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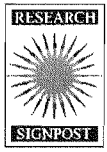


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# 4 Molecular biology and genomics of the nitrogen-fixing tree *Casuarina glauca*

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## Abstract

*Actinorhizal species are non-leguminous perennial plants belonging to 8 angiosperm families. They are able to form 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 Frankia infected cells in the expanded cortex. This chapter reviews the latest knowledge in molecular biology about this original symbiotic process in Casuarina glauca, a tropical actinorhizal tree belonging to the*

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*Casuarinaceae family. Data on several C. glauca genes expressed during the development and functioning of nodules are discussed. We also review current progress in the contribution of genetic transformation of C. glauca and Allocasuarina verticillata (a closely related species of C. glauca) in exploring plant gene function during the different steps of the development of the symbiotic nodule. We describe the genomic approaches developed in our group with the aim of isolating new actinorhizal symbiotic genes.*

# Introduction

Two root nodule symbioses are known between nitrogen fixing soil bacteria and higher plants: Legumes associated with *Rhizobia* (including the non-legume *Parasponia*) and so-called actinorhizal plants that interact with *Frankia*. Inside root nodules, the microsymbionts provide the plant with fixed nitrogen and, in exchange, the bacteria are supplied with carbon by the host plant. Although striking differences are observed between Legume/*Rhizobia* and actinorhizal plants /*Frankia* symbiotic systems [1, 2], it has been suggested that both groups of plants belong to the same clade, and thus share a single origin of the predisposition for root nodule symbiosis [3]. Although the symbiosis between *Rhizobia* and Legumes involves more than 1700 plant species of the *Fabaceae* (Legumes) family, few model legume species have been the subject of extensive and detailed studies that resulted in the discovery of the molecules and transducing signal pathways involved in plant-host recognition [4]. Conversely, the symbiotic association between *Frankia* and actinorhizal plants is still poorly understood at the molecular level [5, 6] because of technical difficulties involved in studying the bacteria. Nevertheless different actinorhizal plant species belonging to different actinorhizal plant clades [7] have been described anatomically, histologically, and physiologically, and provided relevant information [8] that can be analyzed when building a molecular model of how actinorhizal symbiosis evolved its own mechanisms to achieve a functional association.

Actinorhizal plants represent about 200 [8] species distributed among 24 genera and 8 angiosperm families. These plants are distributed worldwide, from cool, high latitudes with strong seasonal influences to warm tropical regions with no pronounced difference between seasons [9]. Actinorhizal plants are perennial dicotyledon angiosperms, and are, with the exception of the genera *Dastica*, woody trees or shrubs. Examples of well known genera include *Alnus* (alder), *Eleagnus* (automn olive), *Hippophae* (sea buckthorn) and *Casuarina* (beef wood). Actinorhizal plants are capable of high rates of nitrogen fixation comparable to those found in Legumes [10]. In Egypt, a nitrogen-fixing potential of 288 kg N $ha^{-1}$ has been reported for *Casuarina* [11]. These plants are able to grow in poor and disturbed soil, they are important pioneer species in plant communities worldwide and play an

essential role in land stabilization and soil reclamation [9]. In addition, some actinorhizal species can grow well under a range of environmental stresses such as high salinity, heavy metal and extreme pH [12]. Recognition of current and potential benefits of actinorhizal plant to forestry and agriculture recently focused research on molecular biological aspects [1, 5, 13].

During the last few years our group has concentrated on understanding the plant molecular mechanisms involved in the symbiosis between *Casuarina glauca* and *Frankia*. Molecular tools including a cDNA nodule library, genetic transformation of *Casuarinaceae* and more recently a root/nodule EST databank have been developed [1, 14]. Such tools allowed us to clone and characterize several *Casuarina* plant genes regulated during the symbiotic process.

Here, we present a brief review of the symbiotic partners - *Casuarina*, the host and *Frankia*, the bacteria - and a short description of the morphological and cytological symbiotic events leading to the development of actinorhizal nodules. We also review the current state of knowledge on the molecular biology and genomics of the symbiotic interaction between the tropical actinorhizal tree *Casuarina* and the actinomycete *Frankia*.

