Plant Cell Rep (2005) 24: 540–548
DOI 10.1007/s00299-005-0963-7

GENETIC TRANSFORMATION AND HYBRIDIZATION

Mariana Obertello · Carole Santi · Mame-Oureye Sy · Laurent Laplaze · Florence Auguy · Didier Bogusz · Claudine Franche

Comparison of four constitutive promoters for the expression of transgenes in the tropical nitrogen-fixing tree *Allocasuarina verticillata*

Received: 16 November 2004 / Revised: 1 March 2005 / Accepted: 8 March 2005 / Published online: 7 June 2005
© Springer-Verlag 2005

Abstract *Allocasuarina verticillata* is an actinorhizal tree that lives in symbiotic association with a nitrogen fixing actinomycete called *Frankia*. In the search for promoters that drive strong constitutive expression in this tropical tree, we studied the organ specificity of four different constitutive promoters (*CaMV 35S*, *e35S*, *e35S-4ocs* and *UBQ1* from *Arabidopsis thaliana*) in stably transformed *A. verticillata* plants. The ß-glucuronidase (*gus*) gene was used as a reporter and expression studies were carried out by histochemical analyses on shoots, roots and actinorhizal nodules. While the *35S* promoter was poorly expressed in the shoot apex and lateral roots, both the *e35S* and *e35S-4ocs* were found to drive high constitutive expression in the transgenic non-nodulated plants. In contrast, the *UBQ1* promoter was very poorly expressed and appeared unsuitable for *A. verticillata*. We also showed that none of the promoters studied were active in the nodule infected cells, whatever the developmental stage studied.

Keywords *Allocasuarina verticillata* · Actinorhizal plant · ß-Glucuronidase · Promoter · Nitrogen-fixing nodules

Abbreviations
BA: 6-Benzylaminopurine;
CaMV: Cauliflower mosaic virus;
ocs: Otopine synthase;
GUS: ß-Glucuronidase;
NAA: α−Naphtalenacetic acid;
UBQ: Ubiquitin;

Communicated by P. Debergh

M. Obertello · C. Santi · M.-O. Sy · L. Laplaze · F. Auguy · D. Bogusz · C. Franche (✉)
Groupe Rhizogénèse Symbiotique, UMR 1098, IRD (Institut de Recherche pour le Développement),
911 avenue Agropolis,
BP 5045, 34394 Montpellier Cedex 5, France
e-mail: franche@mpl.ird.fr
Tel.: +33-4-67-41-62-60
Fax: +33-4-67-41-62-22

Introduction

Allocasuarina verticillata (Lam) L. Johnson is a small tropical tree of the *Casuarinaceae* family which includes about eighty species of shrubs and trees primarily native to the Southern hemisphere, mostly to Australia and the Indo-Pacific region. Like other members of the *Casuarinaceae* family, *Allocasuarina* develops nitrogen-fixing nodules when its roots are associated with the soil actinomycete known as *Frankia*. Field studies have estimated fixation rates of 40–60 kg N/ha per year (Diem and Dommergues 1990). *Allocasuarina* roots are also infected by ectomycorhizal fungi and by arbuscular endomycorhizal fungi. These symbiotic associations contribute to improved growth of *Casuarinaceae* trees on adverse sites and disturbed soils. *Allocasuarina* has been planted in tropical arid and semi-arid regions for rehabilitation of eroded soils, for production of biomass and firewood, and for maintenance of the environment (National Research Council 1984).

Though there are similarities in the infection and nodulation events between actinorhizal and legume–rhizobia symbioses, the actinorhizal nodule is an original structure that resembles lateral roots whereas leguminous root nodules resemble shoots (Pawlowski and Bisseling 1996). The establishment of the actinorhizal symbiosis is a complex process involving many developmental steps. Both plant and actinobacteria undergo physiological and molecular changes during the interaction, but compared to the rhizobium–legume symbiosis, little is known at the molecular level (for recent reviews see Wall 2000; Obertello et al. 2003). The inability to genetically manipulate *Frankia* is a major obstacle to progress in this area (Lavire and Cournoyer 2003). Moreover actinorhizal plants are mostly woody plants, trees or shrubs, and are recalcitrant to molecular biology techniques. Nevertheless progress in nucleic acid isolation allowed the characterization of the first actinorhizal nodulin gene in *Alnus glutinosa* (Goettin-Minesky and Mullin 1994). Since then, several putative symbiotic genes have been isolated from different actinorhizal species (for review see Pawlowski 1997; Obertello et al. 2003).

In recent years, genetic transformation protocols have become available for *A. verticillata,* making it possible to perform a functional analysis of the actinorhizal symbiotic genes. The genetic transformation of *Allocasuarina* was first achieved by Phelep et al. (1991) and transgenic plants were regenerated from roots that were induced by the inoculation of hypocotyl explants with *Agrobacterium rhizogenes*. The transformed plants had an abnormal phenotype characterized by reduced apical dominance of the shoots, and growth of highly branched and agravitropic roots. The disarmed strain of *A. tumefaciens* C58C1 (pGV2260; pBIN19gusint) was then used as biological vector for gene transfer into mature zygotic embryos of *A. verticillata*. Transformed actinorhizal trees exhibiting a normal phenotype were regenerated and used to obtain transgenic nodules after inoculation by *Frankia* (Franche et al. 1997). This protocol has been used to study the pattern of expression conferred by chimeric constructs consisting of *gus* or *gfp* reporter genes placed under the control of promoters from symbiotic actinorhizal or legume genes (Laplaze et al. 2002; Santi et al. 2003b; Svistoonoff et al. 2003).

The *CaMV-35S* promoter is a widely used promoter that exhibits a high level of transcriptional activity in most tissues in a wide variety of plant species (Benfey and Chua 1990). Although it is considered to be a constitutive promoter, its expression can be modulated by photoperiod, temperature and the developmental stage (Benfey et al. 1989; Schnurr and Guerra 2000). In a previous study, we reported a detailed analysis of the *gus* expression pattern conferred by the *CaMV 35S* promoter in *Casuarina glauca*, a close relative of *A. verticillata* (Smouni et al. 2002). The *gus* expression pattern appeared both spatially and developmentally controlled, especially in lateral roots and nodules. In emerging lateral roots, the reporter gene activity was restricted to the apical root cap. The specificity was even more pronounced in transgenic actinorhizal nodules. Intense GUS activity was observed in the vascular bundle, the phellogen, and in strands of uninfected cells filled with polyphenols. *Frankia*-infected cortical cells were completely devoid of ß-glucuronidase enzymatic activity making it impossible to do a functional analysis of the actinorhizal symbiotic genes based on sense and antisense or RNAi strategies with constructs driven by the *CaMV 35S* in *C. glauca*.

