



## Analysis of the expression pattern conferred by the *PsEnod12B* promoter from the early nodulin gene of *Pisum sativum* in transgenic actinorhizal trees of the *Casuarinaceae* family

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### Abstract

To investigate the similarities between nitrogen-fixing symbioses in *Rhizobium*-legumes and *Frankia*-actinorhizal plants, we studied the *gus* expression pattern conferred by the early nodulin promoter *PsEnod12B* from *Pisum sativum* in two transgenic *Casuarinaceae* trees, *Allocasuarina verticillata* and *Casuarina glauca*. Respectively 6 and 13 transgenic lines of *C. glauca* and *A. verticillata* were obtained following genetic transformation with the disarmed strain of *Agrobacterium tumefaciens* C58C1 (pGV2260; pBIN-*PsEnod12B-gus*). In non-symbiotic tissues, the *gus* gene was expressed exclusively in the vascular system of the aerial parts of the plants. In mature nodules, *PsEnod12B* drove some *gus* expression in large *Frankia*-infected cortical cells. This specificity was different from that observed in pea nodules where *PsEnod12B* was found expressed in the infection zone. No reporter gene activity was visible at the early stages of the symbiotic process with *Frankia*, including infected root hairs and prenodules. Whereas some transcription factors of *Allocasuarina* and *Casuarina* are able to interact with the *PsEnod12B* promoter, the pattern of expression is modified in nodules of these non-legume heterologous hosts.

**Abbreviations:** BA – 6-benzylaminopurine; GUS –  $\beta$ -glucuronidase; NAA –  $\alpha$  naphthalene acetic acid; X-gluc – 5-bromo-4-chloro-3-indolyl  $\beta$ -D-glucuronic acid.

### Introduction

Under conditions of nitrogen limitation, soil bacteria of the phylogenetic groups *Rhizobium* and *Frankia* elicit the formation of nodules on the roots of their related host plants (Vessey et al., 2005). The gram-negative rhizobia interact with plant species of the *Leguminosae* family and with

the non-legume genus *Parasponia* of the *Ulmaceae* family. The gram-positive actinomycete *Frankia* develops a symbiotic relationship with plant species belonging to eight families of angiosperms, collectively called actinorhizal plants. Phylogenetic analyses have shown that plants able to establish a root nodule symbiosis with *Rhizobium* and *Frankia* are members of a single clade, the Eurosid I, and that they belong to four subclades interspersed with non-symbiotic plant species. Therefore, it has been proposed that a predisposi-

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tion for nodule formation was acquired by the ancestor of this nitrogen-fixing clade and that the ability to enter a symbiosis with *Rhizobium* or *Frankia* may have arisen several times (Soltis et al., 1995).

In both systems, root nodules result from a complex developmental program that is determined by tightly regulated genes from nitrogen-fixing bacteria and their host plants (Crespi and Galvez, 2000; Obertello et al., 2003; Scheres et al., 1990b; Schultze and Kondorosi, 1998; Vessy et al., 2005). Whereas little data are yet available on the symbiosis between actinorhizal plants and *Frankia*, considerable progress has been achieved in the study of the molecular dialogue between legumes and rhizobia during root infection and nodule ontogenesis. Plant genes that are expressed in the nodule but not in the root tissue of plants capable of symbiotic nitrogen-fixation are called nodulin genes and are referred to as early and late nodulins (for review see Verma, 2000). Protein products of early nodulin genes are often expressed within hours after rhizobial inoculation and are presumably involved in the infection process and nodule ontogenesis. Products of expression of late-nodulin genes such as leghemoglobins are expressed in the mature nodule and are generally responsible for nodule functions.

*Enod12* was one of the first early nodulin genes to be characterized. *ENOD12* sequences have been isolated from several legumes including *Pisum sativum* (*PsEnod12A* and *PsEnod12B*) (Govers et al., 1991; Scheres et al., 1990a), *Medicago sativa* (*MsEnod12A* and *MsEnod12B*) (Allison et al., 1993), *Medicago truncatula* (*MtEnod12*) (Pichon et al., 1992), *Vicia sativa* (*VsEnod12*) (Vijn et al., 1995b) and *Vicia faba* (*VfEnod12*) (Früling et al., 2000). *Enod12* genes encode for repetitive proline-rich proteins (RPRP) that contain an N-terminal signal peptide with a hydrophobic core and are assumed to be structural components of plant cell walls in root nodules.

In pea, two *Enod12* genes have been characterized: *PsEnod12A* and *PsEnod12B* (Govers et al., 1991; Scheres et al., 1990a). The major difference in their sequences is due to the presence of a deletion in the region of the *PsEnod12B* gene encoding the proline rich part of the protein (Govers et al., 1991). Using an antisense *PsEnod12A* RNA probe, it was shown by *in situ* hybridization that *Enod12* transcripts were local-

ized in root cortical cells containing the infection threads, in root cells preparing for infection thread penetration and in the nodule primordia (Scheres et al., 1990a). In mature pea nodules, expression was observed in the region immediately proximal to the apical meristem, corresponding to the distal part of the prefixation zone II. In this region, infection threads continuously infect new meristematic cells. In *M. sativa*, the two *Enod12* genes were found differentially expressed during symbiosis, with *MsEnod12B* being expressed early in roots after inoculation by *Rhizobium*, whereas *MsEnod12A* was first expressed when the developing nodule was visible (Bauer et al., 1994). In *V. sativa*, expression of *VsEnod12* was visible in the whole prefixation zone (Vijn et al., 1995b).

