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Research note:**The 35S promoter is not constitutively expressed in the transgenic tropical actinorhizal tree *Casuarina glauca***

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Abstract. The tropical nitrogen-fixing tree, *Casuarina glauca* Sieb. ex Spreng. was genetically transformed using *Agrobacterium tumefaciens* C58C1(pGV2260; pBIN19GUSINT). We report on the expression pattern conferred by the cauliflower mosaic virus (CaMV) 35S promoter in transgenic *C. glauca* plants grown *in vitro*, and for one year in a greenhouse. Histochemical assays in shoots from *in vitro* plants revealed β -glucuronidase (GUS) staining in apical and axillary buds, and in nearly all tissues near the base of the stem. In roots, the CaMV 35S drove strong GUS expression in the apex and vascular tissue. In 1-year old plants grown in a greenhouse, the CaMV 35S promoter was highly active, except in peripheral suberized tissues. Transgenic *C. glauca* plants were nodulated by the actinomycete *Frankia*. Histochemical assays on vibratome sections of transgenic nodules demonstrated intense GUS activity in the vascular bundle, the phellogen, and in strands of uninfected cells filled with polyphenols. GUS expression was undetectable in *Frankia*-infected cells.

Keywords: *Agrobacterium*-mediated genetic transformation, CaMV 35S promoter, *Casuarina glauca*, transgenic actinorhizal nodules, β -glucuronidase gene.

Introduction

Casuarina glauca Sieb. ex Spreng. is a tropical tree with tall mature habit in the *Casuarinaceae*, a family that originates from Australia (National Research Council 1984). In its natural habitat, *C. glauca* bears nitrogen-fixing root nodules (so called actinorhizal nodules) induced by the actinomycete *Frankia* (Baker and Mullin 1992). Thanks to this symbiotic association, the tree can grow vigorously on soils that otherwise would be too deficient in nitrogen to sustain plant growth (National Research Council 1984). The amount of nitrogen fixed by *Casuarina* is comparable to the amounts fixed by legumes and their *Rhizobium* symbionts (60 kg ha⁻¹ year⁻¹; Diem and Dommergues 1990). *Casuarina* roots also form an association with mycorrhizal fungi that facilitates the uptake of minerals such as phosphorus. In combination with different symbionts, *Casuarina* is an excellent tree species for reforestation of low-fertility lands. It also enhances the quality and growth of adjacent crops (National Research Council 1984).

The possibility of genetically engineering *C. glauca* has great promise with respect to the factors that limit classical

genetic improvement in forestry, such as the large size of the mature plants and the long sexual generation time. Although genetic engineering in trees is still in its infancy, several studies have clearly established its potential use in introducing novel genetic characters such as herbicide tolerance, insect resistance, and modification of lignin content (for reviews see Tzfira *et al.* 1998; Ahuja 2000). Similar strategies could be developed to engineer *Casuarina* trees to resist major pathogens such as the insect *Lymantria xyli* or the fungus *Rhizoctonia solani*. Understanding the regulation of gene expression is an essential requirement for the success of tree biotechnology. Although the list of transgenic tree species is constantly growing, most of the reports available on detailed analysis of expression conferred by promoters have been carried out on the genus *Populus* (for reviews see Jouanin and Pilate 1997; Ahuja 2000). The stability of transgene expression also needs to be assessed over a period of several years because trees are exposed to changing environments.

One major goal of our laboratory is to study the plant genes that are expressed specifically or at enhanced levels in

Abbreviations used: CaMV, cauliflower mosaic virus; GUS, β -glucuronidase; IBA, indole-3-butyric acid; PCR, polymerase chain reaction; X-gluc, 5-bromo-4-chloro-3-indolyl- β -D-glucuronide.

actinorhizal nodules (for review see Franche *et al.* 1998b). To our knowledge, trees in the *Casuarinaceae* are the only actinorhizal plants that can be genetically transformed by *A. tumefaciens* (Le *et al.* 1996; Franche *et al.* 1997). Such transgenic plants are useful systems for exploring the regulation of plant genes involved in the symbiotic process with the actinomycete *Frankia*, using a promoter-reporter gene fusion approach. Transgenic *Casuarina* plants will also serve as valuable tools for up- or down-regulating the symbiotic genes, and will contribute to elucidation of the role of the specific proteins involved in the ontogenesis and functioning of the actinorhizal nodule.

