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Hairy Root Nodulation of *Casuarina glauca*: A System for the Study of Symbiotic Gene Expression in an Actinorhizal Tree

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The purpose of this study was to establish a fast system for producing transgenic actinorhizal root nodules of *Casuarina glauca*. *Agrobacterium rhizogenes* strain A4RS carrying the p35S-*gusA-int* gene construct was used to induce hairy roots on hypocotyls of 3-week-old *C. glauca* seedlings. Three weeks after wounding, the original root system was excised, and composite plants consisting of transgenic roots on untransformed shoots were transferred to test tubes to be inoculated with *Frankia*. The actinorhizal nodules formed on transformed roots had the nitrogenase activity and morphology of untransformed nodules. β-Glucuronidase (GUS) activity was examined in transgenic roots and nodules by fluorometric and histochemical assays. The results indicate that transgenic nodules generated with this root transformation system could facilitate the molecular study of symbiotic nitrogen fixation in actinorhizal trees.

Additional keywords: CaMV 35S promoter, genetic transformation, GUS activity.

Actinorhizal plants have a capacity for root nodule symbiosis with species of the actinomycete *Frankia* as endosymbionts (Tjepkema and Torrey 1979). These root nodules have a very distinctive tissue organization which allows the nitrogen fixation process to occur. Actinorhizal symbiosis is found among 200 species of angiosperms in eight different families (Torrey and Berg 1988). The so-called actinorhizal plant genera are major components of plant communities worldwide and are especially important in low-nutrient soils. Because they can thrive in marginal soils, actinorhizal plants are used for soil reclamation, timber, fuel, and windbreaks.

The establishment of actinorhizal symbiosis is a complex, multistep process. As in the legume-*Rhizobium* symbiosis, both plant and prokaryote undergo biochemical, physiological, and molecular changes during the interaction (Nap and Bisseling 1990; Berry and Sunel 1990). During nodule formation in legumes, several plant genes (nodulin genes) are specifically expressed. Those genes have been defined according to the timing of their expression during nodule development. The expression of early nodulin genes, like *ENOD2* or *ENOD12,* is correlated with the infection process and the development of the nodule structure, while late nodulin genes, like leghemoglobin or uricase, are associated with the nodule function (Nap and Bisseling 1990).

In actinorhizal plants, the proteins which are specifically expressed during nodule development have been described as actinorhizins (Tremblay *et al.* 1986). Although the physiology of actinorhizal symbiosis has received much attention during the past 10 years (reviewed by Berry [1994]), little is known about the molecular aspects of the interaction between actinorhizal plants and *Frankia.* Besides the purification of hemoglobin (Fleming *et al.* 1987), a nodule-specific cysteine proteinase cDNA has been isolated in actinorhizal symbiosis (Goetting-Minesky and Mullin 1994). As in the legume-*Rhizobium* symbiosis, it would be easier to understand the plant's role in the establishment of actinorhizal symbiosis if actinorhizin cDNAs were isolated and an efficient transformation system for the formation of transgenic nodules was made available.

Study of the regulation of nodulin gene expression has been made possible by the use of transgenic legumes containing chimeric genes (de Bruijn *et al.* 1990). The most intensively used transgenic legume, *Lotus corniculatus,* has served as a recipient of legume and nonlegume nodulin genes. Transgenic *L. corniculatus* plants have been spontaneously regenerated from hairy root culture obtained after hypocotyl infection by *Agrobacterium rhizogenes* (Petit *et al.* 1987). By this procedure, it takes about 5 months to analyze transgenic root nodules. In order to accelerate the procedure, transgenic roots on hypocotyls have been nodulated, so that transgenic nodules could be analyzed after approximately 2 months (Hansen *et al.* 1989).

Among the wide variety of plant genera associated with *Frankia,* the genus *Casuarina* is one of the best documented, thanks to numerous physiological, biochemical, and morphogenetic studies (Pinyopusarerk and House 1993). For this reason, we have chosen the Casuarinaceae as a model family for the study of actinorhizal development at the molecular level. We recently established a transformation and regeneration procedure for the casuarinaceous tree *Allocasuarina verticillata* (Phelep *et al.* 1991). Transgenic trees were obtained by culturing hairy roots induced by *Agrobacterium rhizogenes,* and

transgenic root nodules were produced approximately 1 year after wounding (Phelep 1992).