Though *Enod12* was one of the first early nodulin genes characterized in legumes, the role of this putative structural cell wall protein is still obscure. The expression pattern related to infection thread growth in root and nodule cells of pea and Medicago led to the hypothesis that *ENOD12* might be a structural component of the infection threads. *ENOD12* may also be involved in the modification of the root hair cell wall for subsequent penetration of bacteria. However, alfalfa mutants completely devoid of *Enod12* sequences are still capable of forming nitrogen-fixing nodules indicating that this nodulin is not essential in nodule ontogenesis or that other related proteins can replace it during the symbiotic process (Csanadi et al., 1994). *PsEnod12A* is also induced during mycorrhizal infections (Albrecht et al., 1998). This protein may serve to modulate the dynamics of the physical interaction between plant cell wall components and the invading microsymbionts (for review, see Brewin, 2004). Additional studies performed in *M. sativa* showed that the expression of a *MsEnod12A-gus* construct was correlated with meristematic activities, suggesting that *MsEnod12* expression was linked to cell division rather than to the presence of infection threads (Bauer et al., 1997).

In order to improve knowledge of the actinorhizal symbiosis, our laboratory has developed an approach to determine if the promoters from early nodulin genes from legumes retain their expression pattern during the symbiotic process with *Frankia*. Here we report on the  $\beta$ -glucuronidase expression patterns conferred by the *PsEnod12B*

promoter from pea in two actinorhizal trees of the *Casuarinaceae* family, *Casuarina glauca* and *Allocasuarina verticillata*. Transgenic plants were obtained following genetic transformation with the disarmed *Agrobacterium tumefaciens* strain C58C1 (pGV2260). Reporter gene activity was studied in shoots, roots and during actinorhizal nodule development. Our data established that whereas the *PsEnod12B* promoter drove some reporter gene activity in transgenic mature nodules of both *C. glauca* and *A. verticillata*, no expression was visible during the early stages of the symbiotic process with the actinomycete *Frankia*.

## Materials and methods

### Plasmid and bacterial strains

The plasmid construct *Psenod12B-gus* was kindly provided by Dr. T. Bisseling (University of Wageningen, Netherlands). It contains a 2.0 kb fragment corresponding to the promoter region from *Pisum sativum Enod12B* (Vijn et al., 1995a) that is fused to the  $\beta$ -glucuronidase reporter gene (*uidA*) containing the second intron (*IV2*) of the potato *ST-LS1* gene (Vancanneyt et al., 1990) and cloned into the binary vector pBIN19. This construct was introduced by electroporation into the disarmed *A. tumefaciens* strain C58C1 (pGV2260) used for the genetic transformation of *Casuarinaceae* (Vancanneyt et al., 1990). The integrity of the binary plasmid was verified by a Southern blot analysis performed on digested agrobacterial DNA (data not shown). Before cocultivation with the explants, the C58C1 strain was grown for 2 days at 28 °C in Ag medium (Le et al., 1996) supplemented with the appropriate antibiotics.

The strains of *Frankia* Thr (Girgis et al., 1990) and Allo2 (Benoist et al., 1992) used for nodulating *C. glauca* and *A. verticillata* were grown at 28 °C in modified BAP medium (Benoist et al., 1992) and the inocula were prepared as described by Franche et al. (1997).

### Plant material and culture media

*In vitro* seedlings of *A. verticillata* were raised from seeds collected in Australia and obtained

from Versepuy Company (Le Puy-en Velay, France). *C. glauca* seeds were obtained from the Desert Development Center (Saddat City, Egypt). Seeds from both species were scarified for 2 min with 95% sulfuric acid, rinsed for 30 min under tap water, and disinfected with calcium hypochlorite (5% w/v in water) for 30 min. After rinsing three times with sterile water, seeds were sown on a modified Hoagland and Arnon medium (H medium) solidified with 8 g L<sup>-1</sup> Difco bacto-agar (Le et al., 1996). The plantlets were grown for approximately 2 months in a growth chamber maintained at 28 °C with a 16 h photoperiod and a light intensity of 50  $\mu\text{E m}^{-2} \text{s}^{-1}$ .

### Genetic transformation and regeneration of transgenic plants

The chimeric construct *Psenod12B-gus* was genetically transformed into *A. verticillata* and *C. glauca* as described by Franche et al. (1997) and Smouni et al. (2002). Epicotyl fragments of *A. verticillata* and *C. glauca* were cocultivated for 3 days with the *Agrobacterium* strain C58C1 (pGV2260; pBIN-*Psenod12B-gus*). Transgenic calli were selected and propagated *in vitro* on MSC medium (Le et al., 1996) containing 2.5  $\mu\text{M}$  BA, 0.5  $\mu\text{M}$  NAA and respectively 100 mg L<sup>-1</sup> kanamycin for *A. verticillata* and 50 mg L<sup>-1</sup> kanamycin for *C. glauca*. The media were also supplemented with 250 mg L<sup>-1</sup> cefotaxime for 6 months to decontaminate the explants from residual *Agrobacterium*. Untransformed calli obtained from non-coinoculated epicotyls were grown as controls in the same conditions using MSC medium containing growth regulators but deprived of antibiotics.

Transgenic buds were observed within 4–6 months on the calli of *A. verticillata* and within 6–9 months on those of *C. glauca*. Shoots were excised at approximately 3 cm in length and root formation was induced by a 3-day-treatment with 10  $\mu\text{M}$  indole-3-butyric acid for *C. glauca* and by a 1-day-treatment with 25  $\mu\text{M}$  NAA for *A. verticillata*. As previously described, successful transfer of the T-DNA from the binary vector was demonstrated by PCR amplifications using appropriate *gus* and *npIII* primers (Franche et al., 1997) (data not shown).

## Nodulation by *Frankia*

Transgenic and non-transformed control plants of *A. verticillata* and *C. glauca* exhibiting a main root 3 cm in length were transferred to Gibson (1963) tubes which were then placed in the controlled-environment chamber as described above. This experimental system allowed the shoot to grow outside while the root system was immersed in 1/4 strength sterile Hoagland's solution (Hoagland and Arnon, 1938) containing 17 mg L<sup>-1</sup> ammonium sulphate at pH 5.6. The nutrient solution was renewed every week. After 1 month, when the root system had reached the bottom of the tube, the liquid medium was discarded. 2 mL of a dense suspension (25 µg of protein per mL) of *Frankia* were added to each tube (*Frankia* Thr for *C. glauca* and Allo2 for *A. verticillata*). The tubes were kept lying horizontally for 1 h and were then filled up to 5 mm from the top with nitrogen-free Hoagland solution at pH 6.8. Nodule initiation was monitored weekly using a stereomicroscope. Prenodules were visible within 2–3 weeks for *C. glauca* and 3–4 weeks for *A. verticillata*.

## Histochemical β-glucuronidase assay

Plant samples from aerial parts and roots were harvested after a growth period of 6–8 weeks when the plants had reached a height of approximately 10 cm. To assay GUS activity, 1 cm shoots or roots fragments were incubated overnight in a medium containing 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc) in 50 mM sodium phosphate buffer (pH 7.0) (Jefferson et al., 1987). In addition, 1 mM K<sub>3</sub>Fe(CN)<sub>6</sub> and 1 mM K<sub>4</sub>Fe(CN)<sub>6</sub> were added as catalysts to limit the diffusion of blue staining (Santi et al., 2003a). Tissues were fixed in ethanol/formaldehyde/acetic acid (5%/5%/50%) prior to clarification in 70% ethanol.

Alternatively, plant tissues were embedded in a cylinder of 3% agarose solution and cut into 50 µm sections with a vibratome (Leica VT1000E). Sections were stained for β-glucuronidase activity and fixed as described above. They were then dehydrated and mounted on microscope slides in 50% glycerin for photography.

## Results

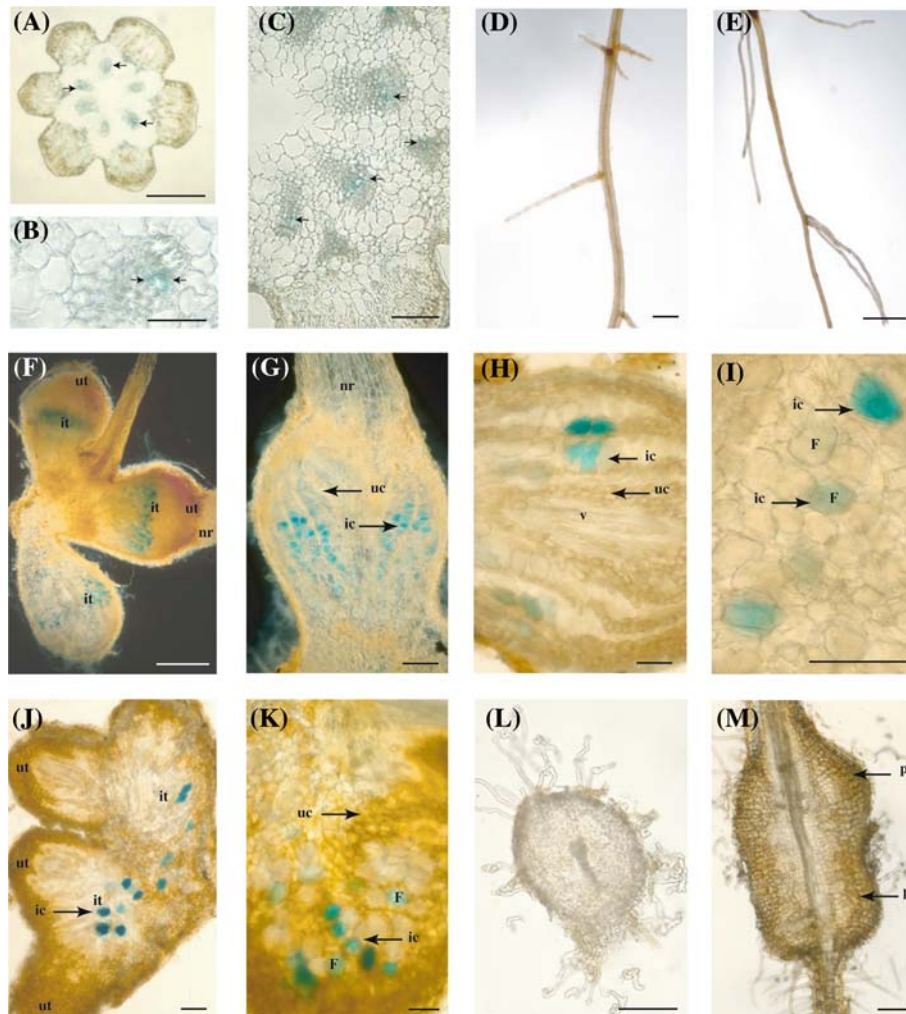
### *Production of transgenic plants*

An average of 200 epicotyl fragments were excised from 45-day-old *C. glauca* and *A. verticillata* plants grown under aseptic conditions and cocultivated with the *Agrobacterium* strain C58C1 (pGV2260; pBIN-*PsEnod12B-gus*). After 6 months of selection on kanamycin, 16 calli of *A. verticillata* were obtained and plants were regenerated from 13 of them. Nineteen antibiotic resistant calli were obtained after genetic transformation of *C. glauca* and shoot differentiation was observed on 6 of them after 9 months following *Agrobacterium* cocultivation. Kanamycin resistant plants were shown to contain the *nptII* and *gus* genes by PCR (data not shown). Although variation in the levels of *gus* expression was observed between independent primary transformants (data not shown), the qualitative patterns were always the same. Control plants obtained from non-transformed calli of *A. verticillata* and *C. glauca* were used as negative controls for histochemical assays.

### *gus expression pattern in non nodulated transgenic plants*

All plants were PCR positive for the *gus* gene and were tested for β-glucuronidase expression in the presence of the substrate X-gluc. Untransformed regenerated plants of *A. verticillata* and *C. glauca* exhibited no detectable *gus* expression in any tissue examined when incubated overnight with X-gluc (data not shown). Staining patterns of representative plants are shown in Figure 1.

β-glucuronidase activity was tested in transgenic plants 2 months after the rooting treatment. Five plants were regenerated from each of the transgenic callus lines of *C. glauca* and *A. verticillata*; a total of 78 plants of *A. verticillata* and 30 plants of *C. glauca* were analyzed. In the aerial part, a faint blue color was observed in whole shoot fragments of *A. verticillata* and *C. glauca*. Vibratome sections established that the blue color was restricted to small cells surrounding central vascular bundles in the stem and leaves (Figures 1 A–C). When the whole root system or 1 cm root



**Figure 1.** Histochemical analysis of  $\beta$ -glucuronidase activity conferred by the *PsEnod12B-gus* construct in transgenic *Casuarinaceae* plants. (A–C) Transverse sections in transgenic shoots of *C. glauca* (A–B) and *A. verticillata* (C). B is a detail of section A. The blue color resulting from the GUS activity is indicated by black arrows. Bar A: 500  $\mu\text{m}$ ; Bar B: 50  $\mu\text{m}$ ; Bar C: 100  $\mu\text{m}$ . (D–E) Whole transgenic roots of *C. glauca* (D) and *A. verticillata* (E) incubated overnight with X-gluc. Bars D–E: 100  $\mu\text{m}$ . (F–I) *gus* expression in longitudinal sections of transgenic *PsEnod12-gus* nodules of *C. glauca*. F and G are dark-field photographs. (J–K) *gus* expression in longitudinal sections of transgenic *PsEnod12-gus* nodules of *A. verticillata*. H, I and K are details of the sections showing higher magnifications of the *Frankia*-infected cells that exhibit some reporter gene activity. Bar F: 500  $\mu\text{m}$ ; Bars G–K: 100  $\mu\text{m}$ . (L) Cross-section of a transgenic root of *A. verticillata* collected 2 days after inoculation with *Frankia* and incubated with X-gluc. (M) Section of a lateral root of *A. verticillata* that shows small external protuberances called prenodule 3 weeks after inoculation with *Frankia*; the section was incubated overnight with X-gluc. Bar L: 50  $\mu\text{m}$ ; Bar M: 100  $\mu\text{m}$ . F: *Frankia*; ic: *Frankia*-infected cells; it: infected tissue; nr: nodular root; uc: uninfected cells; ut: uninfected tissue; v: vascular system; p: prenodule.

fragments were histochemically tested for *gus* expression, no staining was detected (Figures 1D–E). The main and the secondary roots were devoid of reporter gene activity. Careful examination of the root tips and the domes corresponding to the emerging lateral roots did not reveal any blue staining. The same data were obtained when the root system was excised from plants grown

for 2 weeks without any nitrogen (data not shown).

#### *gus* expression pattern in transgenic nodules of *Casuarina glauca*

Five transgenic plants were regenerated from each of the six callus lines, starved of nitrogen for

2 weeks and inoculated with the Thr strain of *Frankia*. Nodules appeared within 2–3 weeks on 95% of the transgenic plants. Due to the lack of penetration of the substrate X-gluc in actinorhizal nodules (Santi et al., 2003a), nodules were first sectioned with a vibratome and these sections (50  $\mu\text{m}$ ) were then used for histochemical GUS staining.

A view of the  $\beta$ -glucuronidase expression pattern observed in a three-lobe-nodule of *C. glauca* is shown in Figure 1F. Blue staining resulting from the GUS activity driven by the *PsEnod12* promoter was clearly visible in mature nodules. A higher magnification of a nodular lobe (Figures 1G–H) showed that the blue cells were organized in strands and were enlarged as a result of infection of the cortical cell by the actinomycete (Figures 1H–I). Previous studies have shown that nitrogen-fixation occurs in these enlarged cells (Gherbi et al., 1997). A gradient in the staining intensity was seen with a decrease in the blue color in the cortical cells closer to the root attachment region. It should be noted that some infected cells in the same region did not stain blue (Figures 1H–I).  $\beta$ -glucuronidase activity was not detected in the non-infected cortical cells, the vascular system, the nodular root, or in the apical region of the nodular lobes (Figures 1F–I). A close up view confirmed that absolutely no reporter gene activity was visible in the nodule cortical cells close to the meristem that are being infected by *Frankia* (Figure 1H).

#### *gus* expression pattern in transgenic nodules of *Allocauarina verticillata*

Ten plants were produced from each line of *A. verticillata* transgenic calli and inoculated with the Allo2 strain of *Frankia*. Nodules developed within 2–3 months on about 75% of the plants. No  $\beta$ -glucuronidase activity was visible in nodule sections resulting from the infection of non-transformed *A. verticillata* plants (data not shown). Typical patterns of  $\beta$ -glucuronidase activity obtained after X-gluc incubation of the nodule sections (50  $\mu\text{m}$ ) are shown in Figures 1J–K. As previously seen with the transgenic nodules of *C. glauca*, intense blue staining was observed in large cortical cells corresponding to *Frankia* infected cells. No reporter gene activity was observed in the apical region of the nodular lobes where the infec-

tion by the actinomycete occurs. A higher magnification confirmed that the indigo blue crystals were localized in some of the enlarged cells (Figure 1K). However, as previously observed with *C. glauca*, not all *Frankia* infected cells stained blue. No staining was seen in the non-infected cortical cells or in the infection zone proximal to the apex. No staining was observed in the nodule vascular system (data not shown). It should be noted that on some sections, short strands of 3–5 blue infected cells were occasionally observed. However, when the staining patterns of the nodules of *A. verticillata* and *C. glauca* were compared at the same stage of development, the number of GUS expressing cells was globally lower on nodule sections of *A. verticillata*.

#### *gus* expression pattern during the early stages of actinorhizal symbiosis

Because it is difficult to produce a large number of transgenic plants with *C. glauca*, a kinetic analysis of  $\beta$ -glucuronidase expression was only undertaken on transgenic roots of *Allocauarina* after inoculation by the actinomycete *Frankia*. Two transgenic lines previously shown to exhibit a high level of *gus* expression in the mature nodules were selected for this experiment. Transgenic plants were starved of nitrogen for 2 weeks and then inoculated by *Frankia* Allo2. For each time point of the kinetic, the root systems of six plants were incubated overnight in the presence of X-gluc. Roots were collected after 4 and 8 h, 1, 2, 4, 7 and 15 days, and when prenodules were visible (about 3–4 weeks). Whatever the time chosen, no blue staining was detected in the root system as can be seen in Figure 1L that shows a cross section of a root collected 2 days after *Frankia* inoculation. The curling observed in the root hairs indicates that the root system was responding to the actinomycete; however, the *PsEnod12B* promoter did not drive any GUS activity in the root hairs or the root epidermis at this stage. No blue color could be seen at the prenodule stage either (Figure 1M).

## Discussion

In this paper, we investigated the  $\beta$ -glucuronidase expression pattern conferred by the early

nodulin gene promoter *PsEnod12B* from *P. sativum* (Vijn et al., 1995a) in two actinorhizal trees, *A. verticillata* and *C. glauca*. Though ENOD12 is a nodulin, both *PsEnod12* genes have been reported to be also expressed in other parts of pea including stem and flowers (Govers et al., 1991; Scheres et al., 1990a). However, expression was lower than in nodules (Govers et al., 1991). Using *in situ* hybridization techniques in pea, *PsEnod12A* mRNAs were localized in stem internode sections, in a zone of cortical cells surrounding the central ring of vascular bundles and the interfascicular cambium cells (Scheres et al., 1990a). Histochemical analysis performed on both *C. glauca* and *A. verticillata* showed that the *PsEnod12B-gus* fusion was also expressed at low levels in the aerial parts of the transgenic plants, in the stem and leaf vascular systems. In the root system of non-inoculated actinorhizal plants, the *PsEnod12B* promoter did not drive any expression. Similar data were obtained in pea where RT-PCR analyses established that no *PsEnod12A* and *PsEnod12B* RNAs were present in uninfected roots (Govers et al., 1991).

In actinorhizal plants, no gene homologous to the *Enod12* genes from legumes has been identified so far in nodule cDNA libraries or by heterologous hybridization. However, our data show that some nodule transcription factors of *Alloca-suarina* and *Casuarina* are able to interact with the *PsEnod12B* promoter. In both *Casuarinaceae* species, GUS activity was observed in large *Frankia*-infected cells and the *PsEnod12B* promoter did not confer any  $\beta$ -glucuronidase expression in the region of infection proximal to the nodule meristem. These data are different from the expression pattern observed in nodules from Legumes. In *P. sativum*, *PsEnod12* transcripts were localized in cells containing infection threads and cells located in front of the growing infection threads (Scheres et al., 1990a). In transgenic *V. hirsuta* root nodules, the *PsEnod12B-gus* construct appeared to have a slightly different specificity and was shown to confer reporter gene activity in the distal part of the nodule, in a region corresponding to the whole prefixation zone II (Vijn et al., 1995a). This pattern of expression was identical to that of the endogenous *VsEnod12* gene, thus indicating that the host plant is involved in determining the exact expression pattern.

The transgenic actinorhizal roots did not show any reporter gene activity after inoculation with *Frankia* whatever the time tested. This is different from the data obtained in Legumes. RT-PCR analyses established that *PsEnod12A* and *PsEnod12B* genes were expressed in infected roots and in root hairs of infected plants 48 h after inoculation (Govers et al., 1991). Nod factors were also shown to induce the expression of *PsEnod12* in root hairs of pea plants within 12 h after addition of the factors (Horvath et al., 1993); however, it remains unclear in the reported data whether or not both *PsEnod12A* and *PsEnod12B* genes were induced. Using specific primers for *PsEnod12A*, Albrecht et al. (1998) clearly established that this gene was expressed 16 h after rhizobial inoculation or Nod factor treatment. In *M. truncatula* and *M. varia*, when transgenic *MtEnod12-gus* plants were tested for  $\beta$ -glucuronidase expression in roots in response to a 3 h treatment with Nod factors, GUS activity was seen throughout the zone of root hair emergence and development (Chabaud et al., 1996; Journet et al., 1994; Pichon et al., 1992).

When using the same approach with the promoter from another early nodulin gene from soybean, *GmEnod40-2* (Roussis et al., 1995), we previously reported that the construct *GmEnod40-2-gus* was not expressed either during the early stages of the symbiotic interaction with *Frankia* (Santi et al., 2003b). So far, there are two symbiotic promoters that retain the same pattern of expression in both Legume and actinorhizal root nodule symbioses. The first one is the promoter from *cg12*, a gene from *C. glauca* encoding a subtilisin-like Ser protease (Laplaze et al., 2000). In transgenic *Casuarinaceae*, the *cg12* promoter confers expression in root hairs and nodule cortical cells that are being infected by the actinomycete (Svistoonoff et al., 2003). When introduced in *M. truncatula*, the construct *cg12-gus* exhibited the same specificity of expression, with reporter gene activity specifically observed in rhizobia infected cells (Svistoonoff et al., 2004). The second promoter was isolated from the nodulin gene *lbc3*, encoding a symbiotic leghemoglobin expressed at high levels in soybean nodules (Stougaard et al., 1987). In transgenic *C. glauca* and *A. verticillata*, the soybean *hb* promoter was found to retain the same expression pattern as that induced by *Rhizobium* in the nodules (Franche et al., 1998).

Using *in situ* hybridization, symbiotic expression patterns of genes expressed at elevated levels in nodules have also been analyzed in two other actinorhizal plants, *Datisca glomerata* and *Alnus glutinosa*, and compared with the expression patterns of the homologous genes in legumes (Pawłowski et al., 2003). The data obtained indicated stronger similarity of gene expression between Legumes (subclade A of Eurosid I) and *Alnus* (subclade B), than between *Alnus* and *Datisca* (subclade E). However, though this gene-by-gene approach based on *in situ* hybridization or promoter-*gus* fusions provides valuable information, a deeper and broader understanding of the evolutionary history of root nodule symbiosis should be acquired in the near future by the comparison of ESTs isolated at different stages of nodule development in legumes and actinorhizal plants representing the four nitrogen-fixing subclades of Eurosid I.

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### References

- Albrecht C, Geurts R, Lapeyrie F and Bisseling T 1998 Endomycorrhizae and rhizobial Nod factors both require SYM8 to induce the expression of the early nodulin genes *PsENOD5* and *PsENOD12A*. *Plant J.* 15, 605–614.
- Allison L A, Kiss G B, Bauer P, Poirier M, Pierre M, Savoure A, Kondorosi E and Kondorosi A 1994 Alfalfa *Enod12* genes are differentially regulated during nodule development by nod factors and *Rhizobium* invasion. *Plant Physiol.* 105, 585–592.
- Bauer P, Crespi M D, Szecsi J, Allison L A, Schultze M, Ratet P, Kondorosi E and Kondorosi A 1994 Alfalfa *Enod12* genes are differentially regulated during nodule development by nod factors and *Rhizobium* invasion. *Plant Physiol.* 105, 585–592.
- Bauer P, Poirier S, Ratet P and Kondorosi A 1997 *MsEnod12A* expression is linked to meristematic activity during development of indeterminate and determinate nodules and roots. *Mol. Plant Microbe Interact.* 1, 39–49.
- Benoist P, Müller A, Diem H G and Schwencke J 1992 High-molecular-mass multicatalytic proteinase complexes produced by the nitrogen-fixing actinomycete *Frankia* strain BR. *J. Bacteriol.* 174, 1495–1504.
- Brewin N J 2004 Plant cell wall remodelling in the *Rhizobium*-legume symbiosis. *Crit. Rev. Plant Sci.* 23, 293–316.
- Chabaud M, Larssonneau C, Marmouget C and Huguet T 1996 Transformation of barrel medic (*Medicago truncatula* Gaertn.) by *Agrobacterium tumefaciens* via somatic embryogenesis of transgenic plants with the *MtENOD12* nodulin promoter fused to the *gus* reporter gene. *Plant Cell Rep.* 15, 305–310.
- Crespi M and Galvez S 2000 Molecular mechanisms in root nodule development. *J. Plant Growth Regul.* 19, 155–166.
- Csanadi G, Szecsi J, Kalo P, Kiss P, Endre G, Kondorosi A, Kondorosi E and Kiss G B 1994 *ENOD12*, an early nodulin gene, is not required for nodule formation and efficient nitrogen fixation in alfalfa. *Plant Cell* 6, 201–213.
- Franche C, Diouf D, Laplaze L, Auguy F, Frutz T, Rio M, Duhoux E and Bogusz D 1998 Soybean (*Ibc3*), *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, Diouf D, Le Q V, N'Diaye A, Gherbi H, Bogusz D, Gobé C and Duhoux E 1997 Genetic transformation of the actinorhizal tree *Allocauarina verticillata* by *Agrobacterium tumefaciens*. *Plant J.* 11, 897–904.
- Früling M, Schröder G, Honjec N, Pühler A, Perlick A M and Küster H 2000 The promoter of the *Vicia faba* L. gene VfENOD12 encoding an early nodulin is active in cortical cells and nodule primordia of transgenic hairy roots of *Vicia hirsuta* as well as in the prefixing zone II of mature transgenic *V. hirsuta* root nodules. *Plant Sci.* 160, 67–75.
- Gherbi H, Duhoux E, Franche C, Pawłowski K, Nassar A, Berry A M and 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 A H 1963 Physical environment and symbiotic nitrogen fixation I The effect of root temperature on recently nodulated *Trifolium subterraneum* L. plants. *Aust. J. Biol. Sci.* 16, 28–42.
- Girgis Z G M, Ishac Z Y, El-Haddad M, Saleh A E, Diem H G and Dommergues R Y 1990 First report on isolation and culture of effective *Casuarina*-compatible strains of *Frankia* from Egypt. In *Advances in Casuarina research and utilisation*. Eds. M H El-Lakany, J W Turnbull and J L Brewbaker. pp. 156–164. Desert Development Center, American University, Cairo, Egypt.
- Govers F, Harmsen H, Heidstra R, Michielsen P, Prins M, van Kammen A and Bisseling T 1991 Characterization of the pea *ENOD12B* gene and expression analyses of two *ENOD12* genes in nodule, stem and flower tissue. *Mol. Gen. Genet.* 228, 160–166.
- Hoagland R and Arnon I 1938 The water culture medium method for growing plants without soil, Circular 347. University of California, College of Agriculture, Agricultural Experiment Station, Berkeley, California.
- Horvath B, Heidstra R, Lados M, Moerman M, Spaink H P, Promé J-C, van Kammen A and Bisseling T 1993 Lipooligosaccharides of *Rhizobium* induce infection related early

- nodulin gene expression in pea root hairs. *Plant J.* 4, 727–733.
- Jefferson R A, Kavanagh T A and Bevan M W 1987 GUS fusion:  $\beta$ -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* 6, 3901–3907.
- Journet E P, Pichon M, Dedieu A, de Billy F, Truchet G and Barker D G 1994 *Rhizobium meliloti* nod Factors elicit cell-specific transcription of the *ENOD12* gene in transgenic alfalfa. *Plant J.* 6, 241–249.
- Laplaze L, Ribeiro A, Franche C, Duhoux E, Auguy F, Bisseling T, Bogusz D and Pawlowski K 2000 Characterization of a *Casuarina glauca* nodule-specific subtilisin-like protease gene, a homolog of *Alnus glutinosa* *ag12*. *Mol. Plant Microbe Interact.* 13, 113–117.
- Le Q V, Bogusz D, Gherbi H, Lappartient A, Duhoux E and Franche C 1996 *Agrobacterium tumefaciens* gene transfer to *Casuarina glauca*, a tropical nitrogen-fixing tree. *Plant Sci.* 118, 57–69.
- Obertello M, Sy M-O, Laplaze L, Santi C, Svistoonoff S, Auguy F, Bogusz D and Franche C 2003 Actinorhizal nitrogen fixing nodules: Infection process, molecular biology and genomics. *Afr. J. Biotechnol.* 2, 528–538.
- Pawlowski K, Swensen S, Guan C, Hadri A-E, Berry A M and Bisseling T 2003 Distinct patterns of symbiosis-related gene expression in actinorhizal nodules from different plant families. *Mol. Plant Microbe Interact.* 9, 796–807.
- Pichon M, Journet E P, Dedieu A, de Billy F, Truchet G and Barker D G 1992 *Rhizobium meliloti* elicits transient expression of the early nodulin gene *ENOD12* in the differentiating root epidermis of transgenic alfalfa. *Plant Cell* 4, 1199–1211.
- Roussis A, van de Sande K, Papadopoulos K, Drenth J, Bisseling T, Franssen T and Katinakis P 1995 Characterisation of the soybean gene *GmENOD40-2*. *J. Exp. Bot.* 46, 719–724.
- Santi C, Svistoonoff S, Constans L, Auguy F, Duhoux E, Bogusz D and Franche C 2003a Choosing a reporter for gene expression studies in transgenic actinorhizal plants of the *Casuarinaceae* family. *Plant Soil* 254, 229–237.
- Santi C, von Groll U, Chiurazzi M, Auguy F, Bogusz D, Franche C and Pawlowski K 2003b Comparison of nodule induction in legume and actinorhizal symbioses: The induction of actinorhizal nodules does not involve *ENOD40*. *Mol. Plant Microbe Interact.* 16, 808–816.
- Scheres B, Van De Wiel C, Zalensky A, Horvath B, Spaink H, Van Eck H, Zwartkruis F, Wolters A M, Gloudemans T, van Kammen A and Bisseling T 1990a The *ENOD12* gene product is involved in the infection process during the pea-*Rhizobium* interaction. *Cell* 60, 281–294.
- Scheres B, van Engelen F, van der Knaap E, van de Wiel C, van Kammen A and Bisseling T 1990b Sequential induction of nodulin gene expression in the developing pea nodule. *Plant Cell* 2, 687–700.
- Schultze M and Kondorosi A 1998 Regulation of symbiotic root development. *Ann. Rev. Genet.* 32, 33–57.
- Smouni A, Laplaze L, Bogusz D, Guermache F, Auguy F, Duhoux E and 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 and 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.
- Stougaard J, Sandal N N, Gron A, Kuhle A and Marker K A 1987 5' analysis of the soybean leghemoglobin *lbc3* gene: Regulatory elements required for promoter activity and organ specificity. *EMBO J.* 6, 3565–3569.
- Svistoonoff S, Laplaze L, Auguy F, Runions J, Duponnois R, Haseloff J, Franche C and Bogusz D 2003 *cg12* expression is specifically linked to infection of root hairs and cortical cells during *Casuarina glauca* and *Allocasuarina verticillata* actinorhizal nodule development. *Mol. Plant Microbe Interact.* 16(7), 600–607.
- Svistoonoff S, Laplaze L, Liang J, Ribeiro A, Gouveia M, Auguy F, Fevereiro P, Franche C and Bogusz D 2004 Infection-related activation of the *cg12* promoter is conserved between actinorhizal and legume-rhizobia root nodule symbiosis. *Plant Physiol.* 136, 1–7.
- Vancanneyt G, Schmidt R, O'Conner-Sanchez A, Willmitzer L and Rocha-Sosa M 1990 Construction of an intron-containing marker gene: splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium* mediated plant transformation. *Mol. Gen. Genet.* 220, 245–250.
- Verma D P 2000 Nodulins: Nodule-specific host gene products, their induction and function in root nodule symbiosis. In *Prokaryotic nitrogen fixation: A model system for analysis of a biological process*. Ed. E W Triplett. pp. 467–487. Horizon Scientific Press, Wymondam, UK.
- Vessey J K, Pawlowski K and Bergman B 2005 Root-based N<sub>2</sub>-fixing symbioses: Legumes, actinorhizal plants, Parasponia sp and cycads. *Plant Soil* 274, 51–78.
- Vijn I, Christiansen H, Lauridsen P, Kardailsky I, Quandt H-J, Broer II, Drenth J, Ostergaard E, van Kammen A and Bisseling T 1995a A 200 bp region of the pea *ENOD12* promoter is sufficient for nodule-specific and nod factor induced expression. *Plant Mol. Biol.* 28, 1103–1110.
- Vijn I, Yang W C, Pallisgard N, Ostergaard Jensen E, van Kammen A and Bisseling T 1995b *VsENOD5*, *VsENOD12* and *VsENOD40* expression during *Rhizobium*-induced nodule formation on *Vicia sativa* roots. *Plant Mol. Biol.* 28, 1111–1119.

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