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MOLECULAR BIOLOGY OF ACTINORRHIZAL SYMBIOSES

1. INTRODUCTION

Two nitrogen-fixing root nodule symbioses between soil bacteria and plants have been described, one between *Rhizobium* and legumes and the other between *Frankia* and actinorhizal plants. The *Rhizobium*/legume symbiosis involves more than 1700 plant species of the *Fabaceae* (*Leguminosae*) family while actinorhizal plants comprise about 260 species belonging to 8 angiosperm families. Legume and actinorhizal nodules differ in their ontogeny and structure. However, recent phylogenetic studies based on *rbcL* gene sequence analysis have shown that all plants able to enter a root nodule symbiosis belong to the same clade suggesting that they share a predisposition for symbiosis (Soltis *et al.*, 1995; Doyle, 1998). The molecular bases of this predisposition are unknown. In that respect, comparison of the genetic program of legume and actinorhizal symbioses is of great interest if we are to transfer the ability to fix nitrogen to crop plants such as cereals.

In legumes, the knowledge of the molecular biology of the symbiotic interaction has progressed considerably during the last decade (for review see Schultze and Kondorosi, 1998). Actinorhizal plants are mostly woody plants, trees or shrubs, and are therefore recalcitrant to molecular biology techniques. However, progress in nucleic acids isolation allowed the characterisation of the first actinorhizal nodulin gene in *Alnus glutinosa* (Goettin-Minesky and Mullin, 1994). Since then, several putative symbiotic genes have been isolated from different actinorhizal species (for review see Pawlowski, 1997; Franche *et al.*, 1998b). The study of actinorhizal symbiotic genes did greatly benefit from the recent development of transformation procedure of actinorhizal trees of the *Casuarinaceae* family (Franche *et al.*, 1997; for review see Franche *et al.*, 1998b; Smouni *et al.*, 2002). This technical breakthrough opened new avenues to study genes involved in actinorhizal symbioses. For instance, it paved the way for the study of the regulatory sequence within the promoters of symbiotic genes (Laplaze *et al.*, 2002). Moreover, transgenic plants are useful tools to study gene function by modulating expression level or pattern. The expression conferred by promoters that might be useful for this kind of experiments such as the cauliflower virus 35S promoter has been characterised in transgenic *Casuarinaceae* (Franche *et al.*, 1998a,b; Smouni *et al.*, 2002). With all these tools available, our understanding of the molecular mechanisms of actinorhizal symbioses has and will continue to improve.

In this chapter, we will try to describe the contribution of plant molecular biology approaches to our understanding of actinorhizal symbioses. We will analyse

what we have learnt about the molecular mechanisms of infection, nodule development and functioning. We will then discuss symbiotic gene evolution in the light of recent heterologous gene expression experiments in transgenic plants. Finally, we will examine new and exciting approaches to study the molecular biology of actinorhizae.

2. INFECTION PROCESS

2.1. Interface between *Frankia* and the plant cell

During the infection of actinorhizal plants by *Frankia*, the bacteria comes in close contact with the plant cell. This interface between the two symbiotic partners is an important zone of exchange of both signals and nutrients. Accordingly, this structure derived from the plant cell wall has some very specific properties (see Wall and Berry, this book) and symbiotic genes that might be involved in its formation and/or functioning have been described.

ag12/cg12 are symbiotic genes from *A. glutinosa* and *Casuarina glauca* respectively (Ribeiro *et al.*, 1995 ; Laplaze *et al.*, 2000b). They both code for proteases of the subtilisin family and they show 85% similarity at the amino acid level. Expression studies showed that those genes are specifically expressed during plant cell infection and that expression turns down when plant cells differentiate to fix nitrogen (Ribeiro *et al.*, 1995; Laplaze *et al.*, 2000b). Recently, we introduced *cg12* promoter-reporter gene fusions in *Allocasuarina verticillata*. Interestingly, expression of the reporter gene was observed during the first steps of the infection process, i.e. when *Frankia* is invading deformed root hairs (Laplaze *et al.*, 2000b; Svistoonoff *et al.*, in preparation). Therefore, expression of these genes is correlated with plant cell invasion by the endosymbiont from the very start of the symbiotic process.

The function of these proteins is still unknown. The presence of a putative signal peptide in the sequence of both proteins indicates that the corresponding enzymes are probably secreted in the extracellular compartment. Since the expression of these genes is restricted to infected cells, it is tempting to speculate that the corresponding proteins are released in the matrix surrounding *Frankia*. Immunolocalisation experiments are underway in our laboratory to try to determine the cellular localisation of *cg12* in *C. glauca* infected cells.

Sequence alignments with other subtilases revealed that those genes belong to the pyrolysin subfamily and are closely related to tomato LeSbt3/4 family (Svistoonoff *et al.*, in preparation). Subtilases can be classified in two classes according to their cleavage specificity: processing and degradative subtilases. Degradative subtilases have poor substrate specificity and are involved in the degradation of a wide-range of proteins. Most Bacterial subtilases belong to this class and also do some plant subtilases like the well studied melon fruit cucumisin (Yamagata *et al.*, 1994; for review see Siezen and Leunissen, 1997). If AG12/CG12 belong to this class they could be involved in general protein digestion associated with the cell wall remodelling that occurs in response to *Frankia* infection. Processing subtilases have high levels of substrate specificity and are generally

involved in maturation of inactive proteins or peptide hormones. Mammalian proprotein convertases that cleave their substrates at paired dibasic residues leading to active hormones or neuropeptides and the yeast KEX-2 subtilase involved in the maturation of the mating pheromone are examples of well studied processing subtilases. In plants, several subtilases have been proposed to belong to this class, including P69B, a potential tomato LRR-protein maturation subtilase (Jorda *et al.*, 1999), and SBP50 that could be involved in prosystemin maturation (Schaller and Ryan, 1994). If AG12/CG12 belong to this class of subtilase, it might be involved in the maturation of unknown proteins or propeptides at the interface between the plant and the bacteria. Biochemical characterisation and cellular localisation of CG12 should help us to have a better idea of the function of these subtilases in the infection process.

agNt84/ag164 are two genes that were isolated from *A. glutinosa* (Pawlowski *et al.*, 1997). These genes are strongly expressed in cells invaded by *Frankia* that are not yet fixing nitrogen. They code respectively for a 10.57 kDa and 9.19 kDa glycine and histidine rich proteins. They both have a signal peptide that would target them to the extracellular compartment, presumably to the interface between the two symbiotic partners. Both proteins have an N-terminal glycine that is potential target to myristilation and have several phosphorylation sites. A fragment of AGNt84 produced in *Escherichia coli* was shown to have an ability to bind to nickel suggesting a role of AGNt84 in the binding of metal ions. Among them, cobalt was suggested as a possible candidate since it is the only mineral ion known to be essential for nitrogen fixing symbiosis (Pawlowski *et al.*, 1997).

2.2. Prenodule formation

In actinorhizal plants that are infected intracellularly, *Frankia* infection triggers cell divisions in the cortical cell adjacent to the infection site. These cell divisions give rise to a small protuberance called prenodule. The endosymbiont invade some of the prenodule cells that subsequently enlarge (Callaham and Torrey, 1977, Wall and Berry, chapter X). The prenodule is an obligatory step of intracellular infection but is not directly involved in nodule formation. Recently, prenodule physiology and function was studied using molecular techniques (Laplaze *et al.*, 2000a). It was shown that *Frankia* can fix nitrogen in prenodule infected cells as demonstrated by the formation of vesicles associated with a strong reducing potential (Angulo Carmona, 1974) and expression of the nitrogenase structural gene *nifH* (Laplaze *et al.*, 2000a). Accordingly, those plant cells differentiate to allow nitrogen fixation as shown by the expression of *cghb*, a symbiotic hemoglobin gene, and cell wall lignification (Laplaze *et al.*, 2000a). Moreover, expression of molecular markers and starch accumulation in uninfected prenodule cells suggest that they display the same characteristics as their nodule counterparts (Callaham and Torrey, 1977 ; Laplaze *et al.*, 2000a). Taken together, these results suggest that the prenodule is formed of two cell types, infected and uninfected cells, that undergo the same differentiation towards symbiotic nitrogen fixation than the corresponding nodule cells. The prenodule is therefore a very simple symbiotic organ and it might be an important rest of the evolution of endophytic nitrogen-fixing symbioses in plants (Laplaze *et al.*, 2000a ; Gualtieri and Bisseling, 2000 ; Sprent and Pawlowski, this book).

3. NODULE DEVELOPMENT

3.1. Nodule formation and structure

After prenodule development and infection, cell divisions are induced in the pericycle opposite to a protoxylem pole that will give rise to a nodule lobe primordium. An apical meristem is responsible for primordium growth toward the root surface in regions non infected by *Frankia*. The primordium does not incorporate the prenodule but gets infected by hyphae coming from the prenodule (Duhoux *et al.*, 1996, Wall and Berry, this book).

Mature actinorhizal nodules are indeterminate and multilobed structures. Each nodule lobe presents a central vascular bundle surrounded by an endoderm, an expanded cortex and a periderm. Only some cortical cells are infected by *Frankia*. Two types of actinorhizal nodule can be defined : the *Myrica* type exhibits a so-called nodule root at the apex of each lobe while the *Alnus* type does not (Duhoux *et al.*, 1996). Nodule roots lack root hairs, have a reduced root cap and are not infected. They show a negative geotropism and present an important aerenchyma. It has been shown that they facilitate the diffusion of gaz (oxygen in particular) in and out of the nodule lobe (Callaham and Torrey, 1977; Tjepkema, 1978 ; Schwintzer and Lancelle, 1983).