This paper describes a fast system for generating transgenic root nodules in *Casuarina glauca,* a tree which nodulates faster than *Allocasuarina verticillata.* It describes the structure of transformed root nodules and examines the expression of the p35S-*gusA-int* gene construct in transgenic *C. glauca* roots and nodules.

RESULTS

Susceptibility to *A. rhizogenes* strain A4RS.

On the basis of previous experiments, we used *A. rhizogenes* strain A4RS (agropine type) to induce hairy root in *C. glauca* after hypocotyl inoculation. The first morphogenetic change, observed 5 days after inoculation, was the development of a small callus at the inoculation site. Within 1 week, the first roots were visible; they grew directly out of the callus (Fig. 1A). The roots induced by strain A4RS showed a typical hairy root phenotype: a high growth rate of 1–3 mm a day, compared to less than 0.5 mm for nontransformed roots, and extensive lateral branching (Fig. 2A). The inoculation protocol induced a high percentage (65–75%) of hypocotyls to produce hairy roots. The wound position slightly affected the frequency of the transformation; the most susceptible part of the hypocotyl was close to the cotyledons. The number of hairy roots produced varied from three to six. Plants inoculated with a sterile needle did not form any roots at the wound site, and an uninoculated control did not form hairy roots.

PCR analysis of transgenic roots.

To show the presence of the Ri plasmid in the hairy root genome, a polymerase chain reaction (PCR) analysis of genomic DNA was performed. We used two pairs of primers specific for a 342-bp fragment of the *rol A* gene and a 512-bp fragment of the agropine gene. The presence of the *rol A* and the agropine gene fragments was demonstrated in DNA from all 20 individual hairy roots analyzed, five of which are shown in Figure 3. No such fragments were detected in DNA from untransformed roots. The absence of amplification in hairy root DNA, which appeared in *Agrobacterium* DNA when internal *virD1* primers were used, demonstrates that amplification was not due to contamination by *Agrobacterium* (data not shown).

Construction of *C. glauca* with transformed roots on untransformed shoots.

The construct p35S-*gusA-int* was used because the β-glucuronidase (GUS) gene (driven by the cauliflower mosaic virus [CaMV] promoter) contains a plant intron and so cannot be expressed by contaminating agrobacteria (Vancanneyt *et al.* 1990).

A histochemical analysis (see Materials and Methods) was performed on hairy roots (3–5 cm long) formed 3 weeks after infection with *A. rhizogenes* (p35S-*gusA-int*). Individual hairy roots were excised and stained for GUS activity; 15 out of a total of 32 hairy roots were stained. This observation indicates that about 50% of the hairy roots were cotransformed with the 35S-*gusA-int* gene from pBin19. Fluorometric GUS assays of 14 cotransformed hairy roots showed GUS activity of 2.3 ± 1.5 μmol of 4-methylumbelliferyl glucuronide (4-MUG) per min per mg of protein. Endogenous GUS activity was 5 ± 2.3 nmol of 4-MUG per min per mg of protein. Variation of GUS expression in transformed roots was probably the result of differences in the position of integration, methylation status, or copy number of the integrated GUS genes (van der Hoeven *et al.* 1994).

The distribution of GUS expression was determined in 15 transgenic roots from separate plants. We observed different expression patterns (Fig. 1C–E). Expression was strongest in the root tip cells, particularly in the meristematic region (Fig. 1C and D). The root caps and hairs (data not shown) were not stained. In three roots the GUS activity appeared to be confined to the central cylinder (Fig. 1E). The large variation in spatial distribution of GUS expression observed in hairy roots from separate plants was comparable to that in hairy roots derived from a single plant.

Nodulation of transformed hairy roots.