The CaMV 35S promoter (Odell *et al.* 1985) has been the most widely used sequence for the introduction of foreign genes into plants, and has proved very effective in dicots and in some monocots. The signals regulating the initiation of 35S expression lie within the DNA sequence that extends approximately 400 bp upstream from the start of transcription (Benfey and Chua 1990). Several studies have already demonstrated the ability of the 35S viral promoter to drive gene expression in stably transformed perennials (for reviews see Jouanin and Pilate 1997; Ahuja 2000). Recent results have shown that expression controlled by the 35S promoter is not always constitutive in trees, and differences in the pattern, level, and inducibility of transgene expression have been observed between *in vitro*, greenhouse, and field environments (Ellis *et al.* 1996; Pilate *et al.* 1997).

To determine whether it is possible to use the 35S promoter to constitutively express valuable traits, or up or down-regulate symbiotic genes in the actinorhizal tree *C. glauca*, the expression pattern conferred by the CaMV sequence was studied in detail in transgenic plants of *C. glauca* obtained following genetic transformation by *A. tumefaciens*. This study, based on the histochemical analysis of GUS activity, was conducted on transformed plants grown *in vitro* and on transgenic plants grown for one year in a greenhouse.

Materials and methods

Plant material and growth conditions

Casuarina glauca Sieb. ex Spreng. seeds were obtained from the Desert Development Center (Saddat City, Egypt). The seeds were scarified with 95% sulfuric acid for 2 min, washed under tap water for 30 min, and surface sterilized for 20 min with calcium hypochlorite (5% w/v in water) as previously described (Le *et al.* 1996). The seeds were then germinated on Hoagland's medium (Le *et al.* 1996) and grown for 1–2 months at 28°C with a 16-h photoperiod (50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$).

Bacterial strains

The disarmed *A. tumefaciens* C58C1(pGV2260) containing the binary plasmid pBIN19GUSINT was used (Vancanneyt *et al.* 1990). Within the T-DNA borders, pBIN19GUSINT contains the coding region for kanamycin resistance under the regulatory control of the *nos* promoter, and an intron-containing the β -glucuronidase (GUS or *uidA*) gene driven by the CaMV 35S promoter. Before co-cultivation with the

explants, the *Agrobacterium* strain was grown for 2 d at 28°C in Ag medium (Le *et al.* 1996) supplemented with the appropriate antibiotics.

The strain of *Frankia* Thr (Girgis *et al.* 1990) was grown at 28°C in modified BAP medium (Benoist *et al.* 1992) and the inoculum was prepared as described by Franche *et al.* (1997).

Transformation procedure and regeneration of transgenic plants

The transformation of *C. glauca* was carried out as described by Le *et al.* (1996) with the following modifications to the protocol. Epicotyls were excised from 30 to 45-d old plants and cut into 1.5–2-cm fragments, followed by 3 d of co-cultivation with the *A. tumefaciens* strain C58C1(pGV2260; pBIN19GUSINT) on Murashige and Skoog medium modified for *Casuarina* (MSC). The agrobacteria were then removed by slowly swirling the explants in sterile water three times for 1 h. The transformed epicotyls were then placed onto MSC medium in petri dishes, which had been supplemented with 0.5 μM NAA, 2.5 μM BA, 50 mg L⁻¹ kanamycin and 250 mg L⁻¹ cefotaxime. After 2 months, one to three calli had developed on about 20% of the explants. When the calli were about 5 mm in diameter, they were placed in glass tubes (2.5 cm in diameter and 15 cm in length), closed by a cotton cap, and subcultured every 3 weeks. After 5–6 months, cefotaxime was omitted from the selection medium; in the absence of this antibiotic, the adventitious regeneration process from the kanamycin-resistant calli improved considerably. Shoots were excised when they were at least 3 cm in height, and root formation was induced by a 3 d treatment with 10 μM indole-3-butyric acid (IBA). Transgenic plants were regenerated within 6–9 months of *Agrobacterium* infection; an average of 17 \pm 5 shoot-producing calli were obtained for 100 plants. As previously reported (Le *et al.* 1996), successful gene transfer in *C. glauca* plants was confirmed by the expression of the reporter gene, and by polymerase chain amplification using *nptII* and *uidA* primers. Additional polymerase chain reactions (PCR) with primers designed for the amplification of the *virD1* gene from *Agrobacterium* were carried out to check that tissues were not contaminated by persisting agrobacteria. Untransformed plants were regenerated from non-transgenic calli and used as negative controls.