3.2. Comparison with lateral root development

Because of their origin and of their structure, actinorhizal nodules are often regarded as modified lateral roots. The formation of a nodule root at the apex of some actinorhizal nodules reinforce this view. However, some important differences can be found. First of all, in *Comptonia* some cortical cells close to the nodule lobe primordia divide and are incorporated in the growing young lobe while lateral root primordia originate only in the pericycle (Callaham and Torrey, 1977). Moreover, the distribution of lateral roots is not changed in nodulated plants thus suggesting that the formation of these two types of organs are regulated independently (Angulo Carmona, 1974 ; Valverde, 2000). Finally, differences in gene expression have been found (Pawlowski, 1997 ; Franche *et al.*, 1998b).

One exciting question is to what extent lateral root and actinorhizal nodule development share common steps. To test that, the *HRGPnt3* gene promoter fused to the β -glucuronidase gene was introduced in transgenic *A. verticillata* plants. The *Nicotiana tabacum* gene *HRGPnt3* encodes a plant cell-wall protein expressed at early stages of lateral root development (Keller and Lamb, 1989; Vera *et al.*, 1994). It is a very good molecular marker of lateral root initiation. Unfortunately, *HRGPnt3-gus* was not expressed during lateral root or nodule development in *A. verticillata* suggesting that the specificity of expression is not maintained in a heterologous environment (Our laboratory, unpublished results). The isolation in actinorhizal plants of homologs of known lateral root development genes from model species should help to answer this question.

Another exciting challenge for the future will be to identify those genes which are responsible for the specific developmental features of nodules. One candidate gene is *dg93*, a nodule-specific gene from *Datisca glomerata* (Okubara *et al.*, 2000).

This gene encodes a 105 a.a. protein with 74% similarity to the soybean early nodulin ENOD93. This gene is the only actinorhizal symbiotic gene known to be expressed in the nodule lobe meristem so far. It is also expressed in infected cells and in the vascular cylinder. The function of the protein is unknown but since it is nodule-specific and it is present in the nodule lobe meristem it might be involved in setting up the specific characteristic of nodule lobes compared to lateral roots. Further studies on DG93 function will be needed to clarify this point.

3.3. Role of plant hormones

Even if the molecular mechanisms responsible for actinorhizal nodule development are poorly known, it is likely that nodulation is linked to a local change in hormonal balance. Auxins that are known to play a central role in lateral root initiation (for review see Malamy and Benfey, 1997) are obvious candidates for regulatory molecules. Actinorhizal nodules have been shown to contain large quantities of auxin and cytokinins (Dullaart, 1970; Henson and Wheeler, 1977; Wheeler *et al.*, 1979). Interestingly, some *Frankia* strains can secrete auxins and cytokinins in culture (Stevens and Berry, 1988; Berry *et al.*, 1989). Moreover, Hamad *et al.* (2002) showed that phenyl-acetic acid (PAA) was released by *Frankia* strains *in vitro*. This molecule is known as an auxin mimetic and was able to induce the formation of nodule-like structures on *Alnus glutinosa* roots. These results suggest that *Frankia* might induce nodule formation by secreting an auxin mimetic molecule.

Recently auxin-responsive genes, such as *gh3* from soybean (Li *et al.*, 1999), have been used as markers to visualise auxin accumulation *in situ*. This molecular marker has been successfully used to study the changes in auxin level or sensitivity during legume nodulation (Mathesius *et al.*, 1998). The soybean *gh3* promoter-*gus* fusion was introduced in *A. verticillata* but no expression was detected in transgenic *Casuarinaceae* even after incubation with auxin. Therefore, an homologue of *gh3* was isolated from a *C. glauca* ESTs library (Our laboratory, unpublished). Its promoter contains auxin response elements. Transfer of a *cggh3* promoter-*gus* fusion in *A. verticillata* is underway and will hopefully be an useful molecular marker to study auxin accumulation *in situ* during actinorhizal nodule development.

Actinorhizal nodules like lateral roots and legume nodules are formed opposite to protoxylem poles. In legumes, it has been shown that a regulator emitted by the stele is responsible for nodule positioning (Libbenga *et al.*, 1973). Further studies suggest that ethylene produced in the region of the pericycle opposite to the phloem poles inhibit cell divisions in the neighbouring cortical cells thus controlling the position of nodule initiation (Heidstra *et al.*, 1997; Penmetsa and Cook, 1997). Mechanisms responsible for lateral root and actinorhizal nodule positioning are not known but it is tempting to speculate that gradients of hormones such as ethylene play a role.

3.4. A role for *enod40* in actinorhizal nodulation ?

enod40 is an early nodulin gene first isolated from soybean (Yang *et al.*, 1993). In legumes, *enod40* is a key gene for nodule organogenesis and a limiting factor in nodule development (Charon *et al.* 1999). It also plays a role in mycorrhizal

symbiosis (Stahelin *et al.* 2001). *enod40* is induced by nodulation factors and its expression precedes the first cortical cell divisions (Fang *et al.*, 1998); it is expressed in the vascular system of roots, stems, mature nodules and in the developing nodule primordia (Crespi *et al.*, 1994). Overexpression of *enod40* results in a significant increase of cortical cell division suggesting that *enod40* action may play a role in initiating nodule morphogenesis (Charon *et al.*, 1999). Identification of homologs in non-leguminous plants suggests that this gene may have a more general biological function. Recent work (Röhrig *et al.*, 2002) has revealed that *enod40* encodes two peptides that bind to sucrose synthase. A function of *enod40* in phloem unloading and/or sink strength determination would be consistent with the effects of its over- and underexpression in *Medicago* (Charon *et al.* 1999; Stahelin *et al.*, 2001).

Homologs from *ENOD40* were isolated from two actinorhizal plants, *A. glutinosa* and *C. glauca*, and were named *agenod40* and *cgenod40* respectively. Southern blot showed that *cgenod40* is encoded by a single gene and contains no intron (Santi *et al.*, in preparation). In the legume genes, two highly conserved regions were distinguished: box I in the 5' end, spanning a conserved ORF, and box II in the central part of the gene which corresponds to non-coding RNA. Both actinorhizal genes do not encode for the conserved peptides found in legume due to insertions, deletions and frameshifts in the box I, contrary to box II that is well conserved. Expression of a *cgenod40-gus* fusion was studied in transgenic *A. verticillata* and *C. glauca*. Expression was found in vascular tissue of the roots, shoots and nodules. No expression was found at earlier stages of the infection by *Frankia* and particularly in prenodules and in nodule primordia or in response to nod factors (Santi *et al.* in preparation).

Contrary to legumes, all actinorhizal plants have a lignified root system, and therefore the mechanisms of carbon transport must be different. A comparison of phloem unloading in *C. glauca* and *Medicago truncatula* using fluorescent tracers indicates that in *Casuarina*, unloading is mostly symplastic while it is mostly apoplastic in *M. truncatula* (K. Pawlowski, personal communication). As these results suggest, *enod40* would be involved in increasing apoplastic phloem unloading to induce nodulation in legume, while in actinorhizal plants, like *C. glauca* with mostly symplastic phloem unloading mechanisms, *cgenod40* does not play a role in nodule induction.

4. NODULE FUNCTIONNING

4.1. Actinorhizal nodule compartmentation

Actinorhizal nodule lobes display two levels of compartmentation. First, because of the presence of a meristem at the apex, the different steps of the symbiotic interaction occur longitudinally in a mature nodule lobe. Accordingly, four zones (figure 1) have been defined based on morphological (Angulo Carmona *et al.*, 1974; Duhoux *et al.*, 1996) and gene expression studies (Ribeiro *et al.*, 1995; Gherbi *et al.*, 1997). (1) The **apical meristem** is free of *Frankia*. In the *Myrica* type, after some time, the meristem undergoes a change leading to the formation of the nodule root

while in the *Alnus* type, it stops its activity. Nothing is known on the signal or molecular mechanisms associated with this change of fate. (2) Adjacent to the meristem is an **infection zone** where some of the young cortical cells coming from the meristem activity are infected by *Frankia*. The bacteria start to proliferate while remaining encapsulated in a plant derived matrix and the plant cell enlarges (3) The subsequent **fixation zone** contains both infected and uninfected cortical cells. Infected cells are hypertrophied and are filled with *Frankia* filaments that differentiate vesicles where nitrogen fixation takes place. The appearance and shape of these vesicles are controlled by the plant. In some species (e.g. *Casuarina*), infected cells have a lignified cell wall. Uninfected cells are smaller and in some species contain amyloplast and phenolic compounds and might be involved in nitrogen and carbon metabolism (see below). Finally, a basal **senescence zone** is observed. Plant cell and bacteria degenerate and nitrogen fixation is switched off.

Recently, a second level of compartmentation was described in *C. glauca* nodules (Laplaze *et al.*, 1999). Accumulation of flavans, a class of flavonoids, occurs in uninfected cells in the endodermis, below the periderm and in the cortex. These cells form layers that delimit *Frankia* infected compartments in the nodule lobe (figure 1). Gene expression studies (Laplaze *et al.*, 1999; Smouni *et al.*, 2002) confirm that those cells represent a third specialised cell type in the cortex of *C. glauca* nodules. This accumulation of tannins is observed in early steps of intracellular infection (Angullo Carmona, 1974; Callaham and Torrey, 1977; Laplaze *et al.*, 1999; Duhoux *et al.*, 2001) and intercellular infection of actinorhizal plants (Torrey, 1976; Miller and Baker, 1985) and *Parasponia rigida* (Lancelle and Torrey, 1984) the only non-legume nodulated by Rhizobia. Interestingly, these deposits do not occur in pseudonodules induced by auxin transport inhibitors (Laplaze *et al.*, 1999) or nodules induced by ineffective *Frankia* strains (Guan *et al.*, 1996). Since phenolic compounds have been shown to influence *Frankia* growth *in vitro* (Perradin *et al.*, 1982) and since *Frankia* hyphae never cross these layers of flavan containing cells, they might be involved in restricting bacterial infection to certain parts of the nodule. Alternatively, they might contribute to limit oxygen penetration in the nodule cortex.

4.2. Late actinorhizal nodulin genes

Two cDNAs encoding genes involved in nitrogen metabolism, glutamine synthetase (GS) and acetylornithine transaminase (AOTA), have been characterized in *A. glutinosa* (Guan *et al.*, 1996). GS is responsible for assimilating the ammonia derived from bacterial nitrogen fixation and AOAT is involved in the synthesis of the nitrogen transport form of *Alnus*, citrulin (Miettinen and Virtanen, 1952). These two genes were shown by *in situ* hybridization to be expressed in the infected cells of the fixation zone and in the pericycle of the vascular system (GS only). Thus, it has been suggested that ammonium assimilation and synthesis occur in these cells (Guan *et al.*, 1996).

Besides *A. glutinosa*, a cDNA encoding asparagine synthetase (AS), an enzyme related to ammonium assimilation was isolated in *Eleagnus umbellata* (Kim *et al.*, 1999). Asparagine was reported to be the major compound of *Eleagnus* nodules (Wheeler and Bound, 1970), this is in good accordance with the high expression of

asparagine synthetase in root nodules (Kim *et al.*, 1999). Furthermore, Kim *et al.*, (1999), showed that AS mRNA were confined in fully infected cells of the fixation zone suggesting that unlike in alfalfa nodules, AS expression in *E. umbellata* is under metabolic control.

A good carbon flux to the nodule is essential to provide energy, reductant and acceptor molecules for fixed nitrogen. A sucrose synthase (SuSy) and enolase cDNAs were isolated from *A. glutinosa*, the corresponding genes turned out to be expressed in infected cells and pericycle of the nodules (van Ghelue *et al.*, 1996). Since SuSy mRNA was not detected in starch-containing noninfected cells these authors proposed that apoplastic invertase instead of SuSy was responsible for starch biosynthesis. A similar pattern of expression has been described for *agthil*, which shares homology with yeast *thi4* encoding an enzyme involved in the biosynthesis of the thiamine precursor thiazol (Ribeiro *et al.*, 1996). Thiamine is a co-factor of both glycolysis and the Calvin cycle. The fact that *agthil* is expressed in the same cells that sucrose synthase and enolase is probably correlated with the high energy-demanding processes taking place in infected cells and pericycle (Ribeiro *et al.*, 1996). More recently, a RuBisCo activase (RCA) cDNA was identified from a *Datisca glomerata* cDNA library (Okubara *et al.*, 1999). Whereas RCA transcripts were detected by *in situ* hybridization in nuclei of infected cells, in some uninfected cells and the vascular cylinder of nodules, the corresponding protein did not accumulate at detectable level. Okubara *et al.* (1999) suggested that inefficient splicing of mRNA and translation of the message were responsible for the absence of protein. RuBisCo activase is involved in photosynthetic carbon reduction *via* the action of RubisCo, thus the significance of an mRNA encoding RuBisCo activase in nonphotosynthetic symbiotic root nodules of *D. glomerata* remains unknown (Okubara *et al.*, 1999).

In free living state, at atmospheric pO₂, all *Frankia* strains are able to fix nitrogen because they can form vesicles that limit O₂ diffusion. In cultured *Frankia*, the increased in numbers of lipid laminae was shown to provide an adaptative barrier to the penetration of oxygen (Parsons *et al.*, 1987). In most actinorhizal plants, *Frankia* in symbiosis forms vesicles whereas except in *Casuarina* and *Allocasuarina* nodules. Various actinorhizal nodule structures are also involved in protecting nitrogenase from O₂ (Silvester *et al.*, 1990; review in Huss-Danell, 1997). However, is to be noted that in contrast to legume nodules, a barrier to the diffusion of gases through the inner cortical zone has not been identified in actinorhizal nodule. In *C. glauca* nodules, an oxygen diffusion barrier is achieved by lignification of the cell wall of the infected and adjacent uninfected cortical cells (Berg and McDowell, 1988). Also, in *Casuarina*, a high amount of the O₂-transport protein hemoglobin (hb) has been found in nodules (Fleming *et al.*, 1987), the purified protein was shown to be similar to the legume leghemoglobin suggesting a similar function (Gibson *et al.*, 1989). This high amount of hb is consistent with the absence of *Frankia* vesicles in *Casuarina* nodules. Symbiotic hb genes (Jacobson-Lyon *et al.*, 1995) and a corresponding cDNA (Gherbi *et al.*, 1996) were isolated from *Casuarina*. Localisation of hb mRNA in nodules by *in situ* hybridization showed that the corresponding Hb symbiotic genes are induced in young infected cells prior of the detection of *Frankia nifH* mRNA suggesting that hb contribute to reduce O₂ tension before *nif* genes expression (Gherbi *et al.*, 1997). In *C. glauca* nodule it has

been demonstrated by immunogold localisation that hb is localised in the cytoplasm and nuclei of infected host cells and not associated with *Frankia* membrane. Thus, in *Casuarina* it seems that, just as in the nodules of legumes, O₂ regulation is mediated by host-derived O₂ diffusion barrier and O₂ transport protein. Furthermore, hb was found in nodules of *Myrica gale* (Pathirana and Tjepkema, 1995) and *A. glutinosa* (Suharjo and Tjepkema, 1995) where *Frankia* vesicles are present. This would suggest that even in presence of vesicles symbiotic hb assure the flow of O₂ within infected cells. It would be instructive to see if the location of hb is similar in nodules whether *Frankia* vesicles are present or absent.

Metallothioneins (MTs) are a group of low-molecular weight cystein-rich proteins which are believed, in animals, to play a role in various biological processes such as detoxification of heavy metals, homeostasis of intracellular metal (Kägi, 1991), defense against intracellular oxidants and regulation of metal-containing enzymes (Andrews, 2000). Although the exact function of plant MTs is not understood, the diversity of MT genes responses suggests that plant MTs might be involved in defense reaction to pathogens, apoptosis, growth development and heavy-metal metabolism. A clone for a type 1 metallothionein (*cgMT1*) was isolated from a *C. glauca* nodule cDNA library. *In situ* hybridization indicated that in nodules *cgMT1* transcripts were present in mature *Frankia*-infected cells and in the pericycle. The promoter region of *cgMT1* was isolated and fused to the β -glucuronidase (GUS) gene. Transgenic *Casuarinaceae* showed that the *cgMT1* promoter was most active in large *Frankia*-infected cells of the nitrogen-fixing zone of nodules, in roots and in the oldest region of the shoot (Laplaze *et al.*, 2002). It has been suggested that *cgMT1* might be involved in metal ion transport required for nitrogenase function or might be also part of the antioxidant defenses against reactive oxygen species (ROS) induced during nodulation (Laplaze *et al.*, 2002). Clearly, further studies are needed to identify *cgMT1* function. Analysis of transgenic plants overexpressing *cgMT1* is underway and should help to understand the physiological function of this gene.

As mentioned previously, in old nodule lobes, a senescence zone is observed where both plant cytoplasm and bacteria undergo degradation. The *ag13* and *Ag-NOD-CPI* cDNAs were found to encode a glutamic acid/proline -rich protein and a cystein proteinase, respectively (Pawlowski, 1997; Goetting-Minesky and Mullin, 1994). Both genes were shown to be expressed in *Frankia* infected cells. The deduced proteins have a putative signal peptide suggesting an extracellular function, probably in the compartment surrounding the endophyte. A role as a defense-related protein and in senescence has been proposed for *ag13* and *Ag-Nod-CPI*, respectively.

Apart from *ag13*, two cDNAs encoding putative proteins structurally related to defense proteins have been isolated from *E. umbellata* root nodules cDNA library (Kim and An, 2002). The two clones, *EuNOD-CHT1* and *Eu-CHT2*, encodes chitinase. From their spatiotemporal expression patterns in nonsymbiotic tissues and during nodule differentiation it was proposed that *EuNOD-CHT1* was involved in defense response while *Eu-CHT2* may be involved in normal plant development and in defense response against external pathogens (Kim and An, 2002).

Acyl carrier protein (ACP) is a component of plant fatty acid synthase, located in chloroplasts. A cDNA corresponding to an ACP was isolated from a *C. glauca* nodule cDNA library (Laplaze *et al.*, 1998). The corresponding protein shows all the characteristic features of plant ACP including a putative chloroplast transit peptide cleavage-site motif and a putative phosphopantetheine attachment site (Laplaze *et al.*, 1998). It has been postulated that ACP may participate in fatty acid biosynthesis occurring during plant cell infection (Laplaze *et al.*, 1998).

Two cDNA encoding S-adenosyl-L-methionine synthetase (*EuSAMS1* and *EuSAMS2*) were isolated from the nodule cDNA library of *Eleagnus umbellata* (Lee *et al.*, 2001). SAMS are housekeeping genes encoding S-Adenosyl-L-methionine which is the major methyl group donor in most transmethylation processes. *In situ* hybridization showed that SAMS genes were differentially expressed in the meristem zone (*EuSAMS1* and *EuSAMS2*), the infection zone (*EuSAMS2*) the infected cells of the fixation zone (*EuSAMS1* and *EuSAMS2*) and in the central vascular system (*EuSAMS1* and *EuSAMS2*) of *E. umbellata* nodules (Lee *et al.*, 2001). A role in nitrogen metabolism and in methylation of cell wall constituents has been posulated for *EuSAMS1* and *EuSAMS2*, respectively (Lee *et al.*, 2001).

5. EVOLUTIONARY ORIGIN OF SYMBIOTIC GENES

The genetic transformation procedures developed for *Casuarinaceae* trees (Diouf *et al.*, 1995; Franche *et al.*, 1997; Smouni *et al.*, 2002) provide valuable tools to investigate the conservation of the mechanisms for nodule-specific expression between legumes and actinorhizal plants. Using this approach, the *gus* reporter gene under the control of promoters from early and late nodulin genes from legumes was 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 (hydroxy)proline-rich protein is one of the best characterized early nodulin genes. 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 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 pattern of expression 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 β -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 actinorrhizal plants. The specificity of expression conferred by this sequence appears to be different in actinorrhizal plants and legumes; whereas *Psenod12* directs expression in the infection zone of legume nodules, it is expressed only in the nitrogen-fixation zone in actinorrhizae indicating that the signals responsible for the early expression are not recognized in this heterologous host plant.

Furthermore, 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 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 O₂ 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.*, 1990). *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 actinorrhizal 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 actinorrhizal symbioses than to legume symbioses, although both legumes and *P. andersonii* are nodulated by the same endosymbiont (rhizobia). Altogether, the fact that legume and actinorrhizal 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. This hypothesis is in accordance with the proposal that legume and *Casuarina hb* genes belong to the same class 2 group of *hb* genes (Hunt *et al.*, 2001).

6. FUTURE DIRECTIONS

6.1. 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 *Agrobacterium 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, *Datisca 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 successful transient expression of a 35S-*gus* construct 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 the host plant, *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 susceptibility 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.*, 2001). 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 actinorrhizal 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). Random sequencing of expressed sequences tags (ESTs) from roots and nodules of *C. glauca* is currently in progress in our laboratory (D. Bogusz, unpublished data). 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.

6.2. Use of model organisms

Symbiotic genes are supposed to have been recruited from non-symbiotic genes. Some of them belong to gene families that have similar properties in different organisms. When homologues of the symbiotic genes can be found in model organisms as *Arabidopsis* or even *E. coli* and yeast, general properties of those genes can be studied much more easily in these organisms.

6.2.1. Use of actinorrhizal nodulin genes homologues in *Arabidopsis*

Ribeiro *et al.*, (1995) identified a homologue of the early actinorrhizal nodulin genes *ag12/cg12*, named *ara12*, in *Arabidopsis*. The corresponding *ara12* protein shares 61% similarity at the a.a. level with *ag12*. In order to understand the role of *ara12*, an *ara12* promoter-*gus* fusion was introduced in *Arabidopsis* (Svistoonoff *et al.*, 2002). Promoter activity was detected in young developing tissues, suggesting a role of *ara12* in protein or polypeptide processing during *Arabidopsis* development (Svistoonoff *et al.*, 2002). Other subtilases have been studied in *Arabidopsis*. For instance, SDD1 is involved in stomata distribution (Berger and Altmann, 2000). Like this *Arabidopsis* subtilases, AG12/CG12 could also be involved in the regulation of some developmental processes induced by *Frankia* penetration.

6.2.2. Characterization of CgENOD40 in *A. thaliana*

enod40 genes are involved in nodule organogenesis in legumes and have been isolated from non-nitrogen fixing plants like tobacco or rice (Kouchi *et al.* 1999). Röhrig *et al.*, (2002), revealed a more general function for *ENOD40*, probably in sucrose metabolism. It has already been shown that the soybean *ENOD40-2* promoter is able to drive the expression of a reporter gene in transgenic *A. thaliana* (Mirabella *et al.*, 1999).

As described previously, the *C. glauca cgenod40* promoter directed reporter gene expression in non-symbiotic condition in actinorhizal plants. In order to understand the role played by *CgENOD40* in nonsymbiotic development, its promoter was fused with *gus* and introduced in a non-fixing plant *Arabidopsis*. Studies of these transgenic *A. thaliana* might help in progressing in the understanding of the non-symbiotic role of *CgENOD40* and, in particular, to check easily the induction by some hormones, like cytokinin, auxin, bacterial factors or inhibitors of auxin transport. In parallel, the sense construct for overexpressing *CgENOD40* was also introduced in the model plant to see a putative effect on development.

6.2.3. Use of *Arabidopsis* root development genes

As described before, lateral roots and actinorhizal nodules share similar initiation and structure. It will be very exciting to study to what extent both developmental processes share common molecular mechanisms. This should help understand the evolution of actinorhizal nodule, a very specialised root organ. In recent years, some key genes involved in lateral root development such as *alf4* (Celenza *et al.*, 1995) have been identified using the model plant *A. thaliana*. This growing knowledge can be exploited to compare lateral root and nodule development in actinorhizal plants. Homologues of these important genes can be isolated in actinorhizal plants using cDNA libraries, RT-PCR or ESTs sequences.

6.3. EST sequencing / genomic

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 (unpublished 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.

6.4. Exploration of actinorhizal nodulin genes 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 N₂-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 β -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 β -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.

7. CONCLUSION

Nodule development is largely under the control of plant genome and is just triggered by bacterial factors. Hence, the mechanisms controlling nodule development might be derived from processes common to all plants. Studies of how these common processes have been altered might provide new means to design strategies by which non-legume plants can be given the ability to establish a symbiosis with a nitrogen-fixing bacteria. Since actinorhizal nodule lobes are modified lateral roots, it is important to use all the tools from *Arabidopsis* root development to investigate nodule formation. The large range of developmental mutants and their corresponding genes in *Arabidopsis* now allows to screen for homologues in actinorhizal plants. The availability of both promoter probes to sense auxins levels and transgenic *Casuarinaceae* should allow to uncover the question of auxin requirement in nodule developmental process.

Finally, it is now a priority to develop genomic approaches of actinorhizal symbioses. A strong international cooperation among groups willing to generate actinorhizal programs should help raising funds dedicated to genomics. In this respect, a model actinorhizal plant should rapidly be recognized and adopted by actinorhizal biologists.

REFERENCES

- Akkermans, A.D.L., Abdulkadir, S., & Trinick, M.J. (1978). N₂-fixing root nodules in *Ulmaceae*: *Parasponia* or (and) *Trema* spp? *Plant and Soil*, 49, 711-715.
- Andersson, C.R., Llewellyn, D.J., Peacock, W.J., & Dennis, E. S. (1997). Cell-specific expression of the promoters of two nonlegume hemoglobin genes in transgenic legume, *Lotus corniculatus*. *Plant Physiol.* 113, 45-57.
- Andrews, G. K. (2000). Regulation of metallothionein gene expression by oxydative stress and metal ions. *Biochem. Pharmacol.* 59, 95-104.
- Angulo Carmona, A.F. (1974). La formation des nodules fixateurs d'azote chez *Alnus glutinosa* (L.). *Acta Bot. Neerl.* 23, 257-303.
- Appleby, C.A. 1992. The origin and functions of haemoglobin in plants. *Sci. Prog.* 76, 365-398.
- Barker, D.G., Bianchi, S., London, F., Dattee, Y., Essad, S., Flament, P., Gallusci, P., Genier, G., Guy, P., Muel, X., Tourneur, J., Dénarié, J., & Huguet, T. (1990). *Medicago truncatula*, a model plant for studying the molecular genetics of the *Rhizobium*-legume symbiosis. *Plant Mol. Biol.* 8, 40-49.
- Bauer, P., Crespi, M.D., Szecsi, J., Allison, L.A., Schultze, M., Ratet, P., Kondorosi, E., & Kondorosi, A. (1994). Alfalfa *enod12* genes are differentially regulated during nodule development by Nod factors and *Rhizobium* invasion. *Plant Physiol.* 105, 585-592.
- Benson, D., & Silvester, A.W. (1993). Biology of *Frankia* strains, actinomycete symbionts of actinorhizal plants. *Microbiol. Rev.* 57, 293-319.
- Berg, R.H., & McDowell, L. (1988). Cytochemistry of the wall of infected cells in *Casuarina* actinorhizae. *Can. J. Bot.* 66, 2038-2047.
- Berry, A.M., Kahn, R.K.S., & Booth, M.C. (1989). Identification of indole compounds secreted by *Frankia* HFPAr13 in defined culture medium. *Plant & Soil*, 118, 205-209.

- Bogusz, D., Appleby, C.A., Landsmann, J., Dennis, E., Trinick, M.J., & Peacock, W. J. (1988). Functioning haemoglobin genes in non-nodulating plants. *Nature*, 331, 178-180.
- Bogusz, D., Llewelyn, D.J., Craig, S., Dennis, E.S., Appleby, C.A. & Peacock, W.J. (1990). Non legume hemoglobin genes retain organ specific expression in heterologous transgenic plants. *Plant Cell*, 2, 633-641.
- Callaham, D., & Torrey, J.G. (1977). Prenodule formation and primary nodule development in roots of *Comptonia (Myricaceae)*. *Can. J. Bot.* 51, 2306-2318.
- Celenza, J.L., Grisafi, P.L., & Fink, G.R. (1995). A pathway for lateral root formation in *Arabidopsis thaliana*. *Genes Dev.* 9, 2131-42.
- Charon, C., Sousa, C., Crespi, M., & Kondorosi, A. (1999). Alteration of *enod40* expression modifies *Medicago truncatula* root nodule development induced by *Sinorhizobium meliloti*. *Plant Cell*, 11, 1953-1965.
- Cook, R.C., VandenBosch, K., de Bruijn, F., & Huguet, T. (1997). Model Legumes get the Nod. *Plant Cell*, 9, 275-281.
- Crespi, M., Jurkevitch, E., Poiret, M., d'Aubenton-Carafa, Y., Petrovics, G., Kondorosi, E., & Kondorosi, A. (1994). *enod40*, a gene expressed during nodule organogenesis, codes for a non-translatable RNA involved in plant growth. *EMBO J.* 13, 5099-5112.
- Diem, H.G., Duhoux, E., Zaid, H., & Arahou, M. (2000). Cluster roots in *Casuarinaceae*: role and relationship to soil nutrient factors. *Ann. Bot.* 85, 929-936.
- Diem, H.G., Gauthier, D., & Dommergues, Y. R. (1982). Isolation of *Frankia* from nodules of *Casuarina equisetifolia*. *Can. J. Microbiol.* 28, 526-530.
- Diouf, D., Gherbi, H., Prin, Y., Franche, C., Duhoux, E., & Bogusz, D. (1995). Hairy root nodulation of *Casuarina glauca*: a system for the study of symbiotic gene expression in an actinorhizal tree. *Mol. Plant Microb. Interact.* 8, 532-537.
- Doyle, J.J. (1998). Phylogenetic perspectives on nodulation : evolving views of plants and symbiotic bacteria. *Trends Plant Sci.* 3, 473-478.
- Duhoux, E., Diouf, D., Gherbi, H., Franche, C., Ahée, J., & Bogusz, D. (1996). Le nodule actinorhizien. *Act. Bot. Gall.* 143, 593-608.
- Duhoux, E., Rinaudo, G., Diem, H. G., Auguy, F., Fernandez, D., Bogusz, D., Franche, C., Dommergues, Y., & Huguenin, B. (2001). Angiosperm *Gymnostoma* trees produce root nodules colonized by arbuscular mycorrhizal fungi related to *Glomus*. *New Phytol.* 149, 115-125.
- Dullaart, J. (1970). The auxin content of root nodules and roots of *Alnus glutinosa* (L.) Vill. *J. Exp. Bot.* 21, 975-984.
- Fang, Y. W. & Hirsch, A. M. (1998). Studying early nodulin gene *enod40* expression and induction by nodulation factor and cytokinin in transgenic alfalfa. *Plant Physiol.* 116, 53-68.
- Flemetakis, E., Kavroulakis, N., Quaedvlieg, N.E.M., Spaink, H.P., Dimou, M., Roussis, A., & Katinakis, P. (2000). *Lotus japonicus* contains two distinct *enod40* genes that are expressed in symbiotic, nonsymbiotic, and embryonic tissues. *Mol. Plant Microbe Interact.* 13, 987-994.
- Fleming, A.I., Wittenberg, J.B., Dudman, W.F. & Appleby, C.A. (1987). The purification, characterization and ligand-binding kinetics of hemoglobins from root nodules of the non-leguminous *Casuarina glauca*-*Frankia* symbiosis. *Biochim. Biophys. Acta.* 911, 209-220.
- Franche, C., Diouf, D. Le, Q.V., N'Diaye, A., Gherbi, H., Bogusz, D., Gobé, C. & Duhoux, E. (1997). Genetic transformation of the actinorhizal tree *Allocauarina verticillata* by *Agrobacterium tumefaciens*. *Plant J.* 11, 897-904.
- Franche, C., Diouf, D., Laplaze, L., Auguy, F., Frutz, T., Rio, M., Duhoux, E., & Bogusz, D. (1998a). Soybean (*lbc3*), *Parasponia*, and *Trema* hemoglobin gene promoters retain symbiotic and nonsymbiotic specificity in transgenic *Casuarinaceae*: implications for hemoglobin gene evolution and root nodule symbioses. *Mol. Plant-Microbe Interact.* 11, 887-894.
- Franche, C., Laplaze, L., Duhoux, E. & Bogusz, D. (1998). Actinorhizal symbioses: recent advances in plant molecular and genetic transformation studies. *Crit. Rev. Plant Sci.* 17, 1-28.
- Gherbi, H., Duhoux, E., Franche, C., Pawlowski, K., Nassar, A., Berry, A., & Bogusz, D. (1997). Cloning of a full-length symbiotic hemoglobin cDNA and *in situ* localization of the corresponding mRNA in *Casuarina glauca* root nodule. *Physiol. Plant.* 99, 608-616.
- Gibson, Q.H., Wittenberg, J.B., Wittenberg, B.A., Bogusz, D., & Appleby, C.A. (1989). The kinetics of ligand binding to plant hemoglobins. *J. Biol. Chem.* 264, 100-107.
- Goetting-Minesky, M.P., & Mullin, B. (1994). Differential gene expression in an actinorhizal symbiosis: evidence for a nodule-specific cysteine proteinase. *Proc. Natl. Acad. Sci. U.S.A.* 91, 9891-9895.

- Govers, F., Harmsen, H., Heidstra, R., Micheielsen, P., Prins, M., van Kammen, A., & Bisseling, T. (1991). Characterization of the pea *enod12B* gene and expression analyses of the two *enod12* genes in nodule, stem and flower tissue. *Mol. Gen. Genet.* 228, 160-166.
- Gualtieri, G., & Bisseling, T. (2000). The evolution of nodulation. *Plant Mol. Biol.* 42, 181-194.
- Guan, C., Wolters, D.J., van Dijk, C., Akkermans, A.D.L., van Kammen, A., Bisseling, T., & Pawlowski, K. (1996). Gene expression in ineffective actinorhizal nodules of *Alnus glutinosa*. *Act. Bot. Gall.* 143, 613-620.
- Hamad Y., Nalin R., Marechal J, Fiasson K., Pepin R., Berry A.M., Normand P., Domenach A.-M. (2002). A possible role for phenyl acetic acid (PAA) on *Alnus glutinosa* nodulation by *Frankia*. *Plant & Soil*, in press.
- Handberg, K., & Stougaard, J. (1992). *Lotus japonicus*, an autogamous, diploid legume species for classical and molecular genetics. *Plant J.* 2, 487-496.
- Heidstra, R., Yang, W.C., Yalcin, Y., Peck, S., Emons, A., van Kammen, A., & Bisseling, T. (1997). Ethylene provides positional information on cortical cell division but is not involved in Nod factor-induced root hair tip growth in *Rhizobium*-legume interaction. *Development*, 124, 1781-1787.
- Hendrickson, O.Q., Burgess, D., Perinet, P., Tremblay, F., & Chatatpaul, L. (1995). Effects of *Frankia* on field performance of *Alnus* clones and seedlings. *Plant and Soil*, 150, 295-302.
- Henson, I. E., & Wheeler, C.T. (1977). Hormones in plants bearing nitrogen-fixing root nodules : partial characterization of cytokinins from root nodules from *Alnus glutinosa* (L.) Gaertn. *J. Exp. Bot.* 28, 1076-1086.
- Horvath, B., Heidstra, R., Lados, M., Moerman, M., Spaink, H.P., Prome, J.-C., van Kammen, A., & Bisseling, T. (1993). Lipo-oligosaccharides of *Rhizobium* induce infection-related early nodulin gene expression in pea root hairs. *Plant J.* 4, 727-733.
- Hunt, P.W., Watts, R.A., Trevaskis, B., Llewelyn, D.J., Burnell, J., Dennis, E.S., & Peacock, W.J. (2001). Expression and evolution of functionally distinct haemoglobin genes in plants. *Plant Mol. Biol.* 47, 677-692.
- Huss-Danell, K. (1997). Actinorhizal symbioses and their N₂ fixation. *New Phytol.* 136, 375-405.
- Jacobsen-Lyon, K., Jensen, E.O., Jorgensen, J-E., Marcker, K.A., Peacock W.J., & Dennis, E.S. (1995). Symbiotic and non-symbiotic hemoglobin genes of *Casuarina glauca*. *Plant Cell*, 7, 213-222.
- Jiang, Q., & Gresshoff, P.M. (1997). Classical and molecular genetics of the model legume *Lotus japonicus*. *Mol. Plant-Microbe Interact.* 10, 59-68.
- John, T.R., Rice, J.M., & Johnson, J.D. (2001). Analysis of pFQ12, a 22.4-kb *Frankia* plasmid. *Can. J. Microbiol.* 47, 608-617.
- Jorda, L., Coego, A., Conejero, V., & Vera, P. (1999). A genomic cluster containing four differentially regulated subtilisin-like processing protease genes is in tomato plants. *Proc. Natl. Acad. Sci. U.S.A.* 274, 2360-2365.
- Kägi, J. H. R. (1991). Metallothioneins. *Methods Enzymol.* 205, 613-626.
- Keller, B., & Lamb, C.J. (1989). Specific expression of a novel cell-wall hydroxyproline-rich glycoprotein gene in lateral root initiation. *Genes Dev.* 3, 1639-1646.
- Kim, H. B., & An, C. S. (2002). Differential expression patterns of an acidic chitinase and a basic chitinase in the root nodule of *Eleagnus umbellata*. *Mol. Plant-Microbe Interact.* 15, 209-215.
- Kim, H.B., Lee, S.H., & An, C.S. (1999). Isolation and characterization of a cDNA clone encoding asparagin synthetase from root nodules of *Eleagnus umbellata*. *Plant Sci.* 149, 85-94.
- Kouchi, H., & Hata, S. (1993). Isolation and characterization of novel nodulin cDNAs representing genes expressed at early stages of soybean nodule development. *Mol. Gen. Genet.* 238, 106-119.
- Kouchi, H., Takane, K., So, R.B., Ladha, J.K., & Reddy, P.M. (1999). Rice *ENOD40*: isolation and expression analysis in rice and transgenic soybean root nodules. *Plant J.* 18, 121-129.
- Lancelle, S.A., & Torrey, J.G. (1984). Early development of *Rhizobium*-induced root nodules of *Parasponia rigida* I. Infection and early nodule initiation. *Protoplasma*, 123, 26-37.
- Laplaze, L., Duhoux, E., Franche, C., Frutz, T., Svistoonoff, S., Bisseling, T., Bogusz, D., & Pawlowski K. (2000a). *Casuarina glauca* prenodule cells display the same differentiation as the corresponding nodule cells. *Mol. Plant Microbe Interact.* 13, 107-112.
- Laplaze, L., Gherbi, H., Duhoux, E., Pawlowski, K., Auguy, F., Guermache, F., Franche, C., & Bogusz, D. (2002). Symbiotic and non-symbiotic expression of *cgMTI*, a metallothionein-like gene from the actinorhizal tree *Casuarina glauca*. *Plant Mol. Biol.* 49, 81-92.
- Laplaze, L., Gherbi, H., Franche, C., Duhoux, E., & Bogusz, D. (1998). CDNA sequence for an Acyl Carrier Protein from actinorhizal nodules of *Casuarina glauca* (PGR98-066). *Plant Physiol.* 116: 1605.

- Laplaze, L., Gherbi, H., Frutz, T., Pawlowski, K., Franche, C., Macheix, J.-J., Auguy, F., Bogusz, D., & Duhoux, E. (1999). Flavan-containing cells delimit *Frankia*-infected compartments in *Casuarina glauca* nodules. *Plant Physiol.* 121, 113-122.
- Laplaze, L., Ribeiro, A., Franche, C., Duhoux, E., Auguy, F., Bogusz, D., & Pawlowski, K. (2000b). Characterization of a *Casuarina glauca* nodule-specific subtilisin-like protease gene, a homologue of *Alnus glutinosa ag12*. *Mol. Plant-Microbe Interact.* 13, 113-117.
- Lauridsen, P., Franssen, H., Stougaard, J., Bisseling, T., & Marker, K.A. (1993). Conserved regulation of the soybean early nodulin *ENOD2* gene promoter in determinate and indeterminate transgenic root nodules. *Plant J.* 3, 483-492.
- Lavire, C., Louis, D., Perriere, G., Briolay, J., Normand, P., & Cournoyer, B. (2001). Analysis of pFQ31, a 8551-bp cryptic plasmid from three symbiotic nitrogen-fixing actinomycete *Frankia*. *FEMS Microbiol. Lett.* 197, 111-116.
- Lee, S.H., Kim, H.B., & An, C.S. (2001). Structures and expression patterns of two cDNA clones encoding S-adenosyl-L-methionine synthetase from the root nodule of *Elaeagnus umbellata*. *Aus. J. Plant Physiol.* 28, 951-957.
- Li, Y., Wu, Y.H., Hagen, G., & Guilfoyle, T. (1999). Expression of the auxin-inducible *GH3* promoter/GUS fusion gene as a useful molecular marker for auxin physiology. *Plant Cell Physiol.* 40, 675-682.
- Libbenga, K.R., van Iren, F., Bogers, R.J., & Schraag-Lamers, M.F. (1973). The role of hormones and gradients in the initiation of cortex proliferation and nodule formation in *Pisum sativum* L. *Planta*, 114, 29-39.
- Malamy, J.E., & Benfey, P.N. (1997). Organization and cell differentiation in lateral roots of *Arabidopsis thaliana*. *Development*, 124, 33-44.
- Mathesius, U., Schlaman, H.M., Spaink, H.P., Sautter, C., Rolfe, B.G., Mc Cully, M.E., & Djordjevic, M.A. (1998). Auxin transport inhibition precedes root nodules formation in white clover roots and is regulated by flavonoids and derivatives of chitin oligosaccharides. *Plant J.* 14, 23-34.
- Miettinen, J.K., & Virtanen, A.I. (1952). The free amino acids in the leaves, roots, and root nodules of the alder (*Alnus*). *Physiol. Plant.* 5, 540-557.
- Miller, I.M., & Baker, D.D. (1985). Initiation, development and structure of root nodules in *Elaeagnus angustifolia* L. (*Eleagnaceae*). *Protoplasma*, 128, 107-119.
- Minami, E., Kouchi, H., Cohn, J.R., Ogawa, T., & Stacey, G. (1996). Expression of the early nodulin, *ENOD40*, in soybean roots in response to various lipo-chitin signal molecules. *Plant J.* 10, 23-32.
- Mirabella, R., Martirani, L., Lambertini, A., Iaccarino, M. and Chiurazzi M. (1999). The soybean *ENOD40(2)* promoter is active in *A. thaliana* and is temporally and spatially regulated. *Plant Mol. Biol.* 39, 177-181.
- Mullin, B.C., & An, C.S. (1990). The molecular genetics of *Frankia*. In C.R. Schwintzer & J.D. Tjepkema (Eds.), *The biology of Frankia and actinorhizal plants* (pp. 195-214). Academic Press Inc., San Diego.
- National Research Council. (1984). *Casuarinas : nitrogen-fixing trees for adverse sites*. National Academic Press, Washington DC, USA.
- Okubara, P.A., Fujishige, N.A., Hirsch, A.M., & Berry, A.M. (2000). *Dg93*, a nodule-abundant mRNA of *Datisca glomerata* with homology to a soybean early nodulin gene. *Plant Physiol.* 122, 1073-1079.
- Okubara, P.A., Pawlowski, K., Murphy, T.M., & Berry, A.M. (1999). Symbiotic root nodules of the actinorhizal plant *Datisca glomerata* express rubisco activase mRNA. *Plant Physiol.* 120, 411-420.
- Oldroyd, G.E., & Geurts, R. (2001). *Medicago truncatula*, going where no plant has gone before. *Trends Plant Sci.* 6, 552-4.
- Papadopoulou, K., Roussis, A., & Katinakis, P. (1996) *Phaseolus ENOD40* is involved in symbiotic and non-symbiotic organogenetic processes - expression during nodule and lateral root development. *Plant Mol. Biol.* 30, 403-417.
- Parsons, R., Silvester, W.B., Harris, S., Gruijters, W.T.M., & Bullivant, S. (1987). *Frankia* vesicles provide inducible and absolute oxygen protection for nitrogenase. *Plant Physiol.* 83, 728-731.
- Pathirana, S.M., & Tjepkema, J.D. (1995). Purification of hemoglobin from the actinorhizal root nodules of *Myrica gale* L. *Plant Physiol.* 107, 827-831.
- Pawlowski, K. (1997). Nodule-specific gene expression. *Physiol. Plant.* 99, 617-631.
- Pawlowski, K. (1999). Actinorhizal plants : time for genomics. In J. G. M de Wit, T. Bisseling., & W. J. Stiekema (Eds.), *Biology of plant-microbe interactions*, Vol. 2. (pp. 368-372). International Society for molecular plant-microbe interactions, St. Paul, Minnesota, USA.

- Pawlowski, K., Twigg, P., Dobritsa, S., Guan, C.H., & Mullin, B. (1997). A nodule-specific gene family from *Alnus glutinosa* encodes glycine- and histidine-rich proteins expressed in the early stages of actinorhizal nodule development. *Mol. Plant Microbe Interact.* 10, 656-664.
- Penmetsa, R.V., & Cook, D.R. (1997). A legume ethylene-insensitive mutant hyperinfected by its rhizobial symbiont. *Science*, 275, 527-530.
- Perradin, Y., Mottet, M.J., & Lalonde, M. (1982). Influence of phenolics on *in vitro* growth of *Frankia* strains. *Can. J. Bot.* 61, 2807-2814.
- Ribeiro, A., Akkermans, A.D.L., van Kammen, A., Bisseling, T., & Pawlowski, K. (1995). A nodule-specific gene encoding a subtilisin-like protease is expressed in early stages of actinorhizal nodule development. *Plant Cell*, 7, 785-794.
- Ribeiro, A., Praekelt, U., Akkermans, A.D.L., Meacock, P.A., van Kammen, A., Bisseling, T., & Pawlowski, K. (1996). Identification of *agth11*, whose product is involved in biosynthesis of the thiamine precursor thiazole, in actinorhizal nodules of *Alnus glutinosa*. *Plant J.* 10, 361-368.
- Ridge, R.W., Ridge, K.M., & Rolfe, B.G. (1993). Nodule-like structures induced on the roots of rice seedlings by addition of the synthetic auxin 2,4 dichloro-phenoxy-acetic acid. *Aust. J. Plant Physiol.* 20, 705-717.
- Röhrig, H., Schmidt, J., Miklashevichs, E., Schell, J., & John, M. (2002). Soybean *ENOD40* encodes two peptides that bind to sucrose synthase. *Proc. Natl. Acad. Sci. U.S.A.* 99, 1915-1920.
- Roussis, A., van de Sande, K., Papadopoulou, K., Drenth, J., Bisseling, T., Franssen, H., & Katinakis, P. (1995). Characterization of the soybean gene *GmENOD40-2*. *J. Exp. Bot.* 46, 719-724.
- Savka, M.A., Liu, L., Farrand, S.K., Berg, R.H., & Dawson, J.O. (1992). Induction of hairy roots or *pseudoactinorhizae* on *Alnus glutinosa*, *A. acuminata* and *Eleagnus angustifolia* by *Agrobacterium rhizogenes*. *Acta Oecologica*, 13, 423-431.
- Schaller, A., & Ryan, C.A. (1994). Identification of a 50-kDa systemin binding protein in tomato plasma membranes having Kex2p-like properties. *Proc. Natl. Acad. Sci. U. S. A.* 91, 11802-11806.
- Schultze, M., & Kondorosi, A. (1998). Regulation of symbiotic nodule development. *Annu. Rev. Genet.* 32, 33-57.
- Schwencke, J., Bureau, J. M., Crosnier, M. T., & Brown, S. (1998). Cytometric determination of genome size and base composition of three species of three genera of *Casuarinaceae*. *Plant Cell Rep.* 18, 346-349.
- Schwintzer, C.R., & Lancelle, S.A. (1983). Effect of watertable depth on shoot growth, root growth, and nodulation of *Myrica gale* seedlings. *J. Ecol.* 71, 489-501.
- Shimamoto, K. (1998). *Molecular biology of rice*. Springer, 304 p.
- Siezen, R.J., & Leunissen, J.A.M. (1997). Subtilases: the subtilisin-like serine proteases. *Protein Sci.* 6, 501-523.
- Silvester, W.B., Harris, S.L., & Tjepkema, J.D. (1990). Oxygen regulation and hemoglobin. In Schwintzer and Tjepkema (Eds). *The biology of Frankia and Actinorhizal Plants* (pp. 157-176). Academic Press, Inc.
- Simon, L., Stein, A., Côté, S., & Lalonde, M. (1985). Performance of *in vitro* propagated *Alnus glutinosa* (L.) Gaertn. clones inoculated with *Frankia*. *Plant and Soil*, 87, 125-133.
- Smouni, A., Laplaze, L., Bogusz, D., Guermache, F., Auguy, F., Duhoux, E., & Franche C. (2002). The 35S promoter is not constitutively expressed in the transgenic tropical actinorhizal tree, *Casuarina glauca*. *Funct. Plant Biol.* 29, 649-656.
- Soltis, D.E., Soltis, P.S., Morgan, D.R., Swensen, S.M., Mullin, B.C., Dowd, J.M., & Martin, P.G. (1995). Chloroplast gene sequence data suggest a single origin of the predisposition for symbiotic nitrogen fixation in angiosperms. *Proc. Natl. Acad. Sci. U.S.A.* 92, 2647-2651.
- Stahelin, C., Charon, C., Boller, T., Crespi, M., & Kondorosi, A. (2001). *Medicago truncatula* plants overexpressing the early nodulin gene *enod40* exhibit accelerated mycorrhizal colonization and enhanced formation of arbuscules. *Proc. Natl. Acad. Sci. U.S.A.* 98, 15366-15371.
- Stevens, G.A., & Berry, A.M. (1988). Cytokinin secretion by *Frankia* sp. HFPAr13 in defined medium. *Plant Physiol.* 87, 15-16.
- Suharjo, U.K.J., & Tjepkema, J.D. (1995). Occurrence of hemoglobin in the nitrogen-fixing root nodules of *Alnus glutinosa*. *Physiol. Plant.* 95, 247-252.
- Svistoonoff, S., Laplaze, L., Auguy, F., Santi, C., Fontanillas, E., Duhoux, E., Franche, C., & Bogusz, D. (2002). Expression pattern of *ara12*, an Arabidopsis homologue of the nodule-specific actinorhizal subtilases *cg12/ag12*. *Plant & Soil*, in press.
- Taylor, E.R., Nie, X.Z., MacGregor, A.W., & Hill, R.D. (1994). A cereal haemoglobin gene is expressed in seed and root tissues under anaerobic conditions. *Plant Mol. Biol.* 24, 853-862.

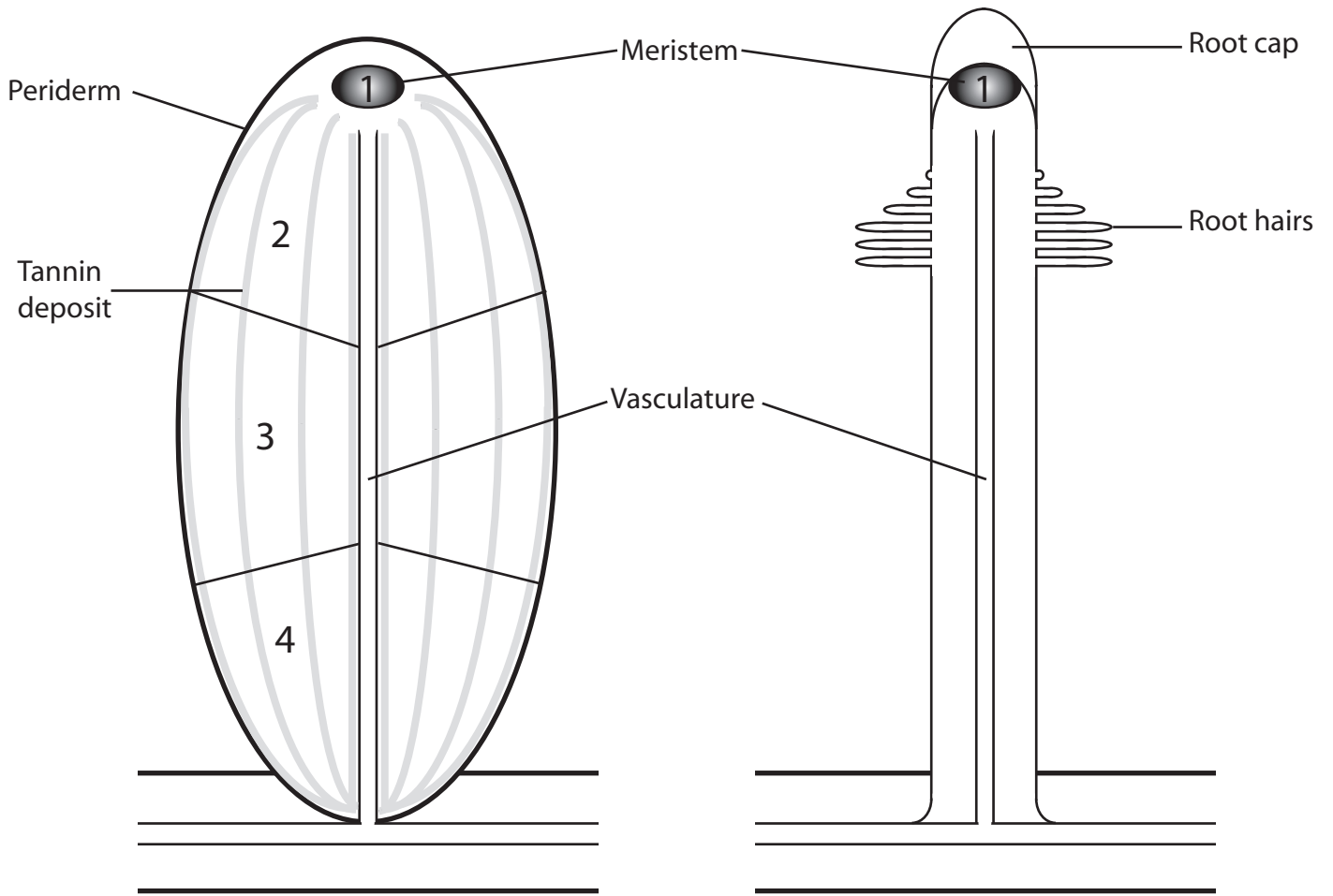
- Terada, R., Ignacimuthu, S., Bauer, P., Kondorosi, E., Schultze, M., Kondorosi, A., Potrykus, I., & Sautter, C. (2001). Expression of early nodulin promoter gene in transgenic rice. *Current Sci.* 81, 270-276.
- Tjepkema, J. (1978). The role of oxygen diffusion from the shoots and nodule roots in nitrogen fixation by root nodules of *Myrica gale*. *Can. J. Bot.* 56, 1365-1371.
- Torrey, J.G. (1976). Initiation and development of root nodules of *Casuarina* (*Casuarinaceae*). *Am. J. Bot.* 63, 335-345.
- Trevaskis, B., Watts, R.A., Andersson, C.R., Llewelyn, D.J., Hargrove, M.S., Olson, J.S., Dennis, E.S., & Peacock, W.J. (1997). Two haemoglobin genes in *Arabidopsis thaliana*: the evolutionary origins of leghemoglobins. *Proc. Natl. Acad. Sci. USA.* 94, 12230-12234.
- Trinick, M.L. (1979). Structure of nitrogen-fixing nodules formed by *Rhizobium* on roots of *Parasponia andersonii*. *Appl. Environ. Microbiol.* 55, 2046-2055.
- Valverde, C. (2000). La symbiosis *Discaria trinervis-Frankia*. Regulacion de la nodulacion radicular. PhD thesis, Facultad de ciencias exactas, Universidad Nacional de La Plata, La Plata, Argentina.
- van Ghelue, M., Ribeiro, A., Solheim, B., Akkermans, A.D.L., Bisseling, T., & Pawlowski, K. (1996). Sucrose synthase and enolase expression in actinorhizal nodules of *Alnus glutinosa*: comparison with legume nodules. *Mol. Gen. Genet.* 250, 437-46.
- Vera, P., Lamb, C., & Doerner, P.W. (1994). Cell-cycle regulation of hydroxyproline-rich glycoprotein HRGPnt3 gene expression during the initiation of lateral root meristem. *Plant J.* 6, 717-727.
- Vijn, I., Yang, W.C., Pallisgard, N., Jensen, E.Ø., van Kammen, A., & Bisseling, T. (1995). *VsENOD5*, *VsENOD12* and *VsENOD40* expression during *Rhizobium*-induced nodule formation on *Vicia sativa* roots. *Plant Mol. Biol.* 28, 1111-1119.
- Wang, H.Y., & Berry, A.M. (1996). Plant regeneration from leaf segments of *Datisca glomerata*. *Acta Bot. Gallica*, 143, 609-612.
- Wheeler, C.T., & Bond, G. (1970). The amino acids of non-legume root nodules. *Phytochemistry*, 9, 705-708.
- Wheeler, C.T., Henson, I.E., & Mc Laughlin, M.E. (1979). Hormones in plants bearing actinomycetes nodules. *Bot. Gaz. (Chicago)*, 140, 52-57.
- Xu, X.D., Kong, R.Q., de Bruijn, F.J., He, S.Y., Murry, M.A., Newman, T., & Wolk, P. (2002). DNA sequence and genetic characterization of plasmid pFQ11 from *Frankia alni* strain Cp11. *FEMS Microbiol. Lett.* 207, 103-107.
- Yamagata, H., Masuzawa, T., Nagaoka, Y., Ohnishi, T., & Iwasaki, T. (1994). Cucumisin, a serine protease from melon fruits, share structural homology with subtilisin and is generated from a large precursor. *J. Biol. Chem.* 269, 32725-32731.
- Yang, W. C., Katinakis, P., Hendriks, P., Smolders, A., de Vries, F., Spee, J., Kammen, A., Bisseling, T. & Franssen, H. (1993). Characterization of *GmENOD40*, a gene showing novel patterns of cell-specific expression during soybean nodule development. *Plant J.* 3, 573-585.