Three weeks after inoculation of the hypocotyls with *A. rhizogenes,* the seedlings were washed with cefotaxime (500 μg/ml) as described in Materials and Methods, and the original root was cut off. Composite plants consisting of transgenic roots on untransformed shoots were transferred to glass tubes containing a nutrient solution (Hoagland and Arnon 1938) supplemented with 17 mg of NH_4SO_4 per liter. Six weeks later, the growth tubes were replenished with N-free solution (Hoagland and Arnon 1938), and the plants were inoculated with *Frankia* strain Thr (Girgis *et al.* 1990). By 10–15 days after inoculation, infected roots zones swelled as a result of the proliferation and hypertrophy of root cortical cells. Infection tests showed that all inoculated nontransformed roots and 40% of the inoculated transformed roots formed nodules (two to five nodules per plant) within 3 weeks. A *rol A* fragment and an agropine fragment were amplified from six roots harboring nodules, indicating that both the TL and the TR regions of the Ri plasmid were present (data not shown).

After 6 months of growth, there was no significant difference between the lengths of the aerial parts of nodulated composite plants and those of composite plants grown in N medium. This suggested that the N_2-fixing potential of transformed nodules supplied sufficient nitrogen for plant growth. Nontransformed aerial systems with transformed roots exhibited distinctive traits in comparison to normal *C. glauca.* Figure 2 shows a 3-month-old composite plant with a highly branched aerial system. Generally, 6-month-old wild-type *C. glauca* plants were 10–16 cm tall, whereas composite plants were 4–7 cm tall. The number of branchlets on nontransformed plants was 17 ± 10; on composite *C. glauca,* the number was 64 ± 10.

Characterization of transgenic nodules.

No major difference in size and shape was observed between nodules developed on transformed roots (Fig. 1B) and those on untransformed roots (data not shown). Transformed and untransformed 4-week-old nodules exhibited comparable acetylene reduction activities (45 μmol of C_2H_4 per g [dry weight] of nodules per h.

The distribution of GUS expression in six randomly chosen transgenic nodules was determined histochemically. There was a high level of staining in the vascular bundle and in the