Plant inoculation by Frankia

Transgenic and control plants exhibiting a main root 3 cm in length were transferred to Gibson tubes (Gibson 1963), which were then placed in a controlled-environment chamber as described above. This experimental system allows the shoot to grow outside while the root system is immersed in 1/4 strength sterile Hoagland's liquid medium (Hoagland and Arnon 1938) containing 17 mg L⁻¹ ammonium sulfate 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. A 2 mL aliquot of a dense suspension (25 $\mu\text{g protein mL}^{-1}$) of *Frankia* Thr was added to each tube. Tubes were kept lying horizontally for 1 h before being filled to within 5 mm of the top with nitrogen-free Hoagland's solution at pH 6.8. Nodule initiation was monitored weekly using a stereomicroscope.

Histochemical GUS assays and histochemistry

Four lines exhibiting a high level of GUS activity, Cg35S-8B, Cg35S-7A, Cg35S-5B and Cg35S-5F, were used for detailed cytological analyses. Plant samples (shoots and roots) from *in vitro* propagated transgenic and control plants were taken 4–5 weeks after rooting treatment, when plants had reached a height of approximately 8 cm. Two months after plant inoculation by *Frankia*, nodules displaying different stages of development were harvested. To assay GUS activity, whole explants or sectioned tissues were flooded with a staining solution containing 1 mM 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-gluc), and incubated for 16 h at 37°C, as recommended by Jefferson (1987). To confine the localization of the blue staining,

0.5 mM $K_3Fe(CN)_6$ and 0.5 mM $K_4Fe(CN)_6$ were added as catalysts. Plant samples were fixed and cleared, as previously reported, and examined under a stereomicroscope (Le *et al.* 1996).

For higher resolution analysis, samples were embedded in 3% agarose and sliced into 45–50 μ m thick longitudinal or transverse sections on a vibratome (Leica VT1000E, Wetzlar, Germany) as described by Franche *et al.* (1998a). After incubation in X-gluc, the Wiesner reaction was performed on some nodule sections according to standard protocols (Strivastava 1966). Sections were immersed in 10% phloroglucinol in absolute ethanol, then mounted in 50% HCl.

Results

Genetic transformation of C. glauca

Epicotyls of *C. glauca* were genetically transformed with the disarmed strain of *A. tumefaciens* C58C1(pGV2260; pBIN1935SGUSINT) as described in 'Materials and methods'. From three separate transformation experiments, 62 calli growing on kanamycin were obtained, and shoot differentiation occurred within 6–9 months on 21 of these. *Agrobacterium* gene transfer was routinely scored in transgenic plants by the expression of the GUS gene, and by a PCR analysis based on the amplification of the *uidA* and *nptII* genes and the absence of amplification of the agrobacterial *virD1* gene (data not shown) (Le *et al.* 1996). Although different levels of reporter gene activity were noted at a macroscopic level in the lines of transgenic *C. glauca* plants micropropagated *in vitro* and incubated with X-gluc, all showed similar GUS activity patterns. Untransformed regenerated plants showed no detectable *gus* expression in any tissue examined when incubated with X-gluc (data not shown).

35S–GUS expression in in vitro cultivated C. glauca plants

GUS expression pattern in aerial parts of C. glauca

The aerial vegetative structure of *C. glauca* plants cultivated *in vitro* consists of assimilatory branchlets composed of nodes and internodes. Whorls of reduced scale leaves are inserted at the nodes, concrescent with the stem. At the node above this leaf insertion, the leaves become detached from the stem and are visible in a cross section of the axis.

The CaMV 35S promoter was found to be active in *C. glauca* branchlets, although its expression was controlled both spatially and developmentally. When the shoot apices were incubated in the substrate X-gluc, a heterogeneous staining pattern was observed in the scales (Fig. 1A). Cell-specific expression was examined more precisely on longitudinal and transverse sections obtained with a vibratome. In the vegetative apex and axillary buds of the apical shoot (Figs 1B, C), GUS activity was found to be restricted to meristematic apical and axillary zones and distal parts of the scales. Shoot samples were taken every 2 cm throughout the plants; the blue staining generally increased with distance from the shoot apex. In the middle part of the shoot, GUS activity occurred in the ridges and in

the stem vascular bundles (Fig. 1D). In older regions (Figs 1E, F), strong GUS expression was observed in mesophyll tissue. In the sections close to the nodes, vascular bundles exhibited an intense blue staining (Fig. 1D), whereas none was visible in the medullar parenchyma (Fig. 1E).