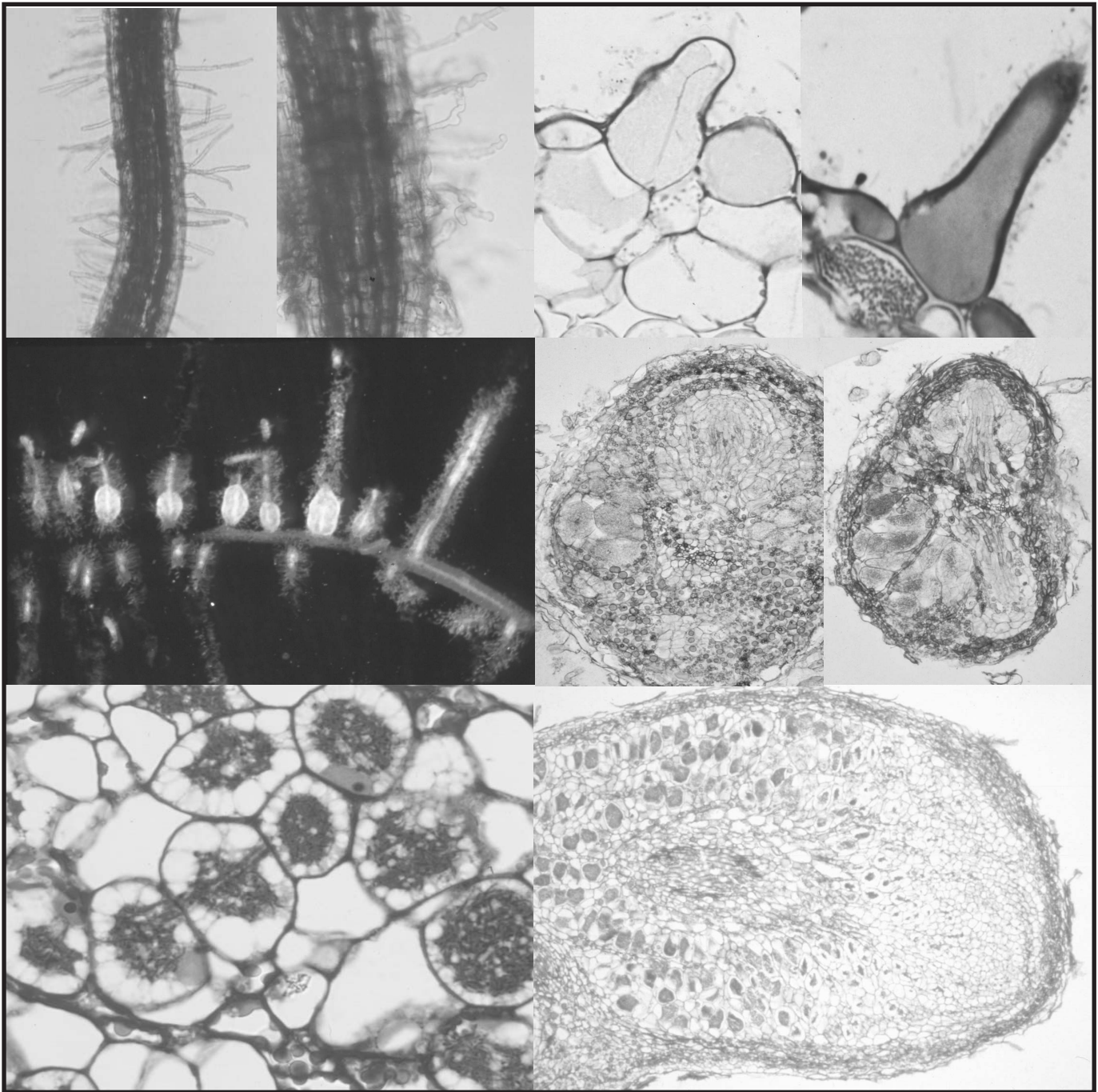
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Table 1 Actinorhizal genes expressed in nodules

Name	Description	Expression profile in nodules
Dg93^a	Similar to a soybean early nodulin gene (<i>GmENOD93</i>)	Lobe meristem, early infection zone, periderm, cells of vascular cylinder
Ag12^b/ Cg12^{c,d}	Similar to plant subtilisin-like proteases	Root hairs post-inoculation, strong in young infected cells of the infection zone; weak in infected cells of the fixation zone
AgNt84^e/ Ag164^e	Glycine- and histidine-rich protein Possibly a metal-binding protein	Young infected cells of the infection zone
Ag11^f	Glutamine synthetase	High level in infected cells of the fixation zone and in pericycle
Ag118^f	Acetylornithine transaminase	High level in infected cells of the fixation zone
EuNOD-AS1^g	Asparagine synthetase	Similar expression as <i>Ag118</i>
AgPgh1^h	Enolase	Infected cells of the N ₂ fixation zone and the pericycle; weak in infected cells of the infection zone
AgSus1^h	Sucrose synthase	Similar expression as <i>AgPgh1</i>
Agthi1ⁱ	Involved in thiazole biosynthesis	Similar expression as <i>AgPgh1</i> and <i>AgSus1</i>
EuSAMS1^j/ EuSAMS2^j	S-adenosyl-L-methionine synthetase	Meristem, infected cells of the N ₂ fixation zone, central vascular system; also <i>EuSAMS2</i> is expressed in the infection zone
EuNOD-CHT1^k/ EuNOD-CHT2^k	Chitinase	EuNOD-CHT1: elevated in meristem, weak in outer cortex layer and uninfected cells of the fixation zone EuNOD-CHT2: elevated in infected cells of the N ₂ fixation zone and central vascular system, weak in the senescence zone
Dgrca^l	Rubisco activase	High level in nuclei of infected cell, weak in uninfected cortical cells adjacent to the periderm and vascular cylinder
CgCHS1^m	Chalcone synthase	Apex of young nodule lobes; also in phenolic containing cells of cortex
hb-Symⁿ hb-Cg1F^o	Hemoglobin	High levels in infected cells of the fixation zone, weak in infected cells of infection zone
CgMT1^p	Metallothionein	High levels in infected cells of the fixation zone and in the pericycle
Ag40^q/ Cg40^r	Similar to legume early nodulin gene <i>ENOD40</i>	High level in the vascular bundles of mature nodule lobes
AgNOD-CPI^s	Cystein protease	Nodule-specific
Ag13^t	Proline- and glutamic acid-rich protein	Infected cells of the senescence zone and in the pericycle

a, Okubura *et al*, 2000 (*Datisca glomerata*); b, Ribeiro *et al*, 1995 (*Alnus glutinosa*); c, Laplaze *et al*, 2000 (*Casuarina glauca*); d, Svistoonoff *et al*, submitted (*C glauca*); e, Pawlowski *et al*, 1997 (*A glutinosa*); f, Guan *et al*, 1996 (*A glutinosa*); g, Kim *et al*, 1999 (*Eleagnus umbellata*); h, van Ghelue *et al*, 1996 (*A glutinosa*); i, Ribeiro *et al*, 1996 (*A glutinosa*); j, Lee *et al*, 2001 (*E umbellata*); k, Kim and An, 2002, (*E umbellata*); l, Okubura *et al*, 1999 (*D glomerata*); m, Laplaze *et al*, 1999 (*C glauca*); n, Jacobson-Lyon *et al* 1995 (*C glauca*); o, Gherbi *et al*, 1996 (*C glauca*); p, Laplaze *et al*, 2002 (*C glauca*); q, Pawlowski *et al*, (*A glutinosa*) in preparation; r, Santi *et al*, (*C glauca*) in preparation; s, Goetting-Mineski and Mullin, 1994 (*A glutinosa*); t, Guan *et al*, 1997 (*A glutinosa*)





Infection process :;

Laplaze Laurent, Svistoonoff Sergio, Santi Carole, Auguy
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