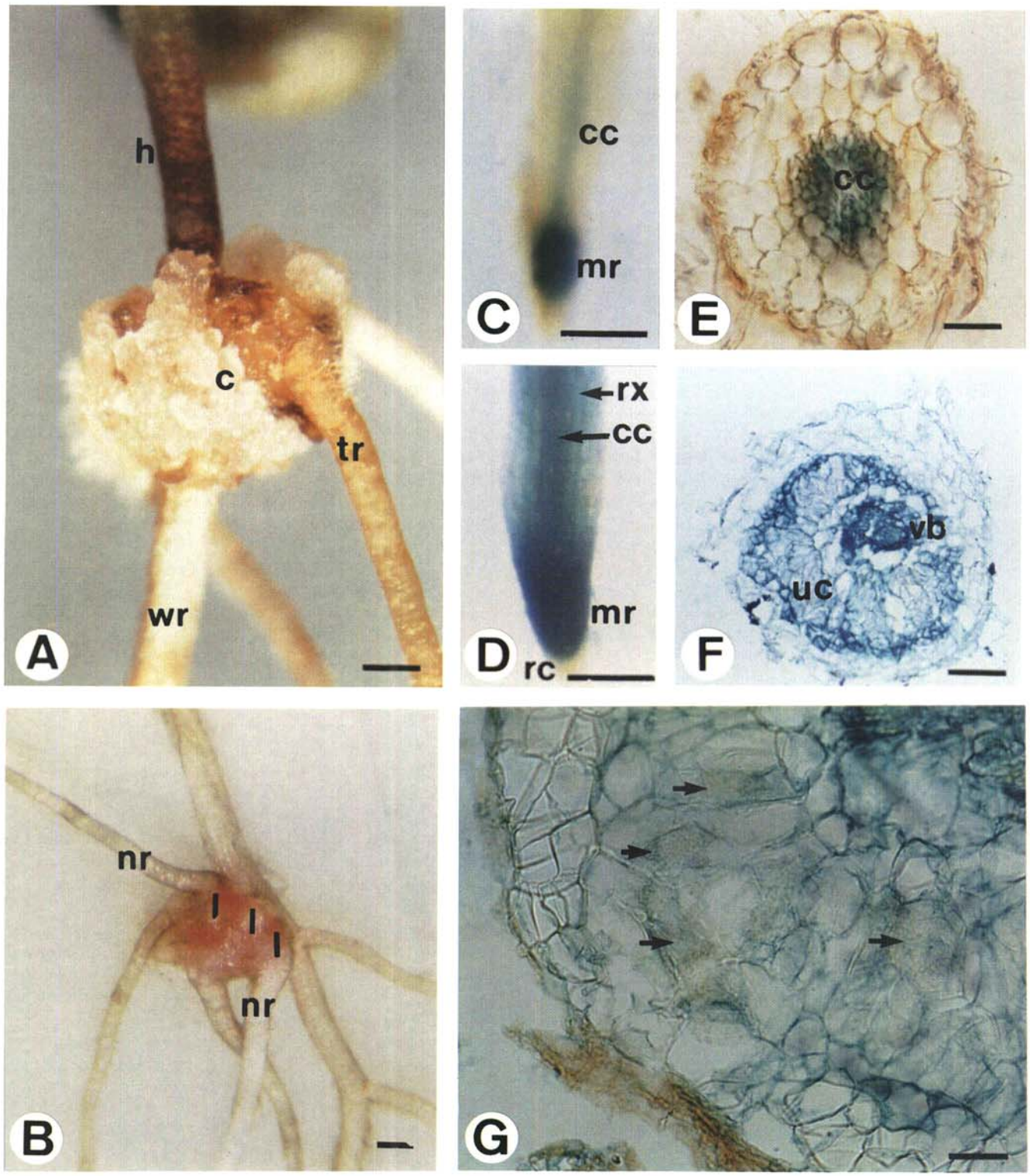

Fig. 1. A, Transgenic hairy root (tr) emerging from the callus (c) on a wounded *Casuarina glauca* hypocotyl (h), with an untransformed wild-type root (wr). Bar = 1 mm. **B,** *C. glauca* root nodule formed on a transgenic root. Plants were inoculated with *Frankia* strain Thr. The nodule has a multiple-lobe structure, with nodular roots (nr) growing from the lobes (l). Bar = 1 mm. **C–E,** Histochemical localization of β-glucuronidase (GUS) gene expression in transgenic roots of *C. glauca* transformed with the p35S-*gusA-int* gene construct. The blue staining identifies cells or tissues actively expressing the chimeric GUS gene. Various transgenic roots from *C. glauca* were stained for GUS enzyme activity. **C,** Strong staining in the meristematic region (mr) and central cylinder (cc). Bar = 1 mm. **D,** Strong staining in the meristematic region (mr), central cylinder (cc), and root cortex (rx). No GUS activity is visible in the root cap (rc). Bar = 1 mm. **E,** Cross section of a transgenic root with staining in the central cylinder (cc). Bar = 200 μm. **F** and **G,** Histochemical localization of GUS gene expression in a nodular lobe of a transgenic 3-week-old *C. glauca*. **F,** Low-magnification light micrograph of whole young nodule. Strong staining is observed in the vascular bundle (vb) and in nodule cortical cells (uc). Bar = 400 μm. **G,** High-magnification micrograph of young nodule section. No staining is visible in *Frankia*-infected cells (arrows). Bar = 100 μm.

cortical zone but not in the infected cells (Fig. 1F and G). No staining was observed in the rhizodermis (Fig. 1F and G).

The specific GUS activity in tissue extracts from six transgenic root nodules was 2.5 ± 1.1 μmol of 4-MUG per min per mg of protein; this is comparable to the levels of GUS expression in cotransformed hairy roots.

DISCUSSION

The wounding of *C. glauca* hypocotyls with *A. rhizogenes* strain A4RS produced fast-growing, highly branched roots. The roots were shown to contain both TL- and TR-DNA sequences. We established the possibility of nodulating the transformed roots after inoculation with *Frankia* strain Thr. About 50% of the transgenic roots were cotransformed with the *gusA-int* reporter gene from pBin19. The expression of the reporter gene under the control of the 35S promoter was studied in transgenic roots and nodules. The possibility of obtaining transformed nodules within 4 months offers a significant shortcut, compared to the use of transformed plants obtained through a regeneration cycle. *Allocasuarina verticillata,* another actinorhizal tree in the family Casuarinaceae, was regenerated from hairy root cultures in approximately 1 year.