GUS expression pattern in roots of C. glauca

The expression pattern of the promoter CaMV 35S was found to be different in the main and lateral roots of transgenic *C. glauca* (Fig. 1G). Activity was seen throughout the main root except in the elongation zone (Fig. 1H). Observations of a main root transverse section revealed blue staining to be intense in the vascular system, including xylem, phloem tissues and pericycle (Fig. 1I); the endodermis did not appear to contain any blue precipitate (data not shown). The large, thin-walled cells of the cortex and rhizoderm were lightly stained. As noted in the aerial parts of the plant, the intensity of staining increased with root diameter and distance from the root tip.

In emerging lateral roots, promoter activity was restricted to the apical root cap (Fig. 1G). On the older lateral roots, blue staining was also visible in the proximal region of the vascular tissue.

GUS expression pattern in C. glauca trees grown for one year in a greenhouse

Twenty transgenic *C. glauca* plants representing lines Cg35S-8B, Cg35S-7A, Cg35S-5B and Cg35S-5F, and ten untransformed control plants were transferred to a greenhouse and grown for one year before further analysis. Histochemical analyses were undertaken in the stem and main root with transverse sections collected every 10 cm along the axis of trees whose average aboveground height was about 60 cm. Thanks to the presence or absence of photosynthetic tissues in the adult tree, we could distinguish assimilatory branchlets and branches, and stem, respectively. The stem, branches and main root developed secondary growth typical of woody plants with no interfascicular regions (Fig. 2). GUS activity was found mainly in primary and secondary xylem, cambium, and secondary phloem of the stem. In the apical and young zones of the stem (Figs 2A, B), GUS expression was seen at the periphery of the stem section, mostly in the ridges and phellogen. In the basal zone of the stem (Fig. 2C), blue staining was observed in the wood and liber, but was absent in the cork (phellem). In the photosynthetic branchlets of the 1-year old transgenic *C. glauca* trees, the CaMV 35S promoter was found to confer the same expression pattern as in the *in vitro*-cultivated transgenic trees (data not shown).

In the main root, GUS expression was visible in meristematic tissues (cambium and phellogen) and secondary xylem and phloem, but not in phloem fibres (Figs 2D, E). Variation in GUS activity occurred among different lateral

roots of the same transformant line. No or little expression was found in the lateral roots at the distal part of the root system, whereas blue staining was seen in young lateral roots beneath the soil surface (Fig. 2*F*). The heterogeneous pattern of blue staining observed when lateral roots were incubated with X-gluc could be the result of poor

penetration of the GUS substrate into the lignified whole roots (Fig. 2*F*).

GUS expression pattern in transgenic nodules

Actinorhizal nodules are clusters of modified lateral roots with central vascular tissue and bacterially-infected cells in