With the aim of obtaining a highly efficient promoter that could drive constitutive expression in the tropical tree *A. verticillata* and in actinorhizal nodules, we introduced *via A. tumefaciens* four different promoters fused to the ß-glucuronidase reporter gene. Here we present the assessment in shoots, roots and actinorhizal nodules, of the specificity of expression conferred by the *CaMV 35S* promoter, the enhanced double *35S* (*e35S*) promoter (Odell et al. 1988), the *e35S-4ocs* promoter which contains four copies of the *ocs* element (Ellis et al. 1987), and the ubiquitine 1 (*UBQ1*) promoter from *Arabidopsis thaliana* (Callis et al. 1990).

Materials and methods

Plasmids

The gene constructs used in this study are shown in Fig. 1. They contain the ß-glucuronidase reporter gene (*uidA* commonly referred as *gus*) (Jefferson et al. 1987) and the 3′ *nos* fragment of 0.3 kb originating from the nopaline synthase gene of *A. tumefaciens* (Bevan et al. 1983). The plasmids containing the *35S-gus* and *e35S-gus* fusions were respectively obtained from R. Jefferson (CAMBIA, Canberra, Australia) and R. Beachy (Danforth Plant Science Center, St Louis, USA).

The entire *35S* promoter consists in the DNA region extending from −800 to +8 (Benfey and Chua 1990). Two different *35S* promoter derivatives were studied. The enhanced *35S* (*e35S*) contains a duplication of the *35S* region between nucleotides −343 and −90 (Kay et al. 1987). The second construct named *e35S-4ocs-gus* is derived from the *e35S* and contains four copies of a 40 nucleotide DNA sequence (−206 to −166) isolated from the octopine synthase gene of *Agrobacterium tumefaciens* (Ellis et al. 1987). Tetramers of the *ocs* element kindly provided by J. Ellis (CSIRO, Canberra, Australia) were obtained with a *Bam*HI site on the 5′ end and a *Bgl*II site on the 3′ end. This fragment was cloned into the *e35S* promoter after *Eco*RV digestion (−90 of the *35S* promoter) and attachment of *Bam*HI linkers. The construct P*UBQ1-gus* kindly provided by J. Callis includes 2500 pb corresponding to the promoter region of the *UBQ1* gene from *Arabidopsis thaliana* (Callis et al. 1990).

Bacterial strains

The *Agrobacterium tumefaciens* strain C58C1(pGV2260) containing the ß-glucuronidase constructs in the binary vector pBIN19 was used for genetic transformation of *Allocasuarina verticillata* (Vancanneyt et al. 1990). The presence and the integrity of the chimeric gene fusions introduced into electrocompetent agrobacteria was verified by Southern blot analysis of digested bacterial DNA (data not shown). Before cocultivation with the explants, *Agrobacterium* strains were grown overnight at 28°C in Ag medium (Le et al. 1996) supplemented with appropriate antibiotics.

The *Frankia* Allo2 strain was grown at 26°C in a modified BAP medium and used for inoculation of transgenic *A. verticillata* plants (Benoist et al. 1992).

Plant material and culture media

A. verticillata seeds were collected in Australia and obtained from Versepuy Company (Le Puy-en Velay, France). Disinfected seeds were germinated in sterile conditions on H medium solidified with 8 g./l Difco bacto-agar (Le et al.

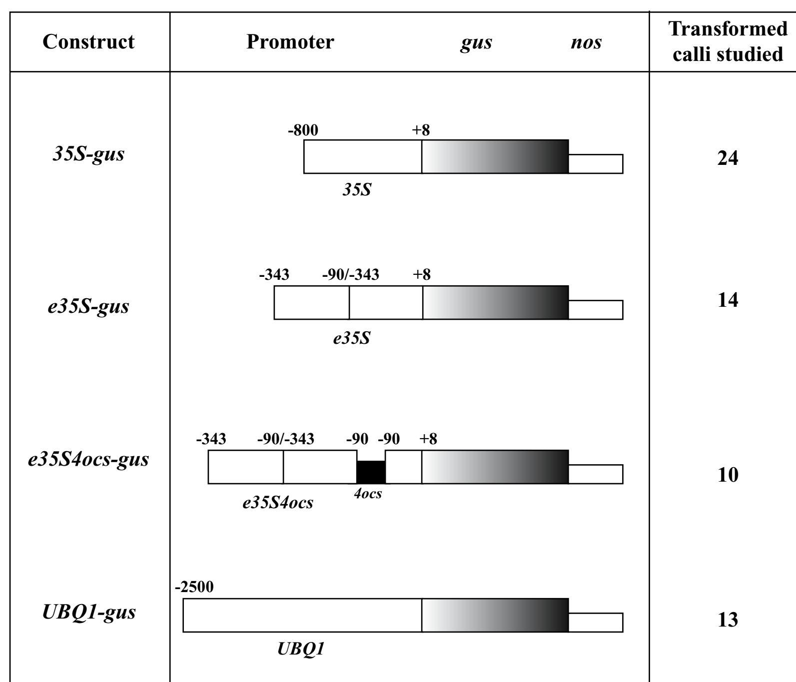


Fig. 1 Schematic representation of the gene constructs that control ß-glucuronidase expression in transgenic *A. verticillata* plants. *35S*: promoter region (−800 to +8) of cauliflower mosaic virus (Benfey and Chua, 1990); *e35S*: enhanced *35S* promoter containing a duplication of the region extending from −343 to −90 (Kay et al. 1987); *4ocs*: tetramer of a 40 bp fragment (−206 to −166) containing the *ocs* enhancer from the octopine synthase gene of *A. tumefaciens* (Ellis et al. 1987); *UBQ1*: promoter from the *UBQ1* gene isolated in *A. thaliana* (Callis et al. 1990); gus: ß-glucuronidase gene (Jefferson et al. 1987); *nos*: terminator from the nopaline synthase gene of *A. tumefaciens*

1996). The plantlets were grown for 1–2 months at 28°C with a 16 h photoperiod and a light intensity of 50 µE/m^2/s.