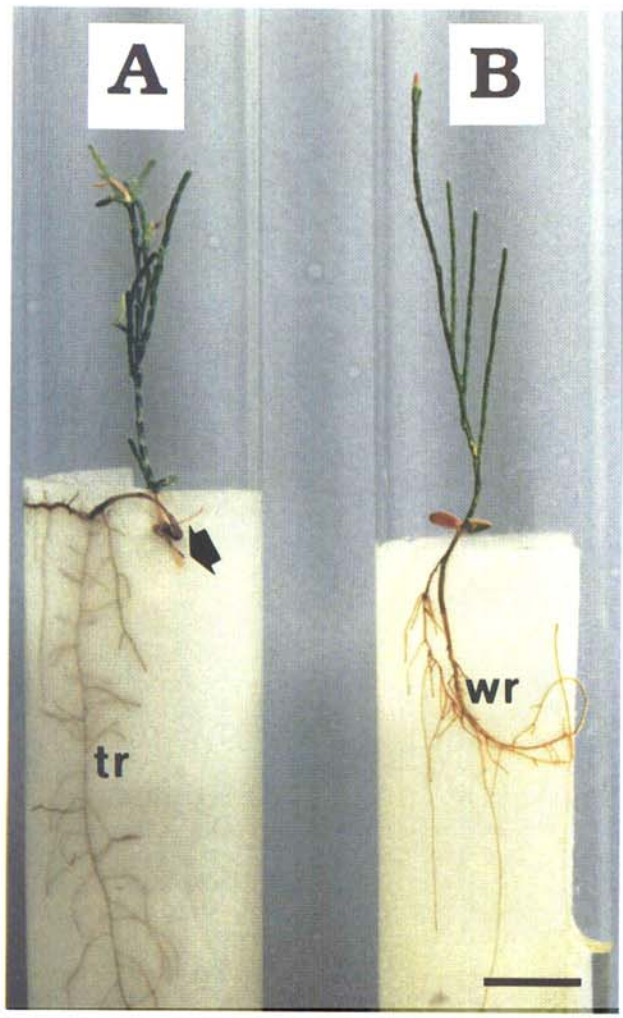

Fig. 2. Three month-old *Casuarina glauca.* **A,** Composite plant, with untransformed aerial system and transformed roots (tr). The arrow indicates where the hypocotyl was cut. **B,** Untransformed *C. glauca,* with wild-type roots (wr). The roots induced by *Agrobacterium rhizogenes* strain A4RS showed a typical hairy root phenotype, with extensive lateral branching. The aerial system of the composite plant exhibited an abnormal phenotype, characterized by a tendency to branching. Bar = 1 cm.

Previous work with legumes and other plants has shown that plant hormones might be involved in root nodulation (Wheeler and Henson 1978; Hirsch and Fang 1994). Among the composite *C. glauca* plants with transformed roots, only 40% formed nodules when inoculated with *Frankia,* whereas nodulation was 100% in the control plants. Two groups of pRi genes are involved in the root induction process, the *rol* genes, located in the TL region, and the genes for auxin and agropine biosynthesis, in the TR region (White *et al.* 1985). We carried out PCR experiments to determine if the presence of the TL or TR region was correlated with altered nodulation frequency in transformed roots. In all individual roots tested, both *rol A* and agropine gene fragments were present. Such transfer of both T-DNAs is also observed in transformed *Brassica napus* (Jouanin *et al.* 1987) and transformed roots of cucumber (Amsalem and Tepfer 1992). Since we have observed both *rol A* and agropine genes in transformed nodulated and unnodulated roots, we cannot conclude that one of the Ri T-DNA segments influences the nodulation frequency.