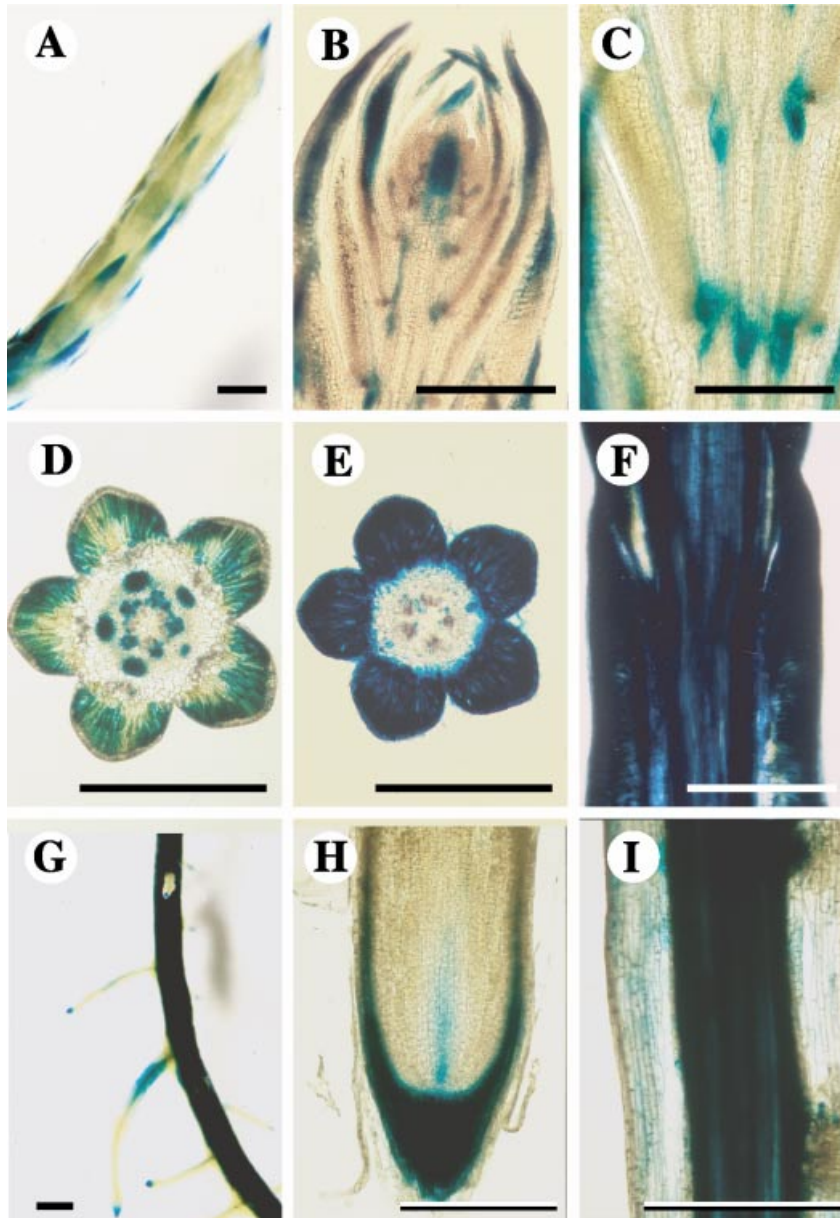


Fig. 1. Localization of 35S-GUS activity in transgenic *Casuarina glauca* plants cultivated *in vitro*. GUS activity was localized by histochemical staining after incubation of whole explants or 50- μ m explant sections in the presence of 1 mM X-gluc. (*A*) Whole shoot; (*B*) longitudinal section through shoot apex; (*C*) close-up of shoot nodes from the apical region; (*D*) transverse section through the middle part of the shoot; (*E*) transverse and (*F*) tangential sections through the stem near the stem/root transition zone; (*G*) whole root; (*H*) longitudinal section through the apex of the main root system; (*I*) longitudinal section through the main root (this sample was taken near the base of the stem). Scale bars, 1 mm (*A*, *B*, *D*, *E*); 500 μ m (*C*, *F*); 2 mm (*G*); 100 μ m (*H*, *I*).

the cortex. Owing to the presence of a meristem at the apex of actinorhizal nodule lobes, a developmental gradient exists, and four different zones have been characterized (for review see Pawlowski and Bisseling 1996). Zone 1 corresponds to the meristem at the apex, zone 2 is the infection zone where the hyphae infect the cortical cells, zone 3 is the fixation zone that is composed of both infected and uninfected cells, and zone 4 is a senescent zone that is only visible in old nodules. The nodules of *C. glauca* are also characterized by nodular roots, which correspond to uninfected roots arising from the apices of nodule lobes. These nodular roots are thought to facilitate oxygen diffusion to the nodule (Silvester *et al.* 1990).

The most efficient nodulation system developed to monitor nodule differentiation on *C. glauca* was based on the method described by Gibson (1963). Using this system, nodules were visible 1–2 months after infection by *Frankia* (Fig. 3A). Amongst 20 control and 50 transgenic *C. glauca* plants (representing the four transgenic lines Cg35S-8B,

Cg35S-7A, Cg35S-5B and Cg35S-5F) inoculated by *Frankia* Thr, 85 and 92%, respectively, had developed an average of three nodules. The shape of transgenic and wild-type nodules was comparable at all developmental stages (data not shown).

Hand-cut and semi-thin vibratome sections of transgenic and control nodules were treated with X-gluc, and GUS activity was localized. As shown in Figs 3B and C, the longitudinal and transverse sections of mature *C. glauca* nodules revealed that the 35S promoter was strongly expressed in the vascular bundle and the phellogen; no GUS activity was observed in the periderm. In the cortical tissue, blue staining was restricted to a few cell layers organized in concentric files that seemed to correspond to uninfected cells. Under the same conditions, no GUS activity was found in untransformed control nodules (data not shown).

In order to further characterize the GUS-expressing cells, transgenic nodule sections previously incubated with X-gluc were stained with phloroglucinol using the Wiesner test

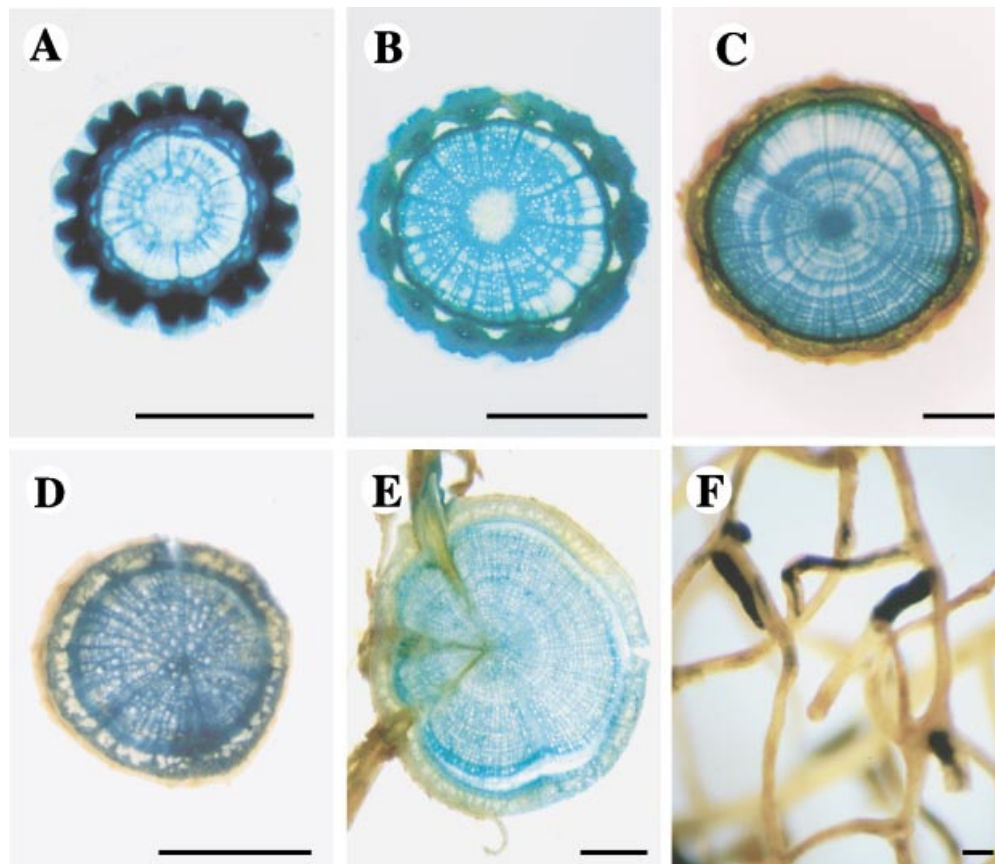


Fig. 2. Localization of 35S–GUS activity in transgenic *Casuarina glauca* plants grown for one year in a greenhouse. GUS activity was localized by histochemical staining after incubation of hand-cut transverse stem sections (A–C), hand-cut transverse main-root sections (D–E), and whole lateral roots (F) in the presence of 1 mM X-gluc. Transverse stem sections were collected 15 (A), 35 (B) and 55 cm from the shoot apex. Transverse main-root sections were taken 35 (D) and 55 cm (E) from the root apex. Lateral roots were taken beneath the soil surface (F). Scale bars, 1 mm.

(Strivastava 1996). This histochemical reagent, which reacts specifically with cinnamaldehyde groups, was used for lignin detection. Phloroglucinol makes possible the discrimination between uninfected cortical cells, which are not lignified, and *Frankia*-infected cells that are characterized by highly lignified walls (Berg and McDowell 1988). The results, which are presented in Fig. 3D, revealed that the lignified red-stained infected cells were surrounded by the blue-cell layers. From these data it can be concluded that the 35S promoter does not drive any expression of the GUS gene in *Frankia*-infected cells of *C. glauca* nodules.

In the nodular root, GUS activity was seen only in the vascular system; staining did not occur in the root cortex nor in the root meristem (Fig. 3E).

Discussion

We have investigated the spatial and temporal expression pattern conferred by the CaMV 35S promoter in the transgenic actinorhizal tree *C. glauca*. In shoots of *in vitro* plants, intense GUS staining was observed in meristematic zones (apical and axillary buds), and in all plant tissue near the base of the stem. In the main root system, the apex and vascular tissue displayed intense GUS activity. In lateral

roots, GUS staining was first restricted to the root cap and then appeared in the vascular tissue as the roots aged. In 1-year old plants transferred to a greenhouse, the main features of the histochemical data were that the CaMV 35S promoter was strongly expressed in vascular structures. It is interesting to note that 1-year old transgenic *C. glauca* trees have retained a high level of GUS activity as indicated by the intensity of the blue staining.