Genetic transformation and regeneration of transgenic plants

The chimeric constructs were genetically transformed into *A. verticillata* as described by Franche et al. (1997). Transgenic calli were maintained and propagated *in vitro* on MSC medium (Le et al. 1996) containing 2.5 µM BA, 0.5 µM NAA and 100 mg/l kanamycin. Untransformed calli obtained from non-inoculated epicotyls were grown as controls in the same conditions. Transgenic plants obtained from independent transformation events were analyzed by PCR amplification using appropriate *gus* and *nptII* primers to confirm that they contained the gene constructs used for the transformations (Franche et al. 1997) (data not shown).

Nodulation by *Frankia*

Transgenic and non-transformed control plants exhibiting a main root of 3 cm in length were transferred to tubes and inoculated with *Frankia* Allo2 as previously described (Santi et al. 2003a). Nodule initiation was monitored weekly using a stereomicroscope.

Histochemistry

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-D-glucuronide (X-gluc) in 50 mM sodium phosphate buffer (pH 7.0) (Jefferson et al. 1987). In addition, 1 mM $K_3Fe(CN)_6$ and 1 mM $K_4Fe(CN)_6$ were added as catalysts to confine the localization 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 ß-glucuronidase activity and fixed as described above. They were then dehydrated and mounted on microscope slides in 50% glycerin for photography.

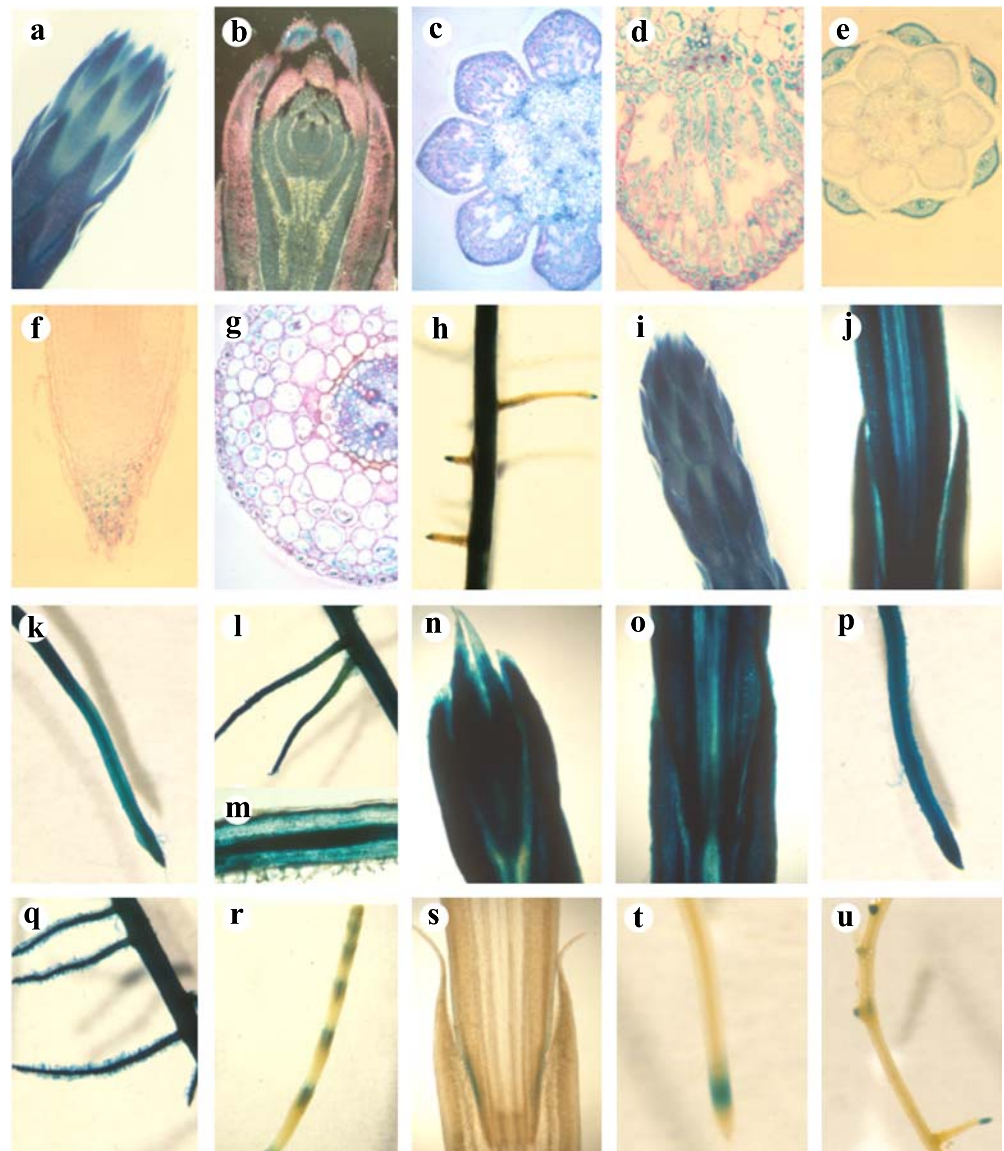
For the structural study, *A. verticillata* nodule was fixed and embedded in paraffin as described previously (Laplaze et al. 1999). Sections (7 µm) were cut with a microtome (Jun GRM 2055, Leica Microsystems, Welzlar, Germany) and stained with 0.025% (w/v) toluidine blue. Sections were mounted in glycerin plus water (15%, v/v) and examined with a light microscope (model DMRB, Leica).