The abnormal phenotype observed in untransformed aerial systems with transformed roots might also be explained by the presence of TL-DNA or TR-DNA (or both) in transformed root tissue. The use of *A. rhizogenes* strains with TL- or TR-deleted Ri plasmids is required before we can hypothesize on how these T-DNAs influence root nodulation and the phenotype of the aerial systems. Beach and Gresshoff (1988) reported that nodulation by *Rhizobium* was inhibited in *A. rhizogenes*–transformed roots of forage legumes. However, when nodules were found on transformed roots of *C. glauca,* the number of nodules per plant and the appearance of macroscopically visible nodules were comparable to those of normal control plants. Nodules from untransformed roots and

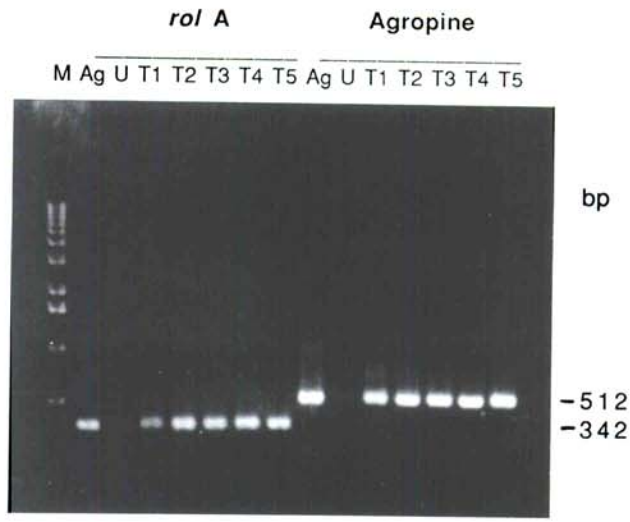

Fig. 3. Agarose gel electrophoresis of amplified *rol A* and agropine gene fragments. Polymerase chain reaction (PCR) products of genomic DNA from *Agrobacterium rhizogenes* strain A4RS (Ag) and from untransformed (U) and transformed *Casuarina glauca* roots (T1–T5). DNAs were primed with oligonucleotides specific to the *rol A* or agropine gene sequences. Lane M is a 1-kbp ladder (Bethesda Research Laboratories, Gaithersburg, MD). The numbers on the right give the size (in bp) of the expected amplified fragments.

those from transformed roots were found to be no different in morphology or in nitrogenase activity.

We localized GUS activity in transgenic roots and nodules carrying the 35S-*gusA-int* gene fusion. In roots, the highest GUS activity was found in cells of the meristematic zone. The cortex and central cylinder also stained intensely, particularly in younger lateral roots. The same staining pattern has been reported for the "constitutive" CaMV 35S promoter with a number of transgenic plants (Jefferson *et al.* 1987; Forde *et al.* 1989; Terada and Shimamoto 1990). In nodules, the strong staining associated with the vascular bundle may reflect the correlation between the 35S promoter activity and the actively dividing tissues (Nagata *et al.* 1987; Jefferson *et al.* 1987; Benfey *et al.* 1990). The staining seen in vascular tissue of transgenic *C. glauca* nodules is similar to that observed in transgenic nodules of the legumes *L. corniculatus* (Forde *et al.* 1989; Szabados *et al.* 1990) and *Vicia hirsuta* (Quandt *et al.* 1993). While 35S GUS activity was detected in *Rhizobium*-infected cells of legume nodules, no GUS activity was observed in *Frankia*-infected cells of *C. glauca.* Further histochemical analysis of *C. glauca* nodules will be required to explain why GUS activity cannot be detected by staining in infected cells.

With the use of *A. rhizogenes,* transgenic nodules can be generated efficiently and quickly on composite *C. glauca.* This procedure will make it possible to study the specific regulatory mechanisms that control the expression of actinorhizal symbiotic genes. We are interested in using this system to determine the expression of symbiotic genes of legumes in transgenic nodules of *C. glauca.* The comparison of the regulatory mechanisms of legume and actinorhizal symbiotic genes may provide new insight into the evolution and the relationship of these two symbiotic systems.

MATERIALS AND METHODS

Agrobacterium strain and plasmid.

A. rhizogenes A4RS (Jouanin *et al.* 1986) was used to induce hairy root. The p35S-*gusA-int* construct, containing the promoter of the 35S transcript of CaMV and the intron-containing GUS gene, was provided by G. Vancanneyt (Vancanneyt *et al.* 1990). The binary vector was introduced into strain A4RS by triparental mating (Koncz and Schell 1986). Transconjugants were selected in the medium described by Petit and Tempé (1978) amended with 50 mg of kanamycin and 50 mg of rifampicin per liter.