Surprisingly, the pattern of expression conferred by the 35S sequence was found to be slightly different in transgenic *C. glauca* compared with previous findings on *Allocasuarina verticillata* (Franche *et al.* 1997). *A. verticillata* is an actinorhizal tree that belongs to the genus *Allocasuarina* of the *Casuarinaceae* family. Whereas the 35S GUS expression pattern observed *in vitro* was similar in roots for transgenic *C. glauca* and *A. verticillata*, a striking difference was observed in the shoot apex. Constitutive expression was generally noted throughout transgenic shoots of *A. verticillata*, even in the young scale-like leaves (Franche *et al.* 1997). In transformed *C. glauca*, an increase in the GUS staining pattern was observed from the apex to the base of the stem. These data indicate that the behaviour of a promoter can differ even in two closely related species.

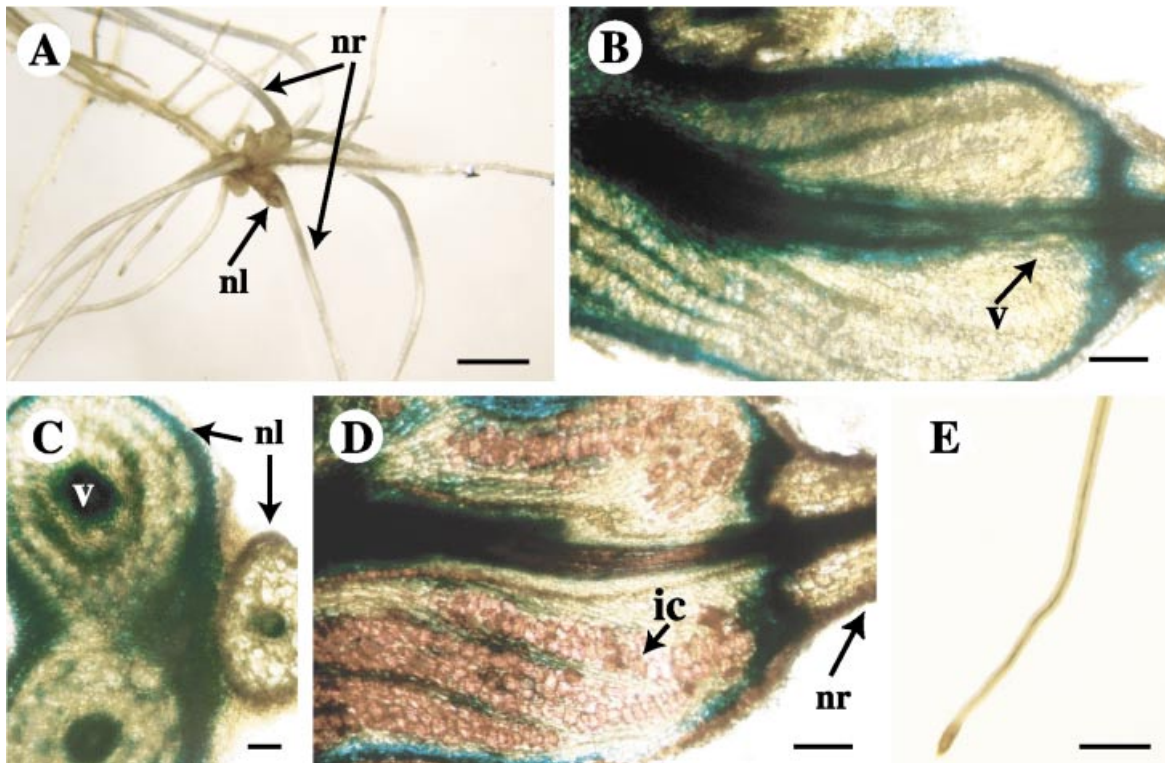


Fig. 3. Localization of 35S-GUS activity in transgenic nodules of *Casuarina glauca* obtained after inoculation by *Frankia*. Nodules were collected 2 months after inoculation by *Frankia*, and exhibited an average of six nodular lobes. (A) Transgenic nodule of *C. glauca*; (B) GUS expression in a longitudinal section of a transgenic nodular lobe; (C) GUS expression in a transverse section of a transgenic nodule with three lobes; (D) longitudinal section of a transgenic nodule incubated with X-gluc and stained using the Wiesner reaction; (E) nodular root incubated in the presence of 1 mM X-gluc. ic, *Frankia*-infected cells; nl, nodular lobe; nr, nodular root; v, vascular bundle. Scale bars, 100 µm.

Several studies have already demonstrated the ability of the 35S viral promoter to drive gene expression in stably-transformed perennials, including angiosperms such as poplar (for review see Jouanin and Pilate 1997), *Liquidambar styraciflua* (Sullivan and Lagrimini 1993), *Liriodendron tulipifera* (Wilde *et al.* 1992), *Eucalyptus camaldulensis* (Ho *et al.* 1998), *Ulmus procera* (Gartland *et al.* 2000), and gymnosperms such as *Picea glauca* (Ellis *et al.* 1993), *Pinus strobus* (Levée *et al.* 1999) and *Picea mariana* (Tian *et al.* 2000). Although expression of the chimeric construct 35S–GUS in trees has been reported, very few detailed analyses are available on the specificity of expression conferred by the CaMV promoter. In both *L. tulipifera* and *U. procera*, strong GUS activity was observed in shoot apices and in leaves, particularly in the blade, whereas roots were reported to stain weakly (Wilde *et al.* 1992; Gartland *et al.* 2000). Histochemical GUS expression in transformed tissues of *E. camaldulensis* revealed intense blue staining throughout the plant, with particularly high reporter gene expression in the vascular tissue of young leaves and roots (Ho *et al.* 1998).

A specific pattern of expression was observed in transgenic nodules of *C. glauca*. Blue staining was restricted to the vascular bundle, the phellogen, and to specific cell layers corresponding to uninfected cells. A previous study performed on *C. glauca* nodules has established that these cell layers accumulate phenolics and limit different areas where *Frankia* infection takes place (Laplaze *et al.* 1999). Since the 35S promoter does not drive any expression in *Frankia*-infected cells, it cannot be used to extinguish the expression of symbiotic genes in this tissue. A different pattern of staining has been described in transgenic nodules from legumes expressing the 35S–GUS construct. In *Vicia hirsuta*, intense GUS activity was detected in the nodule vascular bundle and cells of the symbiotic zone of mature nodules; no 35S–GUS activity was seen in undifferentiated meristematic tissue or in the infection zone (Quandt *et al.* 1993). In transgenic *Lotus corniculatus*, different patterns of GUS staining have been reported. Intense blue was seen in the vascular tissue of the nodule and in *Rhizobium*-infected cells studied by Forde *et al.* (1989). A study published by Carsolio *et al.* (1994) claimed the opposite, i.e. that no GUS activity was observed in infected cells, and expression was restricted to uninfected interstitial cells and vascular tissue. In *Lotus japonicus*, both green fluorescent protein (GFP) and GUS activities were most clearly detectable in the vascular bundles of the 35S–GUSINT/GFP nodules (Quaedvlieg *et al.* 1998). We conclude that, in both *C. glauca* and *A. verticillata*, the promoters from the haemoglobin genes of *Parasponia andersonii* and soybean have been the best sequences for driving expression of foreign genes in nitrogen-fixing cells of actinorhizal nodules (Franche *et al.* 1998a).

Besides the 35S promoter and its derivative, the enhanced 35S (70S or e35S), very few promoters have been tested in stably-transformed perennials compared with annual plants (for review see Ahuja 2000). With the isolation and characterization of a growing number of tree genes, promoters that are more adapted to the needs of genetic engineering in perennials should become more readily available. Our laboratory is currently working on the characterization of *C. glauca* genes expressed in actinorhizal nodules (Franche *et al.* 1998b). This study might lead to the isolation of nodule-specific promoters in *Casuarina*.

An important emerging field of research is the study of the stability of transgene expression in field-grown trees. Analytical data indicate that some modifications in the level and specificity of expression may occur when transformed trees are transferred from the greenhouse to the field. When transgenic poplar hybrids (*P. alba* × *P. grandidentata*) were transferred from the greenhouse to the field, the expression of the GUS gene under the control of the 35S promoter decreased by 10–75% in the leaves (Ellis *et al.* 1996; Pilate *et al.* 1997). Other promoters such as *rbcs* from potato and *rol* from *A. rhizogenes* appeared to be less affected by stresses than the 35S promoter (Ahuja 2000). This information on the stability of transgene expression in field-grown *Casuarinaceae* will be critical in determining the potential of genetic engineering for introduction of valuable new traits into this tropical tree family.

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