Results

Agrobacterium tumefaciens mediated transfer of the ß-glucuronidase constructs into *Allocasuarina verticillata*

Genetic transformation of *A. verticillata* using the disarmed strain of *A. tumefaciens* C58C1(pGV2260) has previously been reported (Franche et al. 1997). An average of 200 epicotyls fragments were cocultivated with the *Agrobacterium*

Fig. 2 Localisation of ß-glucuronidase expression in shoots and roots of transgenic plants of *A. verticillata*. GUS activity was localized by histochemical staining after incubation of explants in the presence of 1 mM X-gluc. The ß-glucuronidase gene was driven by the *35S* (**a–h**), the *e35S* (**i–m**), the *e35S4ocs* (**n–q**) and the *UBQ1* (**s–u**) promoters. *35S* (**a**) whole shoot; (**b**) longitudinal section through shoot apex, in a dark field, *gus* staining appeared pink, (**c**) transverse section in the middle part of the shoot, (**d**) transverse section of a leaf, (**e**) transverse section showing two rows of leaves, (**f**) longitudinal section in the apex of the main root, (**g**) transverse section through the middle part of the main root, (**h**) whole lateral roots. *e35S* (**i**) whole shoot, (**j**) longitudinal section through the middle part of the shoot, (**k**) apex of the main root, (**l**) whole lateral roots, (**m**) longitudinal section through the main root. *e35S4ocs* (**n**) longitudinal section in the shoot apex, (**o**) longitudinal section in the middle part of the shoot, (**p**) apex of the main root, (**q**) lateral roots. *UBQ1* (**r**) whole shoot, (**s**) longitudinal section in the middle part of the shoot, (**t**) apex of the main root, (**u**) lateral roots



strain containing the chosen promoter-ß-glucuronidase fusion. In total, 10–24 kanamycin resistant calli resulting from independent gene transfer events were obtained and plants regenerated from these calli were further studied (see Fig. 1). Although variation in the levels of *gus* expression was observed among independent transformants (data not shown), the qualitative patterns for each construct were always the same. Control plants obtained from non-transformed calli were used as negative controls for histochemical assays.

Pattern of ß-glucuronidase expression in shoots and roots of transgenic *A. verticillata*

The expression of GUS activity during vegetative growth was analysed in axenically grown primary transformants and was localized by examination of sections of shoots and roots which had been treated with the chromogenic GUS substrate X-gluc. The results presented in Fig. 2 are typical of the staining pattern observed in experiments using separate transgenic lines.

Histochemical staining of the aerial part of the *35S-gus A. verticillata* plants revealed a staining that appeared to be mostly constitutive (Fig. 2a–e). However, sectioning of the shoot apex showed that the apex and the first leaves did not exhibit any reporter gene activity (Fig. 2b). Intense staining was observed in vascular tissue in the stem and leaf (Fig. 2c–d). The intensity of staining increased as the leaves were older (Fig. 2e). In the primary root, the root cap was deep blue (Fig. 2f) whereas the elongation zone was not stained (data not shown). Cross sections of the primary root showed expression of the reporter gene in the epidermis and the cortical tissue; the endoderm was not stained (Fig. 2g) and the vascular tissue was deep blue. In lateral roots, the staining was mainly confined to the vascular system and the apical region; occasionally a few cortical cells were stained blue (Fig. 2h).

Compared to the previous construct, the *e35S-gus* plants exhibited a constitutive pattern of expression in both shoots and roots (Fig. 2i–l). The shoot sections were stained deep blue (Fig. 2i–j). The main root exhibited reporter gene activity even in the elongation zone (Fig. 2k); the intense staining of the vascular tissue was still observable. The secondary roots were completely blue (Fig. 2l). Longitudinal sections revealed particularly intense staining in the epidermis, including root hairs, and in the vascular tissue (Fig. 2m). The expression pattern conferred by the *e35S-4ocs-gus* construct was very similar to the *e35S-gus* with the exception of the root hairs which stained deeper blue (Fig. 2n–q).

In contrast, transgenic *A. verticillata* plants harbouring *UBQ1-gus* showed a much lower blue staining that was, in addition, unevenly distributed (Fig. 2r–u). In whole shoots, reporter gene activity was observed in the nodes whereas the internodes remained unstained (Fig. 2r). Longitudinal shoot sections revealed that the indigo staining was restricted to the leaf bases (Fig. 2s). A specialized pattern of expression was also conferred in roots by the *UBQ1* promoter from *A. thaliana*. In the primary root, the ß-glucuronidase activity was confined to the root tip in a region that might correspond to the meristem and the elongation zone (Fig. 2t). The root cap and root differentiated cells did not stain blue. Blue staining was also observed in hemispherical bumps reprensenting lateral root primordia (Fig. 2u). In further developed lateral roots, GUS activity remained localized to the root tip (Fig. 2u).

ß-Glucuronidase expression in transgenic actinorhizal nodules

We also focused on the expression properties of the promoters during actinorhizal symbiosis. The root nodule is a specialized organ in which nitrogen is fixed by the actinobacteria into nitrogenous compounds (for reviews, see Wall 2000; Obertello et al. 2003). During the early steps of the symbiosis, *Frankia* infection triggers cell divisions in the cortical cells adjacent to the infection site. These cell divisions give rise to a small protuberance called a prenodule that appears about 10–15 days post-inoculation. After prenodule development and infection by *Frankia*, cell divisions are induced in the pericycle and give rise to a nodule lobe primordium. Mature actinorhizal nodules are indeterminate multilobed structures consisting of four zones: meristem zone (zone I), prefixation zone (zone II), fixation zone (zone III) and senescence zone (zone IV). Each nodule lobe resembles a lateral root and presents a central vascular bundle surrounded by an endoderm, an expanded cortex and a periderm (Fig. 3a). The cortical tissue of the nodule lobe include bands of *Frankia*-infected cells and uninfected cells. This organization is linked to the infection process by the actinomycete which progresses acropetally through the developing lobe cortex, thus giving rise to a series of infected cells. As *Frankia* hyphae proliferate within, cortical cells considerably enlarge and become lignified. Uninfected cells are smaller and are not lignified (Fig. 3a).

We inoculated ten plants from each transgenic line expressing the *gus* constructs using the *Frankia Allo2* strain. We then studied the pattern of GUS activity during nodule development from the prenodule stage occurring about 2–3 weeks after inoculation to the mature nodule stage 2 months later. Due to poor penetration of the substrate X-gluc (Santi et al. 2003a), the histochemical analysis was performed on transversal and longitudinal vibratome sections of prenodules and nodules. No blue staining was observed in non-transformed nodules as previously reported (Franche et al. 1997).

In prenodules and young nodules exhibiting one lobe, no reporter gene activity was observed when the *gus* gene was driven by the *35S* promoter (Fig. 3b). When investigating longitudinal and transversal sections of mature transformed nodules exhibiting at least two to three lobes, blue staining was restricted to the vascular bundle and to a lesser extent to the phellogen. Absolutely no blue staining was detected in the cortex of the nodule whatever the nodule zone and whatever the cortical cells were infected or not by *Frankia* (Fig. 3c).

GUS staining was observed in prenodules and in young nodules developing on the transgenic *e35S-gus* plants; reporter gene activity was confined to the phellogen and the nodule vasculature (Fig. 3d). In mature nodules, the pattern of expression was similar, but more pronounced (Fig. 3e–f). Isolated blue cells were occasionally observed in the cortical region (Fig. 3e). These cells that were mostly seen at the base of the nodular lobes in the region III do not correspond to the enlarged cortical cells filled of *Frankia* found in the nitrogen-fixing zone of the nodule. They were usually organised in small files of two-three *gus*-expressing cells (Fig. 3e).

The intensity of the staining was found to decrease in the transgenic nodules expressing the *e35S-4ocs-gus* construct. Phellogen staining was still observable whereas no or only a very pale blue colour was observed in the nodule vascular tissue (Fig. 3g–h). As previously observed in the *e35S-gus* transgenic nodules, few small blue cells were sometimes visible in the cortical region (Fig. 3h). As shown on a cross-section performed in the nitrogen-fixing zone III of a transgenic nodular lobe (Fig. 3h), these blue uninfected cortical cells were often seen between the large lignified *Frankia*-infected cells and did not appear to be organized in long files.

The actinorhizal nodules genetically transformed with the *UBQ1-gus* construct exhibited very little ß-glucuronidase activity. The blue staining was restricted to few cells located in the apical region of the nodules corresponding to the meristem (zone I) and the infection zone (zone II) (Fig. 3i). GUS activity was not detected in the nodule vascular tissue, the phellogen and the large cortical cells infected by *Frankia* located in the nitrogen-fixing zone.

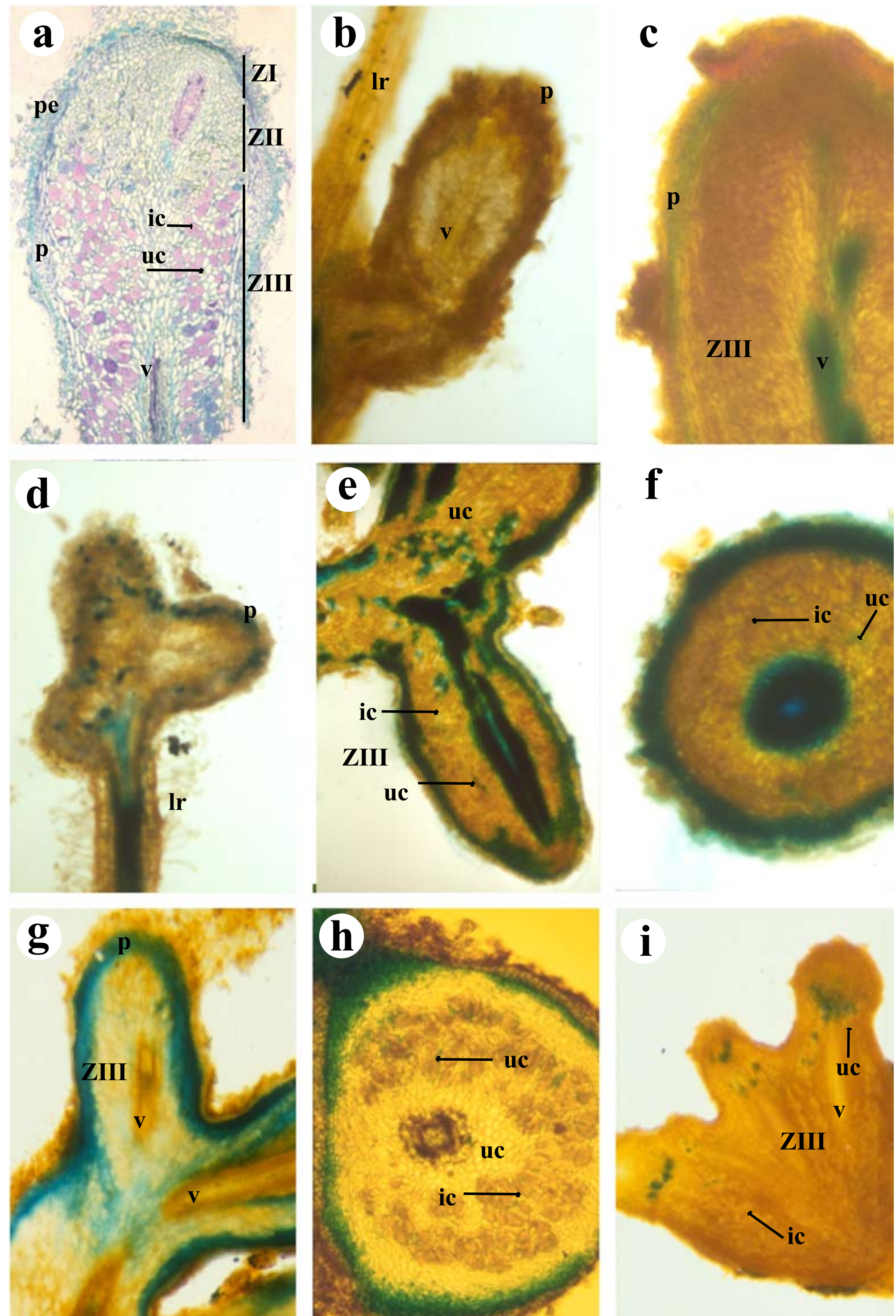


Fig. 3 Localisation of ß-glucuronidase expression in transgenic nodules of *A. verticillata* obtained after inoculation by the strain Allo2 of *Frankia*. (**a**) Longitudinal section of a nodule lobe stained with toluidine blue, *Frankia*-infected cells appear in pink color. *35S* (**b**) longitudinal section through a young nodule developing 3 weeks after inoculation, (**c**) longitudinal section through a 2-month-old nodule. *e35S* (**d**) longitudinal section through a young nodule exhibiting 3 lobes, (**e**) longitudinal section through a 2-month-old nodule, (**f**) transverse section through a mature lobe. *e35S4ocs* (**g**) longitudinal section through a 2-month-old nodule, (**h**) transverse section through a mature lobe. *UBQ1* (**i**) longitudinal section through three nodular lobes of a 2-month-old nodule. GUS activity was localized by histochemical staining after incubation of 50 µm nodule sections in the presence of 1 mM X-gluc. ic *Frankia*-infected cells, lr lateral root, pe periderm, p phellogen, uc uninfected cells, v vascular system, ZI meristem zone, ZII prefixation zone, ZIII nitrogen-fixation zone

Discussion

In this study, we compared the organ specificity of four different putatively constitutive promoters in the tropical nitrogen-fixing tree *A. verticillata*. As a reporter gene, we used the *gus* gene that is well established for use in actinorhizal trees (Santi et al. 2003a). The organ specificity conferred by the promoters was determined by *in situ* staining with X-gluc and a particular interest was focused on reporter gene activity in young and mature transgenic nodules resulting from the symbiotic interaction between *Frankia* and *Allocasuarina*.

The pattern of expression conferred by the *CaMV 35S* in *A. verticillata* was studied first and used as a reference. The *CaMV 35S* promoter is made up of six domains, the A domain (−90 to −46) and five B domains (−343 to

−90), each with different tissue specificity and synergetic interactions (Benfey et al. 1989). The A domain contains a *cis* activation sequence named *as-1* (−65 to −85) (Benfey et al. 1989) that is dynamically regulated by diverse biotic and abiotic stimuli (Qin et al. 1994; Redman et al. 2002). This region is required for root expression in the cortex and meristematic tissues (Benfey et al. 1989). The B domain confers expression in the aerial portions of the plants and contributes to root specific expression only in the vascular tissue (Benfey and Chua 1990). Whereas in whole shoots staining conferred by the *35S-gus* construct initially appeared to be constitutive in transgenic *A. verticillata*, vibratome sections revealed that the shoot apex and the first leaves were poorly stained. Below the shoot apex, blue staining was observed in all cells and the intensity of the colour increased with an increase in the age of the leaves, suggesting a constitutive and high *gus* expression. However, any interpretation in staining should take into account a number of factors, such as cell size, degree of vacuolisation and substrate availability (Jefferson et al. 1987). When compared to the expression pattern observed in the close relative actinorhizal tree *C. glauca* (Smouni et al. 2002), the *35S* promoter appeared more constitutive in the shoots of *A. verticillata* than in those of *C. glauca*. Conversely, the expression pattern in the main and secondary roots was identical with intense staining in the root cap and the vascular system. The high level of GUS staining observed in the shoot and root vascular system, together with the increase in staining in mature tissues compared with young tissues, are in agreement with data previously reported in transgenic tobacco (Benfey and Chua 1990).

The 5'-upstream region of the *35S* promoter extending from −343 to −90 acts as an enhancer sequence in higher plants (Odell et al. 1988). When this upstream region is used in two tandem repeats as an enhancer, data from the literature report that the level of gene expression increases (Kay et al. 1987; Comai et al. 1990). When introduced into *Allocasuarina*, the *e35S-gus* construct resulted in deep blue staining throughout the shoots and the roots. Compared to the histochemical data obtained in the transgenic *35S-gus A. verticillata* trees, the blue staining resulting from the *e35S-gus* construct appeared to be nearly constitutive in the root system. In transgenic tobacco plants, the *e35S* promoter has been reported to drive a ß-glucuronidase activity predominantly associated with leaf vascular tissues. The transformed roots displayed strong GUS activity in all major tissues; however, the staining was more pronounced in the meristematic and vascular tissues (Elmayan and Tepfer 1995).

We then tested the ability of four repeats of a 40 bp *ocs* promoter fragment containing sequences between −206 to −166 to modify the pattern of expression of the *e35S* in transgenic plants. The *ocs* element from the octopine synthase gene of *A. tumefaciens* is a strong enhancer that functions in both dicots and monocots (Ellis et al. 1987). It belongs to the family of *as-1* elements discovered in the A domain of the *35S* promoter. Fromm et al. (1989) demonstrated that the *ocs* enhancer binds the activation sequence factor ASF-1, a factor that also interacts with *as-1*. Intense dark blue staining, similar to that observed in the *e35S-gus* trees, was observed in the transgenic *A. verticillata* grown *in vitro*. Compared to *e35S*, root hair staining was more pronounced. Four copies of the *ocs* element (−211 to −172) have already been used by Last et al. (1991) to construct a recombinant promoter named *pEmu* which is based on a truncated maize *Adh1* promoter. In both monocots and dicots, *pEmu* gave at least a ten-fold increase in expression over the *35S* promoter.

Promoters from plant housekeeping genes like actin or ubiquitin have been used in transgenic plants as an alternative to the *35S* promoter. *UBQ1* is an ubiquitin extension protein gene that was isolated from *Arabidopsis thaliana* (Callis et al. 1990). Our findings established that the *gus* staining conferred by the *UBQ1* promoter in *A. verticillata* was very specific and restricted to the leaf base, the root tip of primary and secondary roots, and few cells located in the apical region of the nodular lobes, making this sequence unsuitable for strong constitutive expression. This specific pattern differs greatly from the one observed in transgenic tobacco plants where near-constitutive expression was reported (Callis et al. 1990; Holtorf et al. 1995).

Though high level of expression is one of the major goal of plant biotechnology, it should be underlined that transgenes designed to overexpress a coding sequence do not always bring to the intended overexpression of gene product. A number of studies have shown that the frequency of silencing positively correlates with the promoter strength driving the introduced genes (Elmayan and Vaucheret, 1996; Que et al. 1997). In a systematic analysis of *Arabidopsis* T-DNA transformants, Schubert et al. (2004) has recently examined to what extent copy number and arrangement of transgene copies, the position of T-DNA integration in the genome, and transgene nature contribute to the variability of transgene expression and to silencing. The results obtained revealed that threshold concentration of transgene transcript was the main factor triggering transgene silencing. The authors suggested that a sense RNA sensing mechanism contributes to eliminate excessively transcribed genes.

Because one aim of our laboratory is to understand the symbiotic process between *Frankia* and *Casuarina*, gene expression conferred by the four promoters was also studied in young and mature transgenic nodules developing after inoculation by the actinomycete. Compared to the transgenic *35S-gus* nodules of *C. glauca* where strands of uninfected cortical cells stained blue (Smouni et al. 2002), the nodule cortical cells of *A. verticillata* were completely devoid of reporter gene activity, irrespective of whether the cortical cells were infected by *Frankia* or not, and whatever the nodule developmental stage tested. The reporter gene activity driven by the *35S* sequence was restricted to the phellogen and the vascular system; this staining was hardly visible in young nodules but was more pronounced as the nodules matured. The blue color was found to increase with the double *35S* promoter in both young and mature nodules, with deep staining in the vascular system; nevertheless, *Frankia*-infected cells did not express the *gus* gene. Some short files of small uninfected cells exhibited some GUS

staining in the nitrogen-fixing zone. Additional studies are necessary to determine if these cells accumulate phenolic compounds. In transgenic nodules of *C. glauca* the histochemical analysis of GUS activity revealed that the *35S* promoter was expressed in layers of phenolic-containing cells that limit cortical areas where *Frankia* infection takes place (Laplaze et al. 1999; Smouni et al. 2002). When four copies of the *ocs* element were added in the *e35S*, the reporter gene activity completely disappeared in the nodule vascular system and blue indigo crystals were exclusively restricted to the phellogen. This modification in the specificity of expression was observed in all transgenic lines studied and may result from a RNA sensing mechanism due to the excessive transcription of the *gus* gene. Fromm et al. (1989) established that one copy of a 59 bp *ocs* fragment (−212 to −154) was able to activate transcription of a heterologous TATA box in transgenic tobacco plants. Moreover, the fragment conferred a specific expression detected principally in cells of the root tip as well as in cells of the shoot apex, with very little activity elsewhere. To our knowledge, this sequence has never been used to increase transgene expression in nitrogen-fixing nodules.

The reason for the poor expression of the *35S* promoter and its derivatives in actinorhizal nodules is unclear. One hypothesis is that transcription factors necessary for efficient *CaMV* promoter activation are not synthesized in the cortex of actinorhizal nodules. In legumes, though different patterns of *35S*-driven expression have been reported in nodules, the *CaMV* promoter does not appear to be specifically regulated as it is in actinorhizal nodules. For example, gene expression has been reported in *Rhizobium*-infected cells of transgenic *35S-gus* nodules from *Vicia hirsuta* (Quandt et al. 1993) and *Lotus corniculatus* (Forde et al. 1989). More recently, the *35S* promoter was shown to be silenced during the interaction with the root-knot nematodes and cyst nematodes (Goddijn et al. 1993). A detailed study undertaken by Bertioli et al. (1999) established that the expression of the *35S* decreased as the nematodes feeding sites matured. These data, together with our results, demonstrate that *cis*-elements from the *CaMV* domains interact with and respond to symbiotic interaction or parasite infection, leading to a silencing of expression. In *Arabidopsis*, it has been clearly established that the regulatory elements *as-1* and *ocs* are dynamically regulated by diverse biotic and abiotic stimuli (Redman et al. 2002; Foley et al. 2004).

The expression of heterologous genes in forest trees is a major focus of tree biotechnology today. The results presented here established that for most applications in the molecular breeding of *A. verticillata*, the expression conferred in shoots and roots by the *CaMV* promoters derivatives including the *e35S* and the *e35S-4ocs* may be sufficient. However, as previously observed for *C. glauca* (Smouni et al. 2002), *CaMV* based promoters are not suitable to obtain transgene expression in *Frankia* induced nodules of *Allocasuarina*. This is a major drawback for developing sense or antisense strategies, or RNAi approaches, aimed at understanding the function of symbiotic genes. One solution for the construction of vectors for efficient expression in actinorhizal nodules might rather lie in the isolation and characterization of endogenous promoters from genes highly expressed in actinorhizal nodules.

Acknowledgements The authors are very grateful to Dr. J. Ellis (CSIRO, Canberra) for providing the *ocs* element and to Dr. J. Callis for providing the *UBQ1* construct. Mariana Obertello is a pHD student from the University of Quilmes (Argentina) supported by a grant from IRD (Institut de Recherche pour le Développement)

References

Benfey PN, Chua NH (1990) The cauliflower mosaic virus *35S* promoter: combinational regulation of transcription in plants. Science 250:959–966

Benfey PN, Ren L, Chua N-H (1989) The *CaMV 35S* enhancer contains at least two domains, which can confer different developmental and tissue-specific expression patterns. EMBO J 8:2195–2202

Benoist P, Müller A, Diem HD, Schwencke J (1992) High-molecular-mass multicatalytic proteinases complexes produced by the nitrogen-fixing actinomycete *Frankia* strain BR. J Bacteriol 174:1495–1504

Bertioli DJ, Smoker M, Burrows R (1999) Nematode-responsive activity of the cauliflower mosaic virus *35S* promoter and its subdomains. Mol Plant-Microbe Interact 12:189–196

Bevan M, Barnes W, Chilton M-D (1983) Structure and transcription of the nopaline synthase gene region of T-DNA. Nucl Acids Res 11:369–385

Callis J, Raash JA, Vierstra RD (1990) Ubiquitin extension proteins of *Arabidopsis thaliana*. J Biol Chem 265:12486–12493

Comai L, Moran P, Maslyar D (1990) Novel and useful properties of a chimeric plant promoter combining *CaMV 35S* and *MAS* elements. Plant Mol Biol 15:373–381

Diem HG, Dommergues YD (1990) Current and potential uses and management of *Casuarinaceae* in the tropics and subtropics. In: Schwintzer CR, Tjepkema JD (eds) The Biology of *Frankia* and Actinorhizal Plants. Academic Press, New York, pp. 317–342

Ellis JG, Llewellyn DJ, Walker JC, Dennis ES, Peacock WJ (1987) The *ocs* element: a 16 pb palindrome essential for activity of the octopine synthase enhancer. EMBO J 6:3203–3208

Elmayan T, Tepfer M (1995) Evaluation in tobacco of the organ specificity and strength of the *rolD* promoter, domain A of the *35S* promoter and the *35S*^{2} promoter. Transgenic Res 4:388–396

Elmayan T, Vaucheret H (1996) Expression of single copies of a strongly expressed *35S* transgene can be silenced post-transcriptionally. Plant J 9:787–797

Foley R, Singh K (2004) *TGA5* acts as a positive and *TGA4* acts as a negative regulator of *ocs* element activity in *Arabidopsis* roots in response to defence signals. FEBS Lett 563:141–145

Forde GG, Day HM, Turton JF, Wen-jun S, Culimore JV, Oliver JE (1989) Two glutamine synthetase genes from *Phaseolus vulgaris* L. display contrasting developmental and spatial patterns of expression in transgenic *Lotus corniculatus* plants. Plant Cell 1:391–401

Franche C, Diouf D, Le QV, N'Diaye A, Gherbi H, Bogusz D, Gobé C, Duhoux E (1997) Genetic transformation of the actinorhizal tree *Allocasuarina verticillata* by *Agrobacterium tumefaciens*. Plant J 11:897–904

Fromm H, Katagiri F, Chua N-H (1989) An octopine synthase enhancer element directs tissue-specific expression and binds ASF-1, a factor from tobacco nuclear extracts. Plant Cell 1:977–984

Goddijn OJM, Lindsey K, van der Lee FM, Klap JC, Sijmons PC (1993) Differential gene expression in nematode-induced feeding structures of transgenic plants harbouring promoter-*gusA* fusion constructs. Plant J 4:863–873

Goetting-Minesky MP, Mullin BC (1994) Differential gene expression in an actinorhizal symbiosis: evidence for a nodule-specific cysteine proteinase. Proc Natl Acad Sci USA 91:9891–9895

Holtorf S, Appel K, Bohlmann H (1995) Comparison of different constitutive and inducible promoters for the overexpression of transgenes in *Arabidopsis thaliana*. Plant Mol Biol 29:637–646

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusion: ß-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907

Kay R, Chan A, Daly M, McPherson J (1987) Duplication of *CaMV 35S* promoter sequences creates a strong enhancer for plant genes. Science 236:1299–1302

Laplaze L, Gherbi H, Frutz T, Pawlowski K, Franche C, Macheix J-J, Auguy F, Bogusz D, Duhoux E (1999) Flavan-containing cells delimit *Frankia* infected compartments in *Casuarina glauca* nodules. Plant Physiol 121:113–122

Laplaze L, Gherbi H, Duhoux E, Pawlowski K, Auguy F, Guermache F, Franche C, Bogusz D (2002) Symbiotic and nonsymbiotic expression of *cgMT1,* a metallothionein-like gene from the actinorhizal tree *Casuarina glauca*. Plant Mol Biol 49:81–92

Last DI, Brettell RIS, Chamberlain DA, Chaudhury AM, Larkin PJ, Marsh EL, Peacock WJ, Dennis E (1991) *pEmu*: an improved promoter for gene expression in cereal cells. Theor Appl Genet 81:581–588

Lavire C, Cournoyer B (2003) Progress on the genetics of the N_2-fixing actinorhizal symbiont *Frankia*. Plant Soil 254:125–137

Le QV, Bogusz D, Gherbi H, Lappartient A, Duhoux E, Franche C (1996) *Agrobacterium tumefaciens* gene transfer to *Casuarina glauca*, a tropical nitrogen-fixing tree. Plant Sci 118:57–69

National Research Council (1984) Casuarinas: nitrogen-fixing trees for adverse sites. National Academic Press, Washington DC, USA

Obertello M, Sy M-O, Laplaze L, Santi C, Svistoonoff S, Auguy F, Bogusz D, Franche C (2003) Actinorhizal nitrogen fixing nodules: infection process, molecular biology and genomics. Afr J Biotechnol 2:528–538

Odell JT, Knowlton S, Lin W, Mauvais CJ (1988) Properties of an isolated transcription stimulating sequence derived from the cauliflower mosaic virus *35S* promoter. Plant Mol Biol 10:263–272

Pawlowski K (1997) Nodule-specific gene expression. Physiol Plant 99:617–631

Pawlowski K, Bisseling T (1996) Rhizobial and actinorhizal symbioses: what are the shared features? Plant Cell 8:1899–1913

Phelep M, Petit A, Martin L, Duhoux E, Tempé J (1991) Transformation and regeneration of a nitrogen-fixing tree, *Allocasuarina verticillata* Lam. Biotechnology 9:461–466

Qin X-F, Holuigue L, Horvath DM, Chua N-H (1994) Immediate early transcription activation by salicylic acid via the cauliflower mosaic virus *as-1* element. Plant Cell 6:863–874

Quandt H-J, Pühler A, Broer I (1993) Transgenic root nodules of *Vicia hirsuta*: a fast and efficient system for the study of gene expression in indeterminate-type nodules. Molecular Plant-Microbe Interact 6:699–706

Que Q, Wang H-Y, English JJ, Jorgensen RA (1997) The frequency and degree of cosuppression by sense chalcone synthase transgenes are dependent on transgene promoter strength and are reduced by premature nonsense codons in the transgene coding sequence. Plant Cell 9:1357–1368

Redman J, Whitcraft J, Johnson C, Arias J (2002) Abiotic and biotic stress differentially stimulate *as-1* element activity in *Arabidopsis*. Plant Cell Rep 21:180–185

Santi C, Svistoonoff S, Constans L, Auguy F, Duhoux E, Bogusz D, 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, Franche C, 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

Schnurr JA, Guerra DJ (2000) The *CaMV-35S* promoter is sensitive to shortened photoperiod in transgenic tobacco. Plant Cell Rep 19:279–282

Schubert D, Lechtenberg B, Forsbach A, Gils M, Bahadur S, Schmidt R (2004) Silencing in *Arabidopsis* T-DNA transformants: the predominant role of a gene-specific RNA sensing mechanism versus position effects. Plant Cell 16:2561–2572

Smouni A, Laplaze L, Bogusz D, Auguy F, Duhoux E, Franche C (2002) The *35S* promoter is not constitutively expressed in the transgenic tropical actinorhizal tree, *Casuarina glauca*. Funct Plant Biol 29:649–656

Svistoonoff S, Laplaze L, Auguy F, Runions CJ, Duponnois R, Haseloff J, Franche C, 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 16:600–607

Vancanneyt G, Schmidt R, O'Connor-Sanchez A, Willmitzer L, 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

Wall LG (2000) The actinorhizal symbiosis. J Plant Growth Regul 19:167–182