Wound site infections.

C. glauca seeds were scarified with 95% sulfuric acid for 5 min, washed with water for 30 min, and disinfected with calcium hypochlorite (5%, w/v, in water) for 30 min. Surface-disinfected seeds were rinsed in sterile water for 5 min and then were germinated in quarter-strength Hoagland and Arnon medium (Hoagland and Arnon 1938) supplemented with 17 mg of $(NH_4)_2SO_4$ per liter and grown on 0.8% agar plates. Petri dishes were sealed with Parafilm and placed in a growth cabinet in a flat position. One month after germination, plants were wounded on the hypocotyls with a needle (12 × 0.45 mm) dipped in *A. rhizogenes* that had been freshly grown on solid AB plates (Petit and Tempé 1978) at 28° C for 2 days. The plants were kept vertical for 3 weeks, in sealed petri dishes, so that hairy roots could develop. The medium consisted of a half-strength Monnier medium (Monnier 1976) solidified with 1.2% agar. Growth conditions were 26° C, a day-night cycle of 16 and 8 h, and light (50 $\mu E \cdot m^{-2} \cdot s^{-1}$) provided by daylight fluorescent tubes (Philips-TL, 40/55 W). Seeds of *C. glauca* were supplied by the Desert Development Center, Saddat City, American University, Cairo, Egypt.

Growth and nodulation of composite *C. glauca.*

Three weeks after wounding of the hypocotyls, hairy roots had grown to a length of 3–5 cm. The plants were decontaminated in half-strength Monnier medium (Monnier 1976) with cefotaxime (500 μg/ml) (Roussel) for 2 days. After being shaken (60 rev/min) in sterile water for 1 h, the primary root system was excised with a scalpel, and the composite plants were transferred to test tubes, thus permitting shoots to grow outside (Vincent 1970). Plant cultivation and inoculation with *Frankia* were carried out as described by Diem *et al.* (1983). Plants were inoculated with inoculum consisting of *Frankia* strain Thr (Girgis *et al.* 1990) grown in modified BAP medium (Benoist *et al.* 1992) for 4 days, washed twice with distilled water, homogenized, and resuspended in sterile water; 1 ml of packed cell per plant was applied by pipetting the *Frankia* suspension directly onto the plant roots. Four weeks after the appearance of nodules, acetylene reduction activity in whole root systems was assessed according to the method described by Hardy *et al.* (1968). Individual root systems and nodules were assayed for GUS activity by the fluorometric method to identify transgenic roots and nodules, which were then analyzed more thoroughly.

Histochemical and fluorometric GUS assay.

Histological assays for GUS expression were performed with X-Gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide) as a chromogenic substrate (Jefferson *et al.* 1987). Freshly harvested transgenic root nodules were cut into 10-μm sections with a cryomicrotome prior to incubation overnight at 28° C in a GUS assay mixture (Bogusz *et al.* 1990). A fluorometric GUS assay was processed as described by Jefferson *et al.* (1987). Protein concentration was determined by the method of Bradford (1976).

PCR analysis of transgenic roots.

The presence of the *rol A* and the agropine gene sequences in transgenic roots was determined by PCR analysis of genomic DNA isolated from individual roots. Genomic DNA was isolated as described by Bousquet *et al.* (1989). For the *rol A* gene, two oligonucleotide primers (5′-GGAATTAG-CCGGACTAAACG-3′ and 5′-CCGGCGTGGAAATGAA-TCG-3′) were designed according to the *rol A* sequence (Slightom *et al.* 1986) and resulted in a 342-bp fragment. For the agropine gene (Bouchez *et al.* 1991), the two primers 5′-GCGCATCCCGAGGCGATG-3′ and 5′-AGGTCTGGCG-ATCGCGAGGA-3′ gave rise to a 512-bp fragment. In order to check for the absence of contaminating bacteria on transformed roots, we carried out PCR using primers designed to amplify a fragment of the *virD1* gene, which is located outside the T-DNA region of the Ri plasmid. The two primers 5′-ATGTCGCAAGGACGTAAGCