle and Torrey, 1984) and that the *Parasponia* nodule is a modified lateral root.

The function of *C. glauca* prenodule has recently been investigated by tracking the differentiation of *Frankia* and plant cells in the prenodule. As markers for differentiation of prenodule cells, the expression of infection-associated genes and symbiotic homologous and heterologous hemoglobin genes were analysed together with infected cell wall lignification. A *Frankia nifH* gene was tracked as a marker for bacterial nitrogen fixation. The results

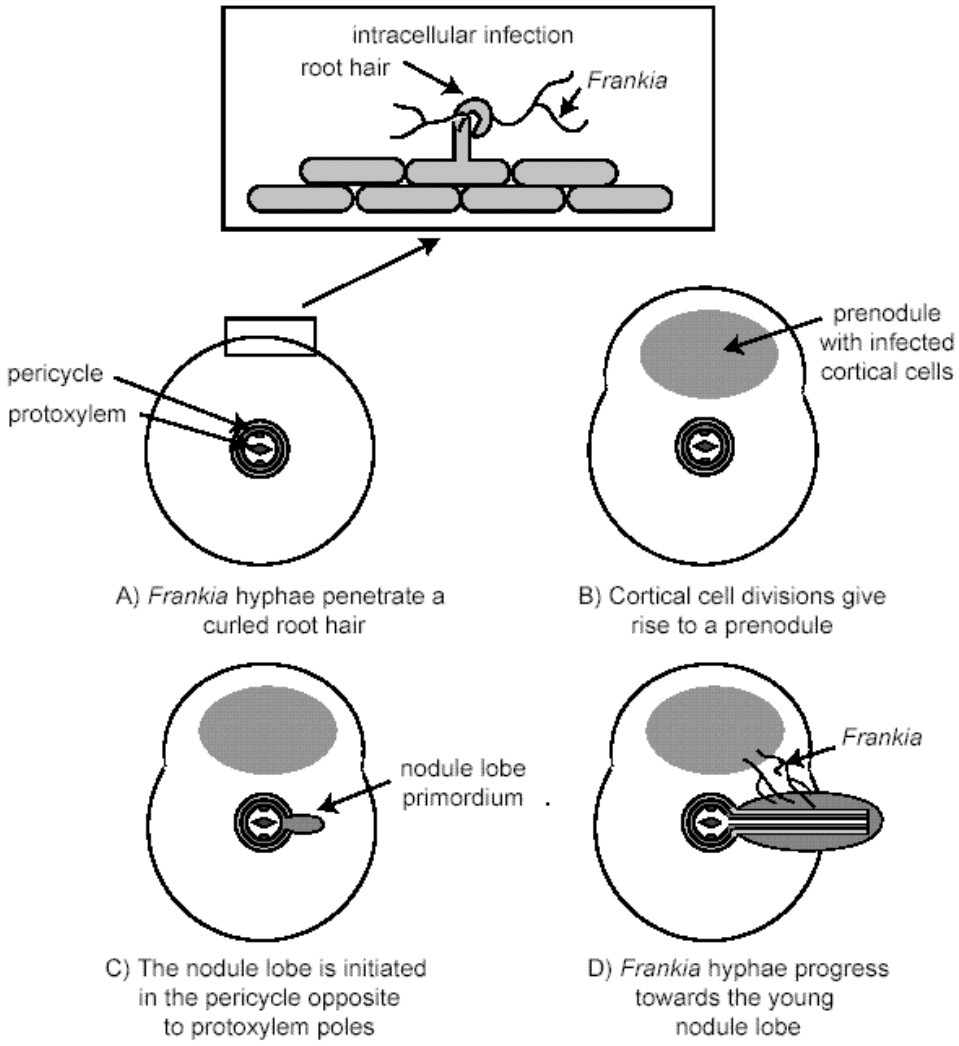

Figure 1. Infection and early organogenesis of a nodule lobe in actinorhizal plants.

strongly suggest the similarity between the differentiation of prenodule cells and that of their nodule counterparts (Laplaze et al., 2000b). For example, infected prenodule cells may fix nitrogen as denoted by expression of the *Frankia nifH* gene. Thus, the prenodule represents a very simple symbiotic organ. Laplaze et al. (2000b) have suggested that prenodule could represent either a nodule precursor or the parallel development of a symbiotic organ.

MOLECULAR RESPONSE TO *FRANKIA* INFECTION

During differentiation of the symbiotic actinorhizal root nodule, a set of genes is activated in the developing nodules (called actinorhizal nodulin genes) (Mullin and Dobritsa, 1996). By homology to legume nodulins, two major types of actinorhizal nodulin genes have been defined by their pattern of expression and function. Early nodulin genes are expressed before the beginning of nitrogen fixation; they are thought to be involved in plant infection or in nodule organogenesis whereas late nodulin genes comprise sequences involved in different metabolic activities necessary for the functioning of the nodule.

Using molecular techniques, actinorhizal nodulin genes were found to be expressed in particular cell types. For example, the earliest symbiotic genes such as *cg12,* were found to be expressed in young infected cells of prenodules and in the nodule infection zone just before onset of nitrogen fixation (Svistoonoff et al., 2003). Cg12 has a strong homology to the subtilisin-like protease families of several plants. Based on the pattern of expression, it has been suggested that Cg12 may play a

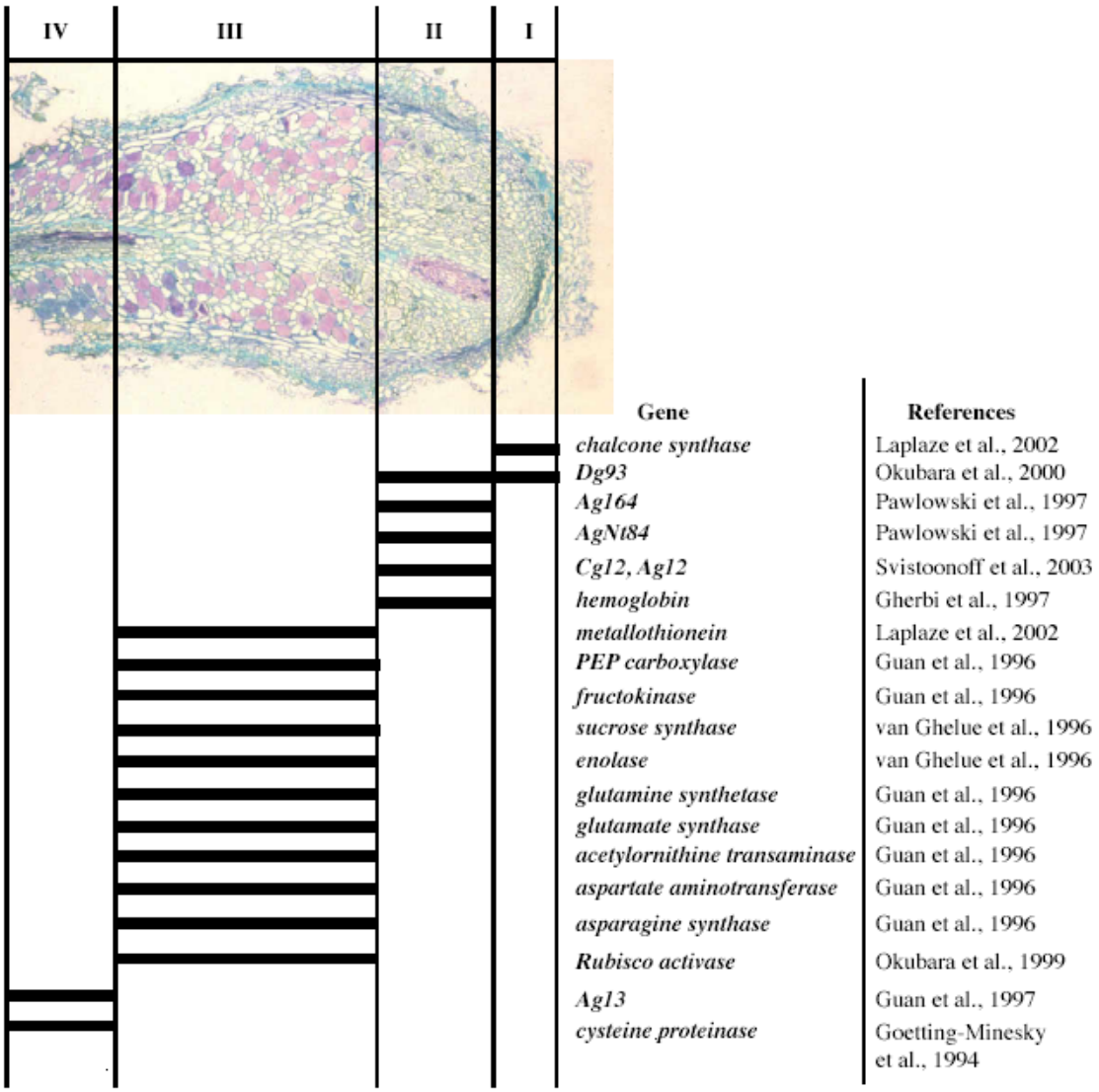

Figure 2. Gene expression map in different zones of actinorhizal root nodules. Nodule zones are indicated: I, meristem zone; II: infection zone; III: nitrogen fixation zone; IV: senescence zone. Black bars indicate the presence of mRNA transcripts. *Dg: Datisca glomerata*; *Ag : Alnus glutinosa* ; *Cg : Casuarina glauca*.

role in the processing of extracellular or the processing of cell wall proteins during early stages of *Frankia* infection (Svistoonoff et al., 2003). Other early actinorhizal genes such as *ag164* and *agNt84* have been characterized in *Alnus glutinosa* and their expression has been observed in young infected cells. The deduced Ag164 and AgNt84 proteins exhibit a putative extracellular localization peptide and an arrangement of glycine and histidine residues that suggests they can bind metal ions (Pawlowski et al., 1997). Ag164 and agNt84 may be involved in transport/storage of metal during the infection process (Figure 2).

Plant hemoglobin is the most studied late nodulins. Hemoglobins genes (*hbs*) were first identified and characterized in nitrogen-fixing nodules of legumes. More recently, hemoglobins have also been found in actinorhizal nodules (Fleming et al., 1987). The role of Hb in symbiosis is to supply oxygen to the bacterial respiration chain while preserving the oxygen-intolerant nitrogenase enzyme complex (Appleby, 1984). A cDNA clone corresponding to *C. glauca* Hb was isolated and the localization of *hb* mRNA in nodules was also studied by *in situ* localization. We found that *hb* transcripts were first detectable in young infected cells adjacent to the apical meristem. The largest amount of *hb* mRNA was present in cells completely filled by the microsymbiont *Frankia* (Gherbi et al., 1997). Several actinorhizal nodulin genes involved in nitrogen and carbon metabolism have

been characterized. For example, the nodule-enhanced sucrose synthase, enolase, as well as *agthi1*, have been shown to be highly expressed in the infected cells of the fixation zone in *Alnus* (Ribeiro et al., 1996; van Ghelue et al., 1996). Genes involved in nitrogen metabolism such as acetylornithine transaminase (AOTA) and a nodule-enhanced glutamine synthetase have also been isolated (Guan et al., 1996).

The involvement of polyphenols in the *C. glauca*-*Frankia* symbiosis has recently been investigated (Laplaze et al., 1999). Histological and histochemical analyses suggest that condensed tannins (flavans) are the major components of the phenolics deposits in the nodule lobe. It has been shown that in *C. glauca* nodules, *Frankia*-infected cells occur in layers surrounded by tannin-containing cell layers located below the periderm, in the endodermis, and in the cortex. The localization of chalcone synthase (*chs*) mRNA by *in situ* hybridization indicated that *chs* is expressed in the flavan-containing cells of the apex of the nodule lobe suggesting that flavonoid synthesis depends on the developmental stage of the cortical cells.

Although our knowledge of molecular mechanisms of actinorhizal symbiosis has increased considerably in the last few years, more work is needed to understand the regulation of actinorhizal nodule development. New tools for genomics that are emerging will certainly exponentially increase the amount of data on genes involved in actinorhizal symbioses.

EVOLUTIONARY ORIGIN OF SYMBIOTIC GENES

Transgenic plants have become a major tool in plant molecular biology and contributed to the rapid progress in basic and applied sciences in disciplines as diverse as developmental biology, physiology, biochemistry and biotechnology. Using *Agrobacterium* as a biological vector for gene transfer, transgenic plants were recovered for both *C. glauca* and *Allocasuarina verticillata* (Diouf et al., 1995; Franche et al., 1997; Smouni et al., 2002) (Figure 3). These transgenic *Casuarinaceae* trees provide valuable tools to investigate the conservation of the mechanisms for nodule-specific expression between legumes and actinorhizal plants. Using the ß-glucuronidase (*uidA* or more commonly named *gus*) reporter gene, chimeric constructs containing promoters from early and late nodulin genes from legumes were introduced in transgenic *Casuarinaceae* and, the regulation of *gus* expression during the ontogenesis of the actinorhizal nodules was investigated.

The *enod12* gene which encodes a hydroxyproline-rich protein is one of the best characterized early nodulin genes from legumes. Two *enod12* genes, *enod12A* and *B*, have been identified in pea (Govers et al., 1991). These two genes are expressed in roots, in response to inoculation with *Rhizobium* or purified Nod factors (Horvath et al., 1993). Expression is found in root hairs of infected pea plants, in root cells containing the infection thread and in cortical cells immediately in front of the infection thread. In the mature pea nodule, expression is confined to the distal part of the infection zone, suggesting that ENOD12 is a cell wall protein involved in the infection process (Bauer et al., 1994). In actinorhizal plants, no homologue of this symbiotic gene has been identified so far. The *gus* gene under the control of the promoter region from the early pea *Psenod12B* nodulin gene (kindly provided by Dr T. Bisseling, Wageningen Agricultural University, The Netherlands) (Vijn et al., 1995) was introduced into *A. verticillata* and *C. glauca*. The expression pattern of the *Psenod12B-gus* was established in transgenic plants regenerated from respectively 13 and 6 transformed calli of *A. verticillata* and *C. glauca* obtained after *A. tumefaciens* gene transfer. In nodulated *Casuarinaceae* plants, no blue staining was observed in roots; in nodules, *Frankia*-infected cells of the nitrogen-fixation zone expressed the reporter gene activity in both *Casuarina* and *Allocasuarina*. A kinetic analysis of the ß-glucuronidase activity in *Frankia*-infected roots established that the *Psenod12B-gus* construct was not expressed during the early stages of the symbiotic process (unpublished data). From these results it can be concluded that, although no homologue of *enod12* has been found in *Casuarinaceae*, *Psenod12* drives a nodule-specific expression in actinorhizal plants. The specificity of expression conferred by this sequence appears to be different in actinorhizal plants and legumes; whereas *Psenod12* directs expression in the infection zone of legume nodules, it is expressed mainly in the nitrogen-fixation zone in actinorhizae indicating that the signals responsible for the early expression are not recognized in this heterologous host plant.

The second construct introduced into *Casuarina* consisted of the *gus* gene driven by the promoter *Gmenod40* from the early nodulin gene *endo40* isolated in soybean (Yang et al., 1993). *enod40* is one of the genes associated with legume nodule development and has a putative role in general plant organogenesis. It is specifically expressed at the early stages of the *Rhizobium*-legume interaction. The results of *in situ* hybridization experiments established that in soybean, *Gmenod40* is induced in the first outer cortical cells that are mitotically activated by *Rhizobium* as well as in the region of the root pericycle facing the nodule primordium. In mature nodules, *enod40* transcripts are detected in the pericycle and vascular bundles, suggesting a role in the late stages of nodule development and/or functioning (Vijn et al., 1995; Roussis et al., 1995). In addition to the expression in symbiotic tissues, *enod40* was also found to be expressed in non symbiotic organs such as stems, lateral and adventitious root primordia and leaf and stipule primordia (Corich et al., 1998). A number of factors including purified Nod factors, cytokinin and

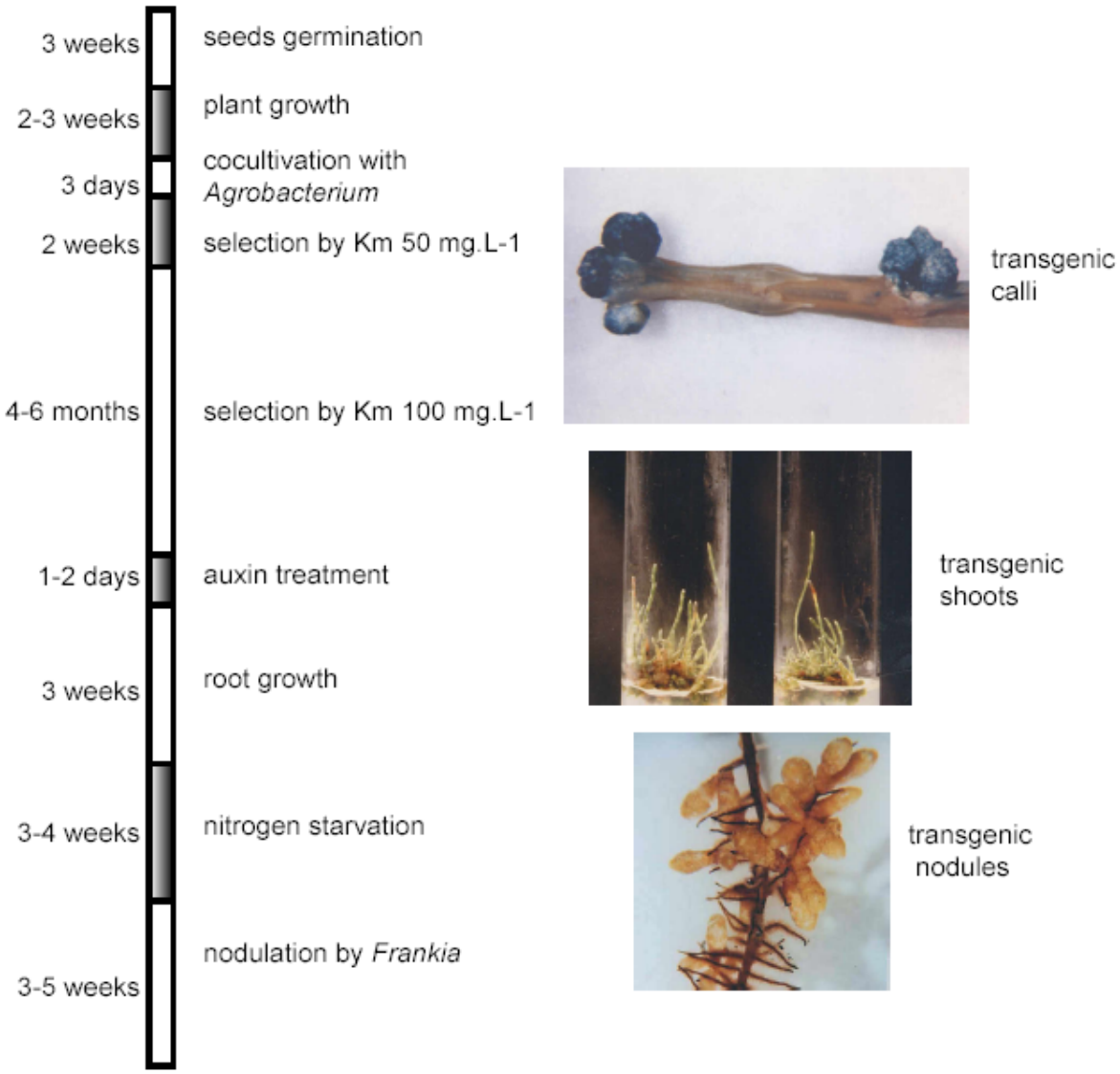

Figure 3. Genetic transformation procedure of *Casuarinaceae* by *Agrobacterium tumefaciens*.

arbuscular mycorrhiza have been shown to induce the transcription of the *enod40* gene in root tissues (Fang and Hirsch, 1998; Mathesius et al., 2000; Staehelin et al., 2001). The *enod40* genes are highly conserved in various leguminous species. They encode transcripts of about 0.7 kb that are characterized by the absence of a long open reading frame; they all contain two conserved regions, named regions I and II (Crespi et al., 1994). A small ORF encoding a peptide of 12 or 13 amino acids has been identified in region I and the translation of an ORF spanning the region II has been demonstrated to be necessary for the biological activity of ENOD40 (Sousa et al., 2001). Recent studies using *in vitro* translation experiment have shown that the soybean *enod40* gene encodes two peptides which bind to soybean nodulin 100 which is a subunit of sucrose synthase, but the exact biochemical function of ENOD40 in nodule organogenesis remains to be determined (Röhrig et al., 2002).

Following genetic transformation with *Agrobacterium*, transgenic plants of *C. glauca* and *A. verticillata* containing the *Gmenod40-gus* construct were obtained. Roots and aerial parts from 16 transgenic lines were stained for GUS activity. In all plants, GUS activity was associated with the vascular system (Santi et al., 2003). In stems, *Gmenod40-gus* was expressed in the procambium/phloem whereas in roots the expression was found not only in the phloem, but also in the xylem parenchyma. *gus* expression was then followed in the course of nodule induction by *Frankia*. Whatever the transgenic lines tested, blue stain was found neither in the prenodule nor in the nodule primordium. Longitudinal

and cross sections of mature actinorhizal nodules showed that blue staining is visible in the pericycle of the nodule vascular bundle, namely at the phloem poles. From these data we can conclude that the signal transduction pathway that leads to the induction of legume *enod40* early in nodule development is not active in actinorhizal plants.

A third set of gene constructs containing the promoters of plant hemoglobin genes were introduced into *A. verticillata* and *C. glauca*. Hemoglobins are widely distributed throughout higher plants and belong to two different families, symbiotic and non symbiotic hemoglobins. Symbiotic hemoglobin is a late nodulin expressed at high concentrations in the nitrogen-fixing nodules of both legumes and non legumes where it facilitates oxygen diffusion to nitrogen-fixing endosymbiotic bacteria (Appleby, 1992). Nonsymbiotic hemoglobins are widespread and have been identified in both symbiotic and non symbiotic plants (Bogusz et al., 1988; Taylor et al., 1994; Trevaskis et al., 1997; Hunt et al., 2001). These non symbiotic proteins are expressed at low level, and their pattern of expression and biochemical properties suggest that they have other functions besides O_2 transport, which are yet to be determined.

Three different hemoglobin sequences were studied in transgenic *Casuarinaceae*: the promoter regions of the hemoglobin genes from soybean *(lbc3)* (Lauridsen et al., 1993)*, Parasponia andersonii* and *Trema* (Bogusz et al., 1988). *lbc3* is a symbiotic gene expressed at high level in soybean nodules (Lauridsen et al., 1993). *P. andersonii*, a nonlegume in the family *Ulmaceae*, lives in symbiotic association with *Rhizobium* (Trinick, 1979); the *Parasponia* hemoglobin sequence is expressed both in the nitrogen-fixing nodules and at low level in the root tissue (Bogusz et al., 1988). *T. tomentosa* is a nonnodulated relative to *P. andersonii* (Akkermans et al., 1978) and the corresponding hemoglobin gene belongs to the non symbiotic family. In transgenic *C. glauca* and *A. verticillata,* the soybean and *P. andersonii* hemoglobin promoters directed expression of the *gus* gene in *Frankia* infected cells; some blue staining was also observed in the root tip of the *Parasponia* construct indicating a recognition of the sequence conferring the non-symbiotic expression. The *T. tomentosa hb* promoter was expressed essentially in the root system (Franche et al., 1998a). Since these different patterns of expression were similar to the endogenous soybean, *P. andersonii* and *T. tomentosa hb* genes, it has been concluded that these promoters retain their cell-specific expression in transgenic *Casuarinaceae*. Conversely, symbiotic *C. glauca* hb promoter retain its nodule specific expression in legume (Jacobsen-Lyon et al., 1995). These findings suggest that, although root nodulation has evolved independently in legumes, *Parasponia* and actinorhizal plants, *hb* genes have maintained regulatory mechanisms through evolutionary convergence. In accordance with results from other groups (Jacobsen-Lyon et al., 1995; Andersson et al., 1997) we showed that *Parasponia* symbiosis seems more related to actinorhizal symbioses than to legume symbioses, although both legumes and *P. andersonii* are nodulated by the same endosymbiont (rhizobia). Altogether, the fact that legume and actinorhizal symbiotic hb gene promoters retain their specific expressions in endophyte infected cells of heterologous nodules suggest that similar transcription factors and DNA regulatory elements are used to regulate these genes.

ACTINORHIZAL NODULIN GENE EXPRESSION IN RICE

Recently rice has become a model for cereals because of the accumulation of molecular information for this species, the efficiency of transformation, its small genome, and the economical importance of this crop which feeds about half of the world's population (Shimamoto, 1998). Research on biological nitrogen fixation and on plant molecular genetics has progressed to the point where it is not unrealistic to design strategies aimed at developing N_2-fixing capacity in cereals. Among the strategies already tested, the introduction of *Rhizobia* into plant roots failed to give significant results, suggesting that the induction of a nodule is necessary to confer the proper environment for nitrogen fixation to occur (Gough et al., 1997). It has also been shown that nodule-like structures called paranodules can be induced in a number of cereals including rice following a 2,4-D treatment (Ridge et al., 1993). More recently, some laboratories have investigated the possibility for non legumes to recognize the LCOs produced by *Rhizobium*. Using transgenic plants containing the *Msenod12A* and *Msenod12B* promoters from the early nodulin gene of *Medicago sativa*, fused to the *gus* reporter gene, Terada et al. (2001) demonstrated that the microballistic application of the Nod factor NodRm-IV (C16:2,S) from *Rhizobium meliloti* changed the ß-glucuronidase activity in transgenic roots exposed to 2,4-D. This result suggests that rice possess receptors that recognize some components of the Nod factors tested.

So far, a sequence from an actinorhizal symbiotic gene has never been expressed in rice. In collaboration with E. Guiderdoni (CIRAD Biotrop, Montpellier, France) we looked for the possibility to express in rice the ß-glucuronidase gene under the control of the promoter of the *cgMT1* metallothionein gene (Laplaze et al., 2002) from *C. glauca*. Transgenic rice plant analyses revealed consistent GUS histochemical staining in root tissues. Staining was mainly observed in root tips, in the elongation zone of the primary and secondary roots and in lateral roots, whereas no GUS activity was detected in the root differentiation zone. Histological investigation of longitudinal and transversal primary, secondary and lateral root sections permitted detection of the presence

of GUS crystals in the endodermis and pericycle cell layers as well as in the vascular system (phloem and xylem cells). As previously observed in transgenic *PcgMT1-gus A. verticillata* plants (Laplaze et al., 2002), the root meristems and the lateral roots exhibited the most intense staining. Histochemical assay of shoot sections of rice plants demonstrated that the immature blade of the innermost rolled leaf did not exhibit detectable staining whereas blade and sheath tissues of leaves of higher rank stained deep blue with a more intense gus signal in the vascular system. The specificity of staining in the vascular system in comparison with other hypodermal parenchyma and sclerified leaf tissues, appears to increase as the leaf matures. In the aerial part of the transgenic *PcgMT1-gus A. verticillata* plants, reporter gene activity was also mostly restricted to the oldest region of the shoots (Laplaze et al., 2002).

These data establish that the promoter from the actinorhizal metallothionein gene *cgMT1* can drive the expression of a reporter gene into *Oryza sativa*. The specificity of expression observed in transgenic rice plants is similar to the one observed in transgenic *cgMT1-gus A. verticillata* trees. The possibility to obtain gus expression in 2,4-D induced paranodules of rice has not been investigated yet.

FUTURE DIRECTIONS

Looking for an actinorhizal model system: In legumes, two species, *Medicago truncatula* and *Lotus japonicus*, have been proposed as model systems to develop the same tools that have fueled breakthroughs in the understanding of *Arabidopsis* plant growth and development. The choice of these species has been based on a number of criteria including their diploid, autogamous nature, short generation times, genome size which is only three to four times that of *Arabidopsis*, and the possibility to genetically transform these species with *A. tumefaciens* (Barker et al., 1990 ; Handberg and Stougaard, 1992; Cook et al., 1997). So far, models have not been identified in actinorhizal plants (Pawloswski, 1999). Nevertheless we will review the characteristics of three species, *D. glomerata*, *A. glutinosa* and *C. glauca* that could make one of these actinorhizal plants to become a model.

Among the actinorhizal plants, *D. glomerata* is the only herbaceous species. The major advantage of *D. glomerata* is its short life cycle (about six months); furthermore, plants are diploid, self pollinating and produce abundant progeny (Wang and Berry, 1996). Compared to *Arabidopsis* which grows vegetatively as a ground rosette of about 2-4 cm and a flowering stem of 20-30 cm, more space is needed to cultivate *Datisca* plants which can extend to a height of 60 cm. So far, no gene transfer has been reported into *Datisca*. Nevertheless, plant regeneration from leaf segments of *D. glomerata* has been published (Wang and Berry, 1996) and a succesful transient expression of a *35S-gus* contruct has been obtained after particle bombardment of *Datisca* leaves (C. Franche, unpublished data). The major drawback with *Datisca* is that so far its microsymbiont *Frankia* has never been cultivated in pure culture.

The genetic studies of *Frankia* have been difficult due a variety of reasons, including low growth rates, multicellular nature, poor germination and lack of genetic markers. Most of the efforts to develop shuttle vectors necessary for the genetic analysis of *Frankia* and for the production of mutants, have been focused on the *Alnus* microsymbionts (for review see Mullin and An, 1990; Benson and Silvester, 1993). To favour the development of specific cloning vectors, several plasmids isolated from *Frankia alni* have been recently sequenced (Lavire et al., 2001; John et al., 2001; Xu et al., 2002). The analysis of the ORFs might open new possibilities for the genetic manipulation of the actinomycete *Frankia alni*. Concerning the valuable characteristics of the host plant, it should be noted that *A. glutinosa* is a diploid tree with a small genome (2C=1.1 pg) (Pawlowski, 1999). *In vitro* micropropagation of *Alnus* has been described in the literature (i.e. Simon et al., 1985; Hendrickson et al., 1995) and the suceptibility of *A. glutinosa* and *A. acuminata* to four strains of *A. rhizogenes* has been established (Savka et al., 1992); but to our knowledge transgenic *Alnus* trees have never been obtained. The major drawback of *Alnus* includes its generation time which is about ten years.

In the *Casuarinaceae* family, transgenic plants have been obtained for two species, *A. verticillata* and *C. glauca,* after gene transfer by either *A. rhizogenes* or disarmed strains of *A. tumefaciens* (for review see Smouni et al., 2000). Nevertheless, it should be noted that the production of transgenic plants is easier with *Allocasuarina* considering the time required for obtaining rooted plants (six months), the large number of transgenic plants produced per transformed calli, and the good rooting ability of the regenerated shoots (Franche et al., 1997). *C. glauca* has so far the smallest genome among actinorhizal plants, with 2C=0.7 pg ; the size of the *A. verticillata* genome is 2C=1.9 pg (Schwencke et al., 1998). However *Casuarinaceae* are small trees, and the production of seeds takes between 2 to 5 years (National Research Council, 1984). Pure cultures of infective *Frankia* strains are available for *Casuarinaceae* (Diem et al., 1982), but no notable effort is being made so far to develop a shuttle vector for the genetic analysis of these strains.

EST sequencing/genomics: For the past few years, several international consortium of researchers have collaborated on projects to provide a full set of genomic tools for the model legumes *Medicago truncatula* and *Lotus japonicus* (Oldroyd and Geurts, 2001; Jiang and

Gresshoff, 1997). Similarly, international effort is necessary for actinorhizal genomics to allow valuable comparison to the model legumes. A relatively rapid way to study the complexity of genes expressed during symbiosis is partial sequencing of cDNAs. Our laboratory has recently started an ESTs project using mRNA isolated from roots and young nodules of *C. glauca*. Several hundreds of ESTs corresponding to novel actinorhizal nodulin genes have already been isolated and sequenced (unpublised data). Comparison between *C. glauca* and legume EST databases will be of great interest to reveal the molecular mechanisms that are common and unique to the two endophytic root nodule symbioses. Furthermore, studies using micro- or macro-arrays should help to get a global understanding of the changes in gene expression induced by the symbiotic interaction.

Conclusions

In the past decade, considerable advances have been made in the identification and characterization of the plant genes involved in the development and functioning of actinorhizal nodules (Franche et al., 1998b; Wall, 2000). The genetic transformation procedures developed in the *Casuarinaceae* family now make it possible to perform functional analysis of the plant symbiotic genes. However, in comparison with the progress achieved in the molecular dissectioning of the communication between *Rhizobium* bacteria and Legumes (Crespi and Galvez, 2000), our understanding of the early steps of the interaction between *Frankia* and the actinorhizal plants lags well behind. Emerging genomics tools may help investigate the early communication between the actinomycete and the host plant in the next years.

Although the different types of nitrogen-fixing root nodules, whether *Rhizobium*- or *Frankia*-induced, share the same function of nitrogen-fixation and ammonia assimilation, there is considerable variation in their development and final morphology (Pawlowski and Bisseling, 1996; Wall, 2000). Actinorhizal and *Parasponia* nodules resemble lateral roots whereas legume nodules have a more stem-like arrangement. Recent phylogenetic data show that all nodule-forming plants belong to a single clade suggesting a single origin of the predisposition for symbiotic nitrogen fixation in the angiosperm (Soltis et al., 1995). Most of the questions with respect to the unique feature of the nodule-forming rosid I clade remain to be answered. One possibility is that all root nodulated plants share common mechanisms for endosymbiosis. The similarity of intracellular infection processes involving *rhizobia* in legumes and *Parasponia* and *Frankia* in nonlegumes is consistent with the conservation of similar mechanisms in *Rhizobium* and *Frankia* infections. A deeper understanding of the different types of nitrogen-fixing nodules will help develop future strategies to modify lateral root development on nonsymbiotic plants to enable some to associate with nitrogen-fixing bacteria.

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