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Contribution of transgenic *Casuarinaceae* to our knowledge of the actinorhizal symbioses

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Abstract The *Casuarinaceae* family is a group of 96 species of trees and shrubs that are tolerant to adverse soil and climatic conditions. In the field, *Casuarinaceae* bears nitrogen-fixing root nodules (so called actinorhizal nodules) resulting from infection by the soil actinomycete *Frankia.* The association between *Casuarina* and *Frankia* is of tremendous ecological importance in tropical and subtropical areas where these trees contribute to land stabilization and soil reclamation. During differentiation of the actinorhizal nodule, a set of genes called actinorhizal nodulins is activated in the developing nodule. Understanding the molecular basis of actinorhizal nodule ontogenesis requires molecular tools such as genomics together with gene transfer technologies for functional analysis of symbiotic genes. Using the biological vectors *Agrobacterium rhizogenes* and *A. tumefaciens*, gene transfer into the two species *Allocasuarina verticillata* and *Casuarina glauca* has been successful. Transgenic *Casuarinaceae* plants proved to be valuable tools for exploring the molecular mechanisms resulting from the infection process of actinorhizal plants by *Frankia*.

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1 The actinorhizal symbiosis *Casuarina-Frankia*

The *Casuarinaceae* family consists of over 96 species of trees and shrubs primarily native to Australia, New Guinea, the Pacific Islands and parts of South East Asia (Wilson and Johnson 1989). *Casuarinaceae* trees have the capacity to enter different endosymbiotic interactions with soil microbes including the filamentous actinomycete *Frankia* and mycorrhizal fungi. Whereas the symbiotic interaction with *Frankia* leads to the formation of nitrogen-fixing actinorhizal root nodules, the interaction with soil fungi results in the establishment of ecto and endo-mycorrhizal associations that contribute to water and phosphate uptake. All these interactions are characterised by bidirectional nutrient transfer; the plant provides carbon compounds to the symbionts that supply nutrients to the host plant. The main significance of *Casuarinaceae* is that, without requiring nitrogen and phosphate fertilizer, they provide wood and fuel, sometimes forage, contribute to improving soil fertility, are used as shelterbelts, and stabilize desert and coastal dunes in many tropical and subtropical areas (Diem and Dommergues 1990).

The establishment of the actinorhizal symbiosis is a complex process that relies on the accurate recognition of signal molecules generated by the plant and the actinomycete. In the tropical tree species *Casuarina glauca*, nodule development is initiated via root hair infection (Callaham et al. 1979). Exchange of still unknown signals between the host plant and *Frankia* leads to the induction of root hair curling. At the side of folding of root hairs, the root hair plasma membrane invaginates and the *Frankia* hypha is internalized, surrounded by a plant-derived encapsulation layer enriched in polygalacturonans. This interfacial matrix is the equivalent of the infection thread wall in legume nodules (Berg 1999). Upon infection, cell divisions occur in the root cortex near the infected hairs, leading to a small external protuberance called a prenodule, which consists of infected and uninfected cells (Berry and Sunell 1990). Since the formation of the nodule primordium does not involve prenodule cells, it is assumed that it could be a primitive symbiotic organ (Laplaze et al. 2000a). The nodule primordium results from mitotic activity in pericycle cells located opposite a protoxylem pole close to the prenodule (Callaham and Torrey 1977). Mature actinorhizal root nodules resemble short lateral roots modified with hypertrophied cortex harbouring large *Frankia*-infected cells. Due to the presence of the apical meristem, actinorhizal nodule lobes have an indeterminate growth pattern, and their tissues are of graded age. Four different zones are observed in a mature nodule lobe: a persistent apical meristem (zone I), an infection zone (zone II), a nitrogen fixation zone (zone III) and, in old nodules, a senescent zone (zone IV) (Duhoux et al. 1996).