# Two partners: *Casuarina* and *Frankia*

## The *Casuarinaceae* family

The *Casuarinaceae* family includes 90 species of trees and shrubs divided into 4 genera: *Casuarina*, *Allocasuarina*, *Ceuthostoma* and *Gymnostoma* [15]. *Casuarinaceae* are primarily native to the Southern hemisphere, and are found from Australia to South East Asia in tropical, subtropical and temperate coastal regions as well as in arid regions. All members of *Casuarinaceae* are characterized by highly reduced leaves and photosynthetic deciduous branchlets that limit loss of water by evapotranspiration and allow their survival in hot dry areas [15]. They are pioneer species able to colonize severely disturbed sites and contribute to the rehabilitation of these sites by stabilizing the soil and building up its nitrogen content. Some species like *Casuarina glauca* can even grow in a wide variety of soil types, including sandy and saline soils, which has led to the introduction of species belonging to the *Casuarina* and *Allocasuarina* genera in most tropical and sub-tropical areas worldwide and particularly in coastal areas to anchor dunes and to protect crops from wind [16]. Their rapid growth combined with their tolerance of poor fertility and low soil moisture makes them very useful for agroforestry and land reclamation, as well as being valuable sources of fuel wood and charcoal, and generating income for smallholders in tropical countries like India, China and Vietnam [11, 16].

The ability of *Casuarinaceae* to adapt to a range of environmental conditions is due to the exceptional plasticity of their root system, which

enables them to adapt to different environmental stresses. The symbiotic association with the actinomycete *Frankia* allows them to grow in soil deficient in nitrogen. In addition, *Casuarina* roots have a symbiotic relationship with endo- and ectomycorhizal fungi that facilitate the uptake of minerals, notably phosphorus. Furthermore, in phosphorus or iron deficient soil, *Casuarina* roots produce short, densely clustered lateral roots called proteoid (or cluster) roots, which help to absorb phosphorus and other vital minerals insoluble nutrients required for growth and nitrogen fixation [17].

### The actinomycete *Frankia*

The microsymbiont *Frankia* is a filamentous, branching, gram-positive actinomycete and is characterized by a slow growth rate, and high G+C DNA content [18, 19]. The first successful isolation of *Frankia* in culture was reported in 1978 [20]. In pure culture, *Frankia* presents three major structures: vegetative hyphae (multiplication form), vesicles that are the site of nitrogen fixation, and sporangia (dissemination form). Due to the lack of genetic tools [21] most aspects of *Frankia* biology, particularly symbiosis, are still unknown [1]. Several trials of genetic transformation, mutagenesis, and functional complementation failed not provide conclusive results [16]. Only the *Frankia* genes involved in nitrogen metabolism have been isolated, and so far, efforts to detect any *Frankia* genes homologous to the *nod* genes of rizobia have failed [22]. Nevertheless preliminary analyses of *Frankia* genome sequences revealed some disperse putative *nod*-like genes although they do not appear to be organized in clusters as in rhizobia and at least *nodA* is not present [P. Normand, personal communication].

## Morphological and cytological description of the development of actinorhizal nodules

The morphological steps in the development of actinorhizal nodules have been described in details in several reviews [2, 23, 24].

### Infection process

Depending on the host plant, two modes of infection of actinorhizal plants by *Frankia* have been described: intercellular root invasion and intracellular root hair infection [6, 24, 25]. Intracellular infection *via* root hairs (e.g. of *Casuarina, Alnus, Myrica*) 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 but the active plant and *Frankia* molecules have not yet been identified [26, 27, 28]. After invagination of growing filaments of *Frankia* into the curled root hairs, infection proceeds intracellularly in the root cortex. *Frankia* hyphae become encapsulated by a

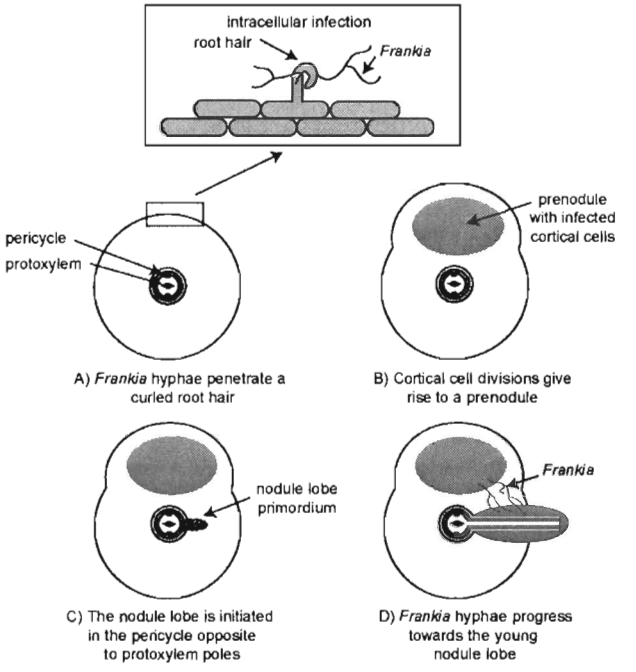


**Figure 1.** Infection and early organogenesis of a nodule lobe in actinorhizal plants.

cell wall deposit that is believed to consist of xylans, cellulose, and pectins of host origin [29, 30]. At the same time, limited cell divisions occur in the cortex near the invading root hair leading to the formation of a small external protuberance called the prenodule [24]. Infection threads consisting of lines of encapsulated *Frankia* hyphae progress intracellularly toward this mitotically active zone and finally invade most cells of the prenodule [31]. *Frankia* filaments inside the infected plant cells are always surrounded by plant plasma membrane.

As the prenodule develops, cell divisions are induced in pericycle cells opposite a protoxylem pole and give rise to the nodule primordium. While cortical cell divisions lead to the formation of a nodule primordium in Legumes, actinorhizal prenodules do not evolve in nodules. The function of the *C. glauca* prenodule is not yet fully understood but a study of the expression of symbiosis-related genes (*cg12*, *cghb*, see above for details) coupled to cellular modification (cell wall lignification) indicated that the prenodule displays the same characteristics as the nodules and can be considered as a very simple symbiotic organ [32]. The prenodule could thus be

a parallel symbiotic organ on its own or the remaining form of a common nodule ancestor for Legumes and actinorhizal plants [32, 33].

Concerning the intercellular root invasion pathway (e.g. *Discaria, Ceanothus, Elaeagnus*, *Hypophae*), *Frankia* hyphae penetrate between two adjacent rhizoderm cells and progress apoplastically through cortical cells within an electron-dense matrix secreted into the intercellular spaces [34, 35, 36, 37]. Unlike the intracellular mode of infection, no prenodule is formed in the root cortex. Once the nodule primordium has developed from the pericycle, intracellular penetration by *Frankia* and the formation of infection threads is initiated acropetally in developing cortical cells of the nodule lobe primordium, following a pattern similar to that described in plant species invaded through root-hairs.

## Nodule development

For both intracellular and intercellular modes of infection, nodule development starts with the induction of mitotic activity in pericycle cells opposite a protoxylem, giving rise to an actinorhizal lobe primordium [10]. An apical meristem is responsible for primordium growth towards the root surface in regions not infected by *Frankia*. The primordium does not incorporate the prenodule but gets infected by hyphae coming from the prenodule [25, 38]. Further development of the primordium gives rise to an indeterminate actinorhizal nodule lobe (Figure 2). New lobes arise continuously to form a coralloid nodule. Mature actinorhizal nodules consists of multiple lobes. In each lobe there

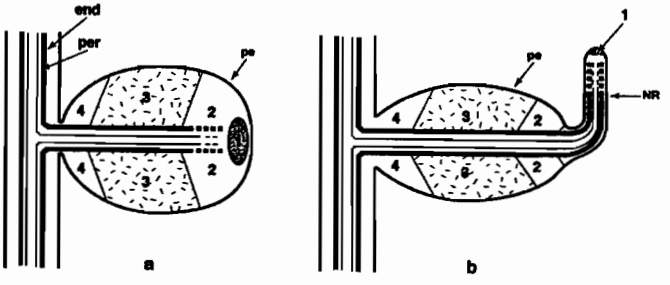


**Figure 2.** Structure of actinorhizal nodule lobe. Nodule consists of discrete or densely packed lobes. Each nodule lobe is a modified lateral root without root cap, including central vascular tissue, cortical parenchyma infected with *Frankia* and a superficial periderm (pe). A zonation of the cortex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

is a central vascular bundle surrounded by an endoderm, an expanded cortex and a periderm. *Frankia* is restricted to the cortical cells. Some species like *Casuarina* or *Myrica* develop a so-called root nodule at the apex of each lobe [25] (Figure 2b). This root nodule lacks root hairs, has a reduced root cap and displays negative geotropism. It might be involved in the diffusion of gas, especially oxygen, in and out of the nodule lobe [39].

## Nodule functioning

Four zones have been morphologically defined in studies of nodules originating from intracellular infection [25, 40] or intercellular root invasion [41] and gene expression [42] (Figure 2). (i) The **apical meristem** is free of *Frankia*. (ii) Adjacent to the meristem is an **infection zone** where some of the young cortical cells resulting from the meristem activity are infected by *Frankia*. The bacterium starts to proliferate but remains encapsulated in a plant-derived matrix, and the plant cells enlarge; (iii) 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 like *Casuarina*, infected cells have a lignified cell wall and there is no vesicle differentiation. Uninfected cells are smaller and in some species contain amyloplast and phenolic compounds, and might be involved in nitrogen and carbon metabolism. Finally a basal **senescence zone** (iv) is observed in old nodules; plant cells and bacteria degenerate and nitrogen fixation is switched off. More recently, a second level of compartmentation was described in *Casuarina glauca* nodules based on the accumulation of flavans, which occurs in uninfected cells in the endodermis and in the cortex. These cells form layers that delimit *Frankia* infected compartments in the nodule lobe and may play a role in restricting bacterial infection to certain zones of the nodule [43].

# Molecular events that occur during *C. glauca-Frankia* symbiosis

During differentiation of the symbiotic actinorhizal root nodule, a set of genes -called actinorhizal nodulin genes- is activated in the developing nodules [44, 45] (Figure 3). Similarly to Legumes, 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.

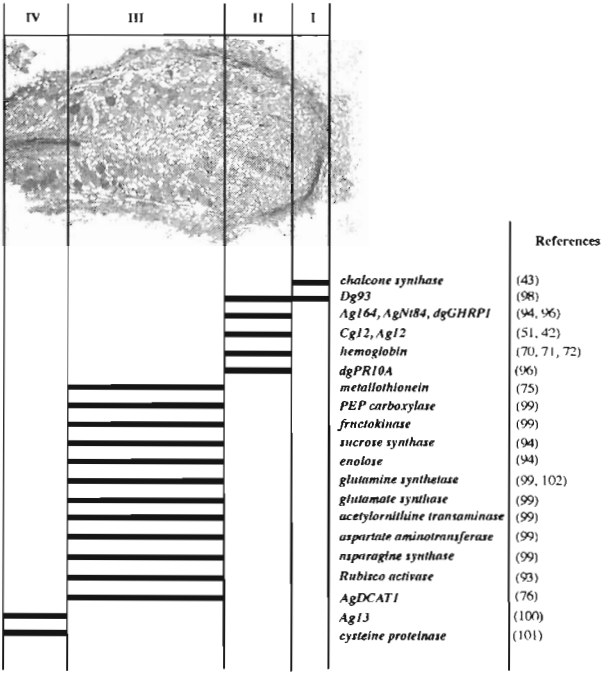


**Figure 3.** Gene expression map in different zones of actinorhizal root nodules. Nodule zones are indicated. I meristem zone, II infection zone, III nitrogen fixation zone, IV senescence zone. Black bars indicate the presence of mRNA transcripts. *Dg: Datisca glomerata, Ag: Alnus glutinosa, Cg: Casuarina glauca.* (modified from Obertello *et al.* (13))