The establishment of the actinorhizal symbiosis involves both specific recognition of symbiotic partners and developmental adaptations of the host plant leading to the nitrogen-fixing nodule. Molecular approaches have been developed to understand changes in gene expression resulting from the interaction with the actinomycete. The use of genomics combined with advanced molecular biology methods recently led to significant progress in knowledge of the molecular mechanisms underlying the interaction between *Frankia* and the most widely-studied actinorhizal species *C. glauca*, *Alnus glutinosa* and *Datisca glomerata* (Hocher et al. 2006; Laplaze et al. 2008; Pawlowski 2002; Pawlowski and Sprent 2008). In 2007, the genome sequencing of three *Frankia* strains was completed (Normand et al. 2007a, b; Rawnsley and Tisa 2007). Efforts to detect genes that are homologous to the *nod* genes of rhizobia in *Frankia* had failed (Cérémonie et al. 1998) until preliminary analysis of the *Frankia* genome revealed some scatered putative *nod*-like genes (Normand et al. 2007a). However, these genes did not appear to be organized in clusters like in rhizobia, and the key *nodA* gene was absent, thus suggesting the signal molecules secreted by *Frankia* are different from the lipochitooligosaccharide signal molecules (LCO), the so-called Nod factors (NFs), produced by rhizobia (Dénarié et al. 1996).

An essential component of plant genomics approaches is a high-throughput transformation system for functional analysis of candidate genes. This review focuses on the methods available to achieve gene transfer into tree species of the *Casuarinaceae* family. The contribution of transgenic Casuarina plants in providing new insights into the molecular basis of actinorhizal symbiosis is also discussed.

2 Progress in genetic transformation of *Casuarinaceae*

2.1 *Allocasuarina verticillata*

In our initial attempts to genetically transform *Casuarinaceae*, we used the biological vector *Agrobacterium rhizogenes* with success. *A. rhizogenes* is a gram-negative soil bacterium capable of transferring a DNA fragment (T-DNA) from its large root-inducing (Ri) plasmid in a large number of dicotyledonous plants. This T-DNA carries a set of genes that encode enzymes which control auxin and cytokinin biosynthesis. The new hormonal balance induces

the formation of proliferating roots, called hairy roots (Veena and Taylor 2007).

In preliminary experiments, three *A. rhizogenes* strains were tested on the *Casuarinaceae* species *Allocasuarina verticillata*: an agropine-type strain, A4 (Petit et al. 1983); a cucumopine-type strain, 2659 (Davioud et al. 1988) and a mannopine-type strain, 8196 (Hansen et al. 1991). All three agrobacteria induced hairy roots on inoculated hypocotyls from one-month-old germinated seedlings (Phelep et al. 1991). Whereas shoot regeneration was only occasionally observed in A4-transformed roots, it was found to occur spontaneously in 90% of the 2659-transformed roots after three months of culture on hormone-free nutrient medium. All shoots rooted after a two-day treatment with indole-3-butyric acid (0.5 µM). However, transgenic plants were characterized by a phenotypic alteration resulting from a modification in the auxin-cytokinin balance. The aerial system exhibited reduced apical dominance with highly branched shoots, and the root system was abundant and exhibited lack of geotropism. After inoculation with *Frankia*, 20% of the transgenic plants developed nitrogen-fixing nodules, whereas nodules appeared on 85% of the untransformed control plants. Due to the morphological alterations resulting from the use of *A. rhizogenes*, we searched for an alternative method based on disarmed *Agrobacterium tumefaciens*.

The first steps in the genetic transformation procedure of *A. verticillata* by *A. tumefaciens* included choosing 1) the plant material; 2) the nutrient medium for adventitious organogenesis and for rooting; 3) the selection stringency and 4) the *A. tumefaciens* strain (Le et al. 1996; Franche et al. 1997). Preliminary experiments were conducted with mature zygotic embryos genetically transformed with the *A. tumefaciens* strain C58C1(pGV2260; pBIN19GUSint) (Vancanneyt et al. 1990). After three days of cocultivation, the plant material was further grown on a selective nutrient medium containing 100 $mg.L^{-1}$ of kanamycin. To prevent untransformed zones in the initial callus, selection pressure was maintained until rooting. After four months, shoots expressing the *GUS* reporter gene encoding the ß-glucuronidase (Jefferson et al. 1987) were obtained on about 70% of the calli growing in the presence of antibiotics and 96% of these shoots were successfully rooted after a two-day-treatment with 1-naphtalene acetic acid (25 µM) (Franche et al. 1997).

Although transgenic plants were successfully regenerated, the major drawback of this method was that generally only 25% of the zygotic embryos were viable; moreover, due to the small size of the seeds (about 1–2 mm), removing the seed coat to wound the embryos with a scalpel was rather tedious. We consequently checked if it was possible to obtain transgenic organogenic calli from hypotoctyls, cotyledons and hypocotyls. Epicotyl fragments collected from 45-day-old plantlets appeared to be the easiest explants to handle for genetic transformation with *A. tumefaciens*. Using this method, we obtained numerous transgenic shoots from transformed *A. verticillata* calli within 4–6 months. For promoter characterisation, in our laboratory, 25 independent transgenic callus lines are usually analysed for each construct. Among these lines, at least five usually express the transferred reporter gene at a high level and continuously produce numerous shoots. Molecular analysis performed on a large number of independent transgenic *35S-GUS* calli established that the T-DNA was fully transferred from the binary vector pBIN19-GUSint in 89% of the kanamycin resistant calli; in 8% of the calli, a deletion was observed in the GUS reporter gene, suggesting a partial deletion near the left T-DNA border, and 3% of the calli that did not contain any transgene appeared to have escaped antibiotic selection. Southern blot analysis further established that 60% of the transgenic shoot lines had only one copy of the T-DNA. In the remaining 40%, two to three copies of the T-DNA were observed (Franche et al. 1997).

2.2 *Casuarina glauca*

In order to establish a fast system for producing transgenic actinorhizal roots and nodules of *C. glauca,* the *A. rhizogenes* A4RS was used to obtain composite plants comprising a transformed root system and a non transgenic shoot (Diouf et al. 1995). Hypocotyls of 3-week-old *C. glauca* seedlings were wounded in the area close to the cotyledons with a needle dipped into a fresh colony of A4RS. After six days of cocultivation, the non-transformed root system was removed and the resulting composite plants were further grown in presence of 300 $mg.L^{-1}$ cefotaxim to eliminate excess agrobacteria. Within three weeks, rapidly growing roots were observed on 75–90% of the inoculated plants. When binary vectors containing the *GUS* (Jefferson et al. 1987) or *GFP* (Niwa et al. 1999) reporter genes driven by the constitutive promoter *35S* were electroporated into A4RS, the frequency of hairy root cotransformation ranged between 30% and 45%. As previously observed with *A. verticillata*, the main drawback resulting from the use of A4RS was the decrease in nodulation efficiency linked to the pronounced hairy-root phenotype, with only about 30% of nodules formed on the transformed root system following the inoculation with *Frankia*. Alternative strains of *A. rhizogenes* such as Arqua1 (Boisson-Dernier et al. 2001) are currently being tested on *C. glauca*.

Genetic transformation of *C. glauca* with *A. tumefaciens* C58C1(pGV2260; pBIN19GUSint) was more difficult to achieve than with *A. verticillata* (Le et al. 1996; Smouni et al. 2002; Diouf et al. 2008). Plant material was found to produce large amounts of phenolic compounds that pre-

vented callus growth and bud differentiation, epicotyls were more sensitive to antibiotic selection, and kanamycin was found to decrease the organogenic potential of the transgenic calli. Moreover, rooting of *C. glauca* shoots using the same procedure as the one described for *A. verticillata* was difficult. We thus tested a wide range of modifications to improve the genetic transformation of *C. glauca*. We found that, after cocultivation, kanamycin had to be added at 25 $mg.L^{-1}$ for two weeks, and then increased to 50 $mg.L^{-1}$ for further callus growth and selection, whereas cefotaxim was added at 300 $mg.L^{-1}$ to prevent the growth of the agrobacteria. To improve the organogenic potential of the putatively transformed calli growing on selective medium, both kanamycin and cefotaxim had to be completely removed four months after contact with *A. tumefaciens*. Another precaution was to transfer the calli on fresh medium every three weeks; otherwise they turned brown and became necrotic if the medium was not renewed in time. With these improvements, one to five shoots were obtained within six to nine months on 10–20% of the transformed calli. About 70% of these shoots rooted after a 3-day treatment with 10 µM indole-3-butyric acid (Smouni et al. 2002). Though the genetic transformation of *C. glauca* by *A. tumefaciens* can be achieved, this technique remains difficult and time consuming. Besides, depending on the batch of seeds, the efficiency of genetic transformation was affected.

2.3 Other *Casuarina* species

In the framework of collaboration between our laboratory and the Research Institute of Tropical Forestry in Guangzhou, attempts to develop genetic transformation procedures for *C. equisetifolia* and *C. cunninghamiana* are in progress. These valuable species proved to be the most effective shelter trees during cyclones in Southern China. Furthermore, *C. equisetifolia* is extremely resistant to salt spray occurring in coastal areas. Preliminary data indicate that *C. equisetifolia* is rather recalcitrant to *A. tumefaciens* and tissue culture, whereas promising results have been obtained for *C. cunninghamiana* (Chonglu Zhong and Claudine Franche, unpublished data).

3 Comparative analysis of root-expressed promoters in actinorhizal nodules and roots of *Casuarina*

In order to investigate if lateral root and nodule lobe development share common molecular mechanisms beyond the morphological features, we also studied the spatiotemporal expression of the *GUS* reporter gene driven by promoters expressed at specific stages of root development during actinorhizal nodule ontogenesis. Prior to the successful isolation of promoters from *C. glauca*, several heterologous promoters including *HRGPnt3* (Keller and Lamb 1989), *cdc2aAt* (Hemerly et al. 1993), *GH3* (Hagen et al. 1991) and *DR5* (Ulmasov et al. 1997) were transferred into Casuarina.

We first studied the *HRGPnt3* promoter from *Nicotiana tabacum*. The corresponding gene encodes a plant cell wall hydroxyproline-rich-glycoprotein (Keller and Lamb 1989). In transgenic tobacco plants, the *HRGPnt3-GUS* transcriptional fusion is expressed at early stages of initiation of lateral and adventitious roots (Hagen et al. 1991). After transfer in *A. verticillata*, expression of the *HRGPnt3-GUS* construct was not observed either in the emerging lateral roots or in prenodule and nodule primordium, suggesting that the regulation of this heterologous promoter from tobacco was not conserved in *Casuarinaceae.*

On the other hand, interesting data were obtained with the cell cycle promoter *cdc2aAt* from *Arabidopsis thaliana* (Hemerly et al. 1993). This gene encodes the P34cdc2 kinase, which is a key component of the cell cycle, acting in the regulation of the G1 to S and G2 to M transitions (Hemerly et al. 1992; Wang et al. 2004). In the model plant *Arabidopsis*, *cdc2aAt* is highly expressed in all meristems including root meristems. It is also expressed in cells that have not yet entered the mitotic cell cycle but have increased competence to do so (Hemerly et al. 1993). In transgenic roots of *A. verticillata*, *cdc2aAt-GUS* was mainly expressed in primary and secondary meristems, whereas in transgenic nodules, *GUS* expression was found to be restricted to the phellogen (Sy et al. 2007). In all transgenic lines, *GUS* expression was not observed either in the emerging prenodule or in the nodular primordium or in the nodule primary meristem. The most striking result was obtained during early stages of the interaction of the root system with the hyphae of *Frankia*. Histological analysis of ß-glucuronidase activity in *cdc2aAt-GUS* roots inoculated for seven days with *Frankia*, revealed that cells from the pericycle located opposite the protoxylem poles were deeply stained. From these data, we concluded that upon *Frankia* infection, cells from the lateral roots, and notably the pericycle cells that give rise to nodule primordium, prepare to re-enter the cell cycle (Sy et al. 2007). We also observed that the addition of supernatants from a two-day-old culture of *Frankia* to *cdc2aAt-GUS* roots, contributed to the induction of the reporter gene, indicating that molecules secreted by *Frankia* are likely to stimulate the cell cycle during the early stages of the interaction. However, this induction was also observed with supernatant from *Frankia* strains that did not induce nodules in the host plant (Mame-Oureye Sy and Claudine Franche, unpublished data). These molecules could be hormones such as indole acetic acid, phenyl acetic acid and cytokinins, which have been reported in *Frankia* culture medium (Berry et al. 1989; Hammad et al. 2003; Stevens and Berry 1988; Wheeler et al. 1979, 1984). Besides, it has been

established in legumes that Nod factors are involved in the activation of the cell cycle during the first stages of the interaction (Yang et al. 1994; Geurts et al. 2005). Unknown non-specific *Frankia* factors could thus also contribute to activation of the cell cycle in actinorhizal roots.

Auxin plays a major role in the initiation of lateral roots in plants (De Smet et al. 2006), and its role has been confirmed during legume-rhizobia symbiosis following the expression of the transcriptional *GH3-GUS* fusion in transgenic plants (Mathesius et al. 1998). *GH3* is an auxin-sensitive promoter from soybean that is a molecular marker for monitoring changes either in auxin concentration or in cellular sensitivity to auxin (Hagen et al. 1991; Guilfoyle 1999). Modifications in reporter gene expression observed in transgenic *GH3-GUS* white clover showed that inoculation by rhizobia leads to transient inhibition of the acropetal transport of auxin at the inoculation site, followed by the accumulation of auxin at the site of nodule initiation (Mathesius et al. 1998; Mathesius 2008). Similarities in *GH3-GUS* patterns of expression were observed in roots and nodules at parallel developmental stages in legumes. In both organs, expression was high in dividing cells of the early primordium, but reduced during later stages of development and differentiation. To check if auxin was also involved in mediating symbiotic response in actinorhizal plants, we monitored the spatiotemporal *GUS* expression conferred by auxin-responsive promoters in response to *Frankia*. Due to the very low activity of *GH3* in *A. verticillata*, we tested the synthetic auxin responsive promoter *DR5* derived from *GH3*. *DR5* comprises tandem repeats of an auxin-responsive element and is widely used to monitor auxin responses in higher plants (Ulmasov et al. 1997; Guilfoyle 1999). Histochemical analysis of transgenic *DR5-GUS* roots revealed *GUS* expression in the apex of primary and lateral roots, and in the vascular system close to the apex. In transgenic roots inoculated with *Frankia*, no modification was observed in the pattern of *GUS* expression. In transgenic actinorhizal nodules, no GUS activity was observed in the meristem.

These data indicate that regulation of heterologous promoters might be affected when transferred into the actinorhizal hosts *C. glauca* and *A. verticillata*. Promoters that have provided valuable insights into nodule ontogenesis in legumes did not provide any or only very poor data in transgenic *Casuarinaceae*. To understand the signalling pathways in the establishment of the actinorhizal symbiosis, the best approah is the use of reporter genes driven by promoters from candidate genes isolated from Casuarina. One example is the characterization of *CgAUX1*, which encodes a high-affinity auxin influx transporter in *C. glauca*. Using the *GUS* gene driven by the *CgAUX1* promoter, it was clearly shown that auxin plays an important role during plant cell infection in actinorhizal symbiosis (Péret et al. 2007). *CgAUX1-GUS* was found to be strongly expressed in *Frankia*-infected root hairs and nodule cortical cells. It was also noticed that *CgAUX1-GUS* was highly expressed in the root primordium, whereas no reporter gene activity was observed in the nodule primordium. This result indicates that molecular mechanisms involved in primordia initiation in lateral roots may differ from those in actinorhizal nodules.

4 RNA silencing for the functional analysis of symbiotic actinorhizal genes

RNA interference (RNAi) refers to a common mechanism of RNA-based post-transcriptional gene silencing in eucaryotic cells (Hannon 2002). When this phenomenon was first discovered, it was termed post-transcriptional gene silencing (PTGS) in plants, quelling in fungi, and RNA interference (RNAi) in animals (Baulcombe 2004). The RNA interference (RNAi) mechanism performs a native role in gene regulation and can also be used for targeted gene silencing (Waterhouse and Helliwell 2003). In both monocots and dicots such as rice and Arabidopsis, RNAi is routinely used to characterize gene function and to engineer novel genotypes (Small 2007). In perennial species, with the exception of poplar, the efficiency and stability of RNAi is still poorly documented (Meyer et al. 2004; Li et al. 2008).

In order to detemine if RNAi could down-regulate plant genes from the tropical *Casuarinaceae* trees, two pHKN gene constructs, pHKN30 and pHKN31, containing hairpin RNA (hpRNA) directed towards the *GUS* reporter gene were introduced by *A. rhizogenes* in the root system of stably transformed *35S-GUS A. verticillata* plants (Kumagai and Kouchi 2003). The pHKN30 hpRNA construct contained inverted repeats of a 582 nt sequence corresponding to the 3' end of the *GUS* coding region, and pHKN31 included a 325 nt sequence at the middle of the reporter gene. Quantitative analysis of GUS activity in the transformed root system from the composite plants established that, in 100% (pHKN30) and 70% (pHKN31) of the GUS-hpRNAi roots, there was a 90% reduction in reporter gene activity (Gherbi et al. 2008b). Histochemical analysis revealed that approximately 70% of the *35S-GUS-hpRNAi* roots exhibited no blue staining. Occasionally, some GUS activity remained in specific locations such as the apical region of the main root and in the vascular system in the elongation zone. Further qPCR analysis confirmed that the level of *GUS* transcripts was downregulated from 46 to 94% in the RNAi roots. These first data on targeted gene silencing in *Casuarinaceae* clearly demonstrated the efficiency of RNAi in reducing both mRNA and enzyme levels.

The RNAi strategy was then used to down-regulate *CgSymRK*, a gene isolated from *C. glauca,* orthologous to

the receptor-like kinase gene *SymRK* required for nodulation in legumes (Endre et al. 2002; Stracke et al. 2002). An hp-construct containing a sense and antisense 365 bp fragment corresponding to the kinase domain of CgSymRK was introduced into A4RS and genetically transformed into *C. glauca* to produce composite plants (Gherbi et al. 2008a). Following analysis of 78 RNAi plants, we found that the frequency of nodulated plants was reduced of 50% compared to control plants. In addition, a range of morphological alterations of the down-regulated CgSymRK-nodules was observed. Whereas mature nodules in the control plants were multilobed, RNAi nodules were dramatically reduced in size and mostly consisted of a single thin lobe. Cytological analysis further showed that these aberrant nodular structures accumulated high levels of phenolic compounds and contained cortical infected cells that were smaller than untransformed nodular lobes in controls. Acetylene reduction activity assays showed that these CgSymRK-RNAi nodules did not have the ability to fix nitrogen. Additional experiments revealed that CgSymRK was also necessary for the establishment of the symbiosis with the arbuscular mycorrhiza *Glomus intraradices*. The knockdown of *CgSymRK* was found to strongly affect the penetration of the fungal hyphae into the root cortex, thus revealing the key role of CgSymRK in root endosymbioses in *C. glauca*, and the conservation of SymRK function between legumes and actinorhizal plants (Gherbi et al. 2008a).

5 Transgenic *Casuarinaceae* as tools for isolation of the signal molecule(s) secreted by *Frankia*

The early steps in the invasion of roots from *Casuarinaceae* members by *Frankia* are characterized by the reciprocal exchange of signal molecules that ultimately allow the bacteria to use the plant root hair as means of entry (Bhuvaneswari and Solheim 2000). But so far, the key molecules that control specific recognition of the actinomycetal partner by the plant have not been characterised.

The first observable event in the infection process of members of actinorhizal plants by *Frankia* is the curling of the root hairs (Callaham et al. 1979; Cérémonie et al. 1999). In legumes, deformation of the root hairs linked to the biological activity of the Nod factors enabled the development of a specific and sensitive root hair-branching bioassay, thereby contributing to purification of Nod factors (Dénarié et al. 1996). In actinorhizal plants, controversial data have been reported concerning the specificity of root-hair deformation by *Frankia* strains, making it difficult to purify the specific actinorhizal signal molecules by testing their effects on roots. For example, it was shown that root hair deformation could be induced in *A. glutinosa* by two strains of *Frankia* isolated from *C. equisetifolia* that did not induce nodules in the host plant (van Ghelue et al. 1997).

As an alternative or complementary approach to the root-hair deformation bioassay, we therefore searched for a promoter induced during the very early stages of the infection process. Promoter regions characterized from putative symbiotic genes of *C. glauca* include *Cg40* (Santi et al. 2002), *CgMT1* (Laplaze et al. 2002), *CgSymRK* (our laboratory, unpublished data) and *Cg12* (Svistoonoff et al. 2003). Among the actinorhizal nodulin genes characterised in *C. glauca*, the gene *Cg12* encoding a subtilisin-like serine protease is one of the earliest induced after inoculation with *Frankia* (Laplaze et al. 2000b). Transcriptional fusion including the *Cg12* promoter and *GUS* and *GFP* reporter genes was introduced via *A. tumefaciens* into *C. glauca* (Svistoonoff et al. 2003). Analysis of transgenic plants revealed that *Cg12* drove expression in *Frankia* infected root hairs and in root and nodule cortical cells containing *Frankia* infection threads. Furthermore when the same constructs were introduced in *M. truncatula*, the *Cg12* promoter was found activated exclusively in cells being infected by *S. meliloti*, indicating that both symbioses share common genetic mechanisms during the early steps of bacterial infection (Svistoonoff et al. 2004). Nevertheless, in both symbiotic systems, no expression was observed in the first hours after inoculation prior to infection. *Cg12* is thus a valuable marker of infection but cannot be used as a marker of perception of pre-infection signalling molecules. Nevertheless, we are pursuing this approach with the characterisation of new candidate genes putatively involved in the first hours of the symbiotic interaction.

6 Future directions

Great efforts are currently underway to identify the changes in the global patterns of gene expression occuring during different stages of the symbiotic interaction by using microarray technologies in the actinorhizal plants *C. glauca*, *A. glutinosa* and *D. glomerata* (Hocher et al. 2006; Laplaze et al. 2008). The goal is to identify key genes involved in nodule development, and to perform comparative analysis of the symbiotic process in actinorhizal plants and legumes. RNAi technology, which was recently developed for *Casuarinaceae*, will be a valuable tool to generate down-regulated transgenic lines and identify the function of candidate genes during endosymbiotic processes. Alternatively, when actinorhizal genes have homologs in *Medicago truncatula*, *Lotus japonicus* or Arabidopsis, the molecular mechanisms in which they are involved will be analysed using the knowledge already available on these model plants. Another possible approach is complementa-

tion experiments using available mutants in these plants (Gherbi et al. 2008a; Péret et al. 2007).

The access to unprecedented amounts of ESTs from actinorhizal plants together with the progress in RNAi technology and the sequence information from different whole genomes of *Frankia*, should lead to significant progress in deciphering the molecular basis of the actinorhizal symbiosis in the coming years. This knowledge, together with the data obtained to understand endosymbiotic accomodation of AM fungi and rhizobia in legumes (Vessey et al. 2005; Sprent 2007; Sprent and Euan 2007), will undoubtely contribute to developing future strategies for the transfer of nodulation to non-nodulating plants.

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