*Casuarina glauca* is a good model for studying symbiotic gene expression as it is the only actinorhizal species with *Allocasuarina* that can be genetically transformed. Using *Agrobacterium* as a biological vector for gene transfer, transgenic plants have been recovered for both *C. glauca* and *Allocasuarina verticillata* [46, 47, 48, 49]. These transgenic *Casuarinaceae* trees provide valuable tools, first, to investigate the molecular mechanisms involved in actinorhizal symbiosis and, second, to establish comparisons with Legumes [13].

## *cg12* an early expressed symbiotic gene in *C. glauca*

*cg12* is an actinorhizal symbiotic gene isolated from *C. glauca* [50] that is homologous to *ag12* previously described for *A. glutinosa* [42]. As described for *ag12*, *cg12* encodes for a subtilisin-like serine protease (subtilases) and is specifically expressed during plant cell infection in young prenodule and

nodule infected cells just before plant cells differentiate to fix nitrogen [50]. The regulation of *cg12* expression and its possible role during actinorhizal nodule infection were investigated with a transgenic approach by introducing *cg12* promoter-reporter fusions in *Allocasuarina verticillata* and in *Casuarina glauca*. Expression of the reporter gene was observed during the first steps of the infection process, i.e. when *Frankia* was invading deformed root hairs and in root and nodule cortical cells containing growing infection threads. *cg12* expression seems to be correlated with plant cell invasion by the endosymbiont from the very start of the symbiotic process [51]. A study of the promoter expression of *ara12*, the *Arabidopsis* homologue of *cg12*, revealed expression in roots and shoots and in developing trichomes and siliques suggesting a role in cell elongation and/or differentiation [52].

Subtilases are a super-family of proteases and are thought to play a role in several different aspects of plant development including epidermal surface formation and stomatal density and distribution in *Arabidopsis* [53, 54], response to pathogens [55], lateral root development [56], and microsporogenesis [57]. Using anti-CG12 polyclonal antibodies our group recently investigated CG12 cytolocalization. The CG12 protein was only detected in nodules in *Frankia*-infected cells. Furthermore, microscopical observations showed that CG12 was associated with the plant cell wall and the polysaccharidic matrix surrounding *Frankia* filaments. Although the implication of the localization of CG12 in this compartment is not yet understood, it has been suggested that CG12 may play a role in the maturation of a polypeptide involved in signalling cascades activated upon *Frankia* infection [58].

## *enod 40*: Involvement in actinorhizal symbiosis

*enod40* is an early nodulin gene first isolated from soybean [59]. In Legumes, *enod40* genes are highly conserved and are key genes for nodule organogenesis and a limiting factor in nodule development [60]. They also play a role in mycorrhizal symbiosis [61]. *enod40* genes encode transcripts of about 0.7 kb that are characterized by the absence of a long open reading frame (ORF); they all contain two conserved regions, named regions I and II [62]. 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 region II has been demonstrated to be necessary for the biological activity of *ENOD40* [63]. In Legumes, *enod40* expression is induced at a very early stage by nodulation factors, and is localized in the vascular system of roots, shoots, mature nodules as well as in nodule primordia [60, 62]. Recent work has revealed that *enod40* encodes two peptides that bind to sucrose synthase which suggests a role in increasing phloem unloading and/or sink strength determination to induce nodulation [60, 64].

A homolog of *enod40* was isolated from *C. glauca* (*cgenod40*) [65]. Sequence comparison with other *ENOD40* from Legumes and non Legumes revealed that in addition to significant similarities, the ORF peptide in region I was lacking in both *C. glauca* and *A. glutinosa,* another actinorhizal tree. RNA gel blot analysis revealed a lower level of *cgenod40* expression in actinorhizal nodules than that observed in Legume nodules. Expression of *cgenod40-gus* fusion was then studied in transgenic *A. verticillata* and *C. glauca* and expression in the vascular tissue of the roots, shoots and nodules was observed. However expression was found neither in the early stages of infection by *Frankia* including prenodules and nodule primordia, nor in response to nod factors [65]. These results are different from the scheme described in Legumes and suggest *enod40* plays a different role in actinorhizal plants. In Legumes, phloem unloading is mostly apoplastic in the root nodulation zone, but mostly symplastic (due to a lignified root system) in actinorhizal species, thus explaining why *enod40* is not involved in nodule induction [65]. However the role of *enod40* in actinorhizal symbiosis is not yet understood.

## Late actinorhizal nodulins

### Hemoglobin

Biological nitrogen fixation is an ancient biochemical process that evolved before photosynthesis and it is absolutely $O_2$ sensitive. At the same time the reduction of $N_2$ to $NH_3$ consumes a lot of energy in the cell, so $O_2$ is useful to generate ATP. Different strategies have evolved in nature to handle this paradox, and examples can be found in the diversity of nitrogen fixing microorganisms. Actinorhizal symbiosis also show different nodule anatomy development as different solutions for this physiological problem [39]. Except in *Casuarina* and *Allocasuarina*, when associated with actinorhizal plants, *Frankia* forms vesicles that limit $O_2$ diffusion to protect nitrogenase. In *C. glauca* nodules, an oxygen diffusion barrier is created by lignification of the cell wall of the infected and adjacent uninfected cortical cells [66]. A large amount of the $O_2$-transport protein hemoglobin (hb) has also been found in *Casuarina* nodules [67]; the purified protein was shown to be similar to the legume leghemoglobin, thus suggesting a similar function [68]. The large amount of hb and the lignified cell walls of infected cells are consistent with the absence of *Frankia* vesicles in *Casuarina* nodules. Symbiotic hb genes [69] and a corresponding cDNA were isolated from *Casuarina glauca*. Localisation of hb mRNA in nodules by *in situ* hybridization showed that the corresponding *Hb* symbiotic genes are induced in young infected cells prior to the detection of *Frankia nifH* mRNA suggesting that hb contributes to reducing $O_2$ tension before *nif* gene expression [70]. In *C. glauca* nodules it has been demonstrated by immunogold localisation that hb is found in the cytoplasm and nuclei of infected host cells and is not associated with *Frankia* membranes. Thus, in

*Casuarina* it seems that, just like in the nodules of Legumes, $O_2$ regulation is mediated by a host-derived $O_2$ diffusion barrier and $O_2$ transport protein. It is interesting to note that hb was found in nodules of *Myrica gale* [71] and *A. glutinosa* [72] where *Frankia* vesicles are present. This suggests that even in the presence of vesicles, symbiotic hb ensures the flow of $O_2$ within infected cells.

**Metallothioneins**

Metallothioneins (MTs) are defined as low molecular weight cysteine-rich proteins that can bind heavy metals and may play a role in their intracellular sequestration and transportation. Although their exact function remains unclear, plant metallothioneins are thought to be involved in response to stresses like wounding, pathogen infection, and leaf senescence [73]. It has recently been argued that they also function as antioxidants and play a role in plasma membrane repair [74].

A clone for type I metallothionein (*cgMT1*) was isolated from a *C. glauca* nodule cDNA library [75]. *In situ* hybridization revealed localisation of the transcripts in mature *Frankia* infected cells and in the pericycle. The *gus* gene under the control of the *cgMT1* promoter was introduced into *Casuarina* and *Allocasuarina*. In transgenic plants the *cgMT1* promoter was shown to be primarily active in large *Frankia* infected cells of the nodule nitrogen-fixing zone, in roots, and in the oldest parts of the shoots. Induction experiments performed on transgenic *Arabidopsis* plants carrying the P*cgMT1-gus* construct revealed that the promoter P*cgMT1* responded to wounding, oxidative stress and pathogen infection. Our current hypotheses is that the metallothionein gene *cgMT1* could be involved in metal ion transport required for nitrogenase function in nodules, in metal homeostasis in roots, and/or in antioxidant defence against reactive oxygen species induced during the symbiotic process [75].

## Other proteins involved in nodule physiology

Several actinorhizal nodulin genes encoding enzymes involved in nitrogen and carbon metabolism have been characterized in different actinorhizal species [For recent reviews see 13, 32, 33]. More recently, the isolation of a nodule-specific dicarboxylase transporter in *A. glutinosa* nodules was reported, which may be involved in carbon metabolism [76]. In *C. glauca*, our group isolated a cDNA encoding for a chalcone synthase (chs), the corresponding mRNA was localized in the flavan-containing cells of the apex of the nodule lobe. Since chalcone synthase is a key enzyme in the flavonoid biosynthesis pathway, our data suggest that flavonoid synthesis depends on the developmental stage of the cells within the nodule lobe [43]. A cDNA corresponding to an Acyl Carrier Protein was also isolated from the *C. glauca*

nodule cDNA library which could be involved in fatty acid biosynthesis occurring during plant cell infection by *Frankia* [77].

# The actinorhizal nodule, a modified lateral root

Legume nodules have a stem-like anatomy with peripheral vascular bundles and infected cells in the central tissue and they originate in the root cortex. In contrast, actinorhizal nodules have the same origin and structure as lateral roots [5]. Thus, we wonder to what extent lateral root and actinorhizal nodule can be compared.

## Comparison between actinorhizal nodule and lateral root development

Actinorhizal nodule and lateral root development have features in common. Both organs originate from divisions in pericycle cells situated in front of a xylem pole. Moreover, both nodule and lateral root vasculatures are central, in contrast with legume nodule vasculature which is peripheral. In addition, some actinorhizal nodules (*e.g. Casuarina glauca* nodules) show a so-called "nodule root" at their apex, highlighting the indeterminate growth characteristic of these nodules. Because of their common developmental origin, their similar structure and the presence of the nodule root, actinorhizal nodules are considered to be modified lateral roots.

Interestingly, the distribution of lateral roots remains unchanged in nodulated *Alnus glutinosa* plants [40] and also in nodulated *Discaria trinevis* [78]. This suggests that the infection by *Frankia* does not "hijack" a young forming lateral root to produce a nodule but induces *de novo* nodule formation. It is very tempting to speculate that part of the lateral root genetic program has been recycled during evolution to create the nodule genetic program. To what extent the lateral root genetic program is used to complete nodule formation remains to be determined. To this end, we are currently studying the expression of genes specifically involved in lateral root development during the course of nodule organogenesis.

One of these genes, *HRGPnt3,* encodes a plant-cell wall protein expressed during early stages of *Nicotiana tabacum* lateral root development [79, 80]. The promoter of this gene was fused to the β-glucuronidase coding sequence and used as a molecular marker for lateral root development in the actinorhizal tree *A. verticillata*. Unfortunately, no GUS activity was detected either in lateral roots or in nodules suggesting that the regulation of this gene is not conserved between the two species. The isolation from actinorhizal plants of homologs of genes known to be involved in lateral root development in model species should help us to further compare nodule and lateral root development.

## Involvement of auxin

Considering the major role of auxin in lateral root formation [81, 82] one would expect this hormone to play a key role in actinorhizal nodule formation. Indeed, it has long been known that nodulated roots contain high levels of auxin compared to non-nodulated roots [83, 84]. Moreover, some *Frankia* strains secrete natural auxins in culture such as phenylacetic acid (PAA) and indolacetic acid (IAA) [85] which are thought to be involved in nodule induction. It has also been suggested that this auxin production by the endosymbiont contributes to the differentiation of the hypertrophied *Frankia* infected cells.

Genes from the AUX1-family are involved in auxin influx transport (entry of auxin into the cell) which is known to be important for lateral root formation [86]. *Arabidopsis* mutants for two of the four members of the gene family, namely *aux1* and *lax3*, have half the number of lateral roots than the wild type. We recently cloned *C. glauca* homologs of *Arabidopsis aux1* and *lax3* and we are in the process of comparing the expression patterns of these genes during lateral root and nodule development. These auxin influx transporters might also play an important role during the early stages of the symbiosis by enabling the perception of bacterial auxins. The use of molecular markers of *in situ* auxin accumulation such as *DR5-gus* [87] and *iaa2-gus* [88] should help to compare the localisation of influx transporters and auxin flux in these organs thus enabling us to better understand the role of this hormone in nodule formation.

# Looking for new early expressed genes: Analysis of *Casuarina glauca* EST banks

The early molecular mechanisms involved in the *Casuarina–Frankia* symbiosis are still poorly understood. Besides the differential hybridization approach, we recently developed a more global non-targeted approach by means of expressed sequence tag (EST) analysis [14].

A total of 3 000 ESTs were obtained from cDNA libraries corresponding to mRNA extracted from (1) young nodules induced by *Frankia* and (2) non infected roots. The raw EST sequences obtained were stored in an in-house database and an automatic treatment pipeline was designed to analyze and annotate them. 70% of the sequences (root and nodule) were considered of high quality and were submitted to a clustering program in order to eliminate redundant ESTs. Each EST or cluster was annotated using the BLAST algorithm by sequence comparison against known proteins of non-redundant database (SWISSPROT, Trembl and PIR) [89]. The e value was fixed at $10^{-5}$. Around 60% of root and 40% of nodule sequences (ESTs and clusters) were annotated and the identified sequences were subsequently assigned to 14

functional categories on the basis of the classification developed for the *Medicago truncatula* EST databank [90, 91]. For both nodulated and non nodulated root ESTs, the largest predominant categories were "protein synthesis" and "primary metabolism". It is worth noting that these categories were also described as being predominant for *M. truncatula* EST [91]. The three largest predominant categories in nodule were: "cell division", "vesicular and cell trafficking", and "defence and cell rescue". This may reflect the development of the nodular structures and the induction of defence genes upon the infection of plant cells by the actinomycete [90, 91, 92]. Not surprisingly, in the nodule EST database, several EST/cluster sequences corresponded to proteins previously described as actinorhizal nodulins. For example, the following ESTs corresponded to identified actinorhizal nodulins genes: hemoglobin [70], metallothioneins [75], subtilisin [51], rubisco activase [93], saccharose synthase [94], glycine and histidine rich proteins [95]. A set of nodule specific sequences was selected and a study of their expression profiles during early symbiotic events is underway. Furthermore, we developed a subtractive hybridization approach using 24 h infected roots *versus* non induced roots to generate nodule sequences of genes that are expressed very early.

# Conclusion

The input of fixed nitrogen by actinorhizal plants on a global scale is enormous; they contribute 15% of symbiotic nitrogen fixation. *Casuarinaceae* species are widely distributed and contribute to maintaining/rehabilitating marginal lands, as well as to providing incomes for smallholders in different tropical and sub tropical countries. Understanding the development and functioning of actinorhizal nodules is thus an important challenge. In the past decade, molecular tools have been developed and considerable advances have been made in the identification and characterization of genes involved in actinorhizal symbiosis. The genetic transformation procedures developed for *Casuarinaceae* made it possible to perform functional analysis of the isolated symbiotic genes. However, our understanding of the early events occurring when the *Casuarina-Frankia* symbiosis takes place is still poor. For example, nodulation signals produced by *Frankia* and plant factors required for the initiation of nodule morphogenesis have not been described to date. Emerging genomic resources such as EST libraries, *Frankia* genome sequences [P. Normand and L. Tisa, personal communication] have profound implications for the study of actinorhizal symbioses and may reveal novel mechanisms of plant-microbe recognition.

Which specific properties permitted actinorhizal plants to form root nodules induced by the nitrogen-fixing actinomycete *Frankia*? Recent phylogenetic studies suggest a single origin for the predisposition to form Legumes/*Rhizobium*

and actinorhizal plants/*Frankia* symbioses [96]. Using the transgenic *Casuarinaceae*/reporter gene approach our group has shown that common mechanisms of transcriptional gene regulation activated during bacterial infection and nodule functioning may be part of the common heritage [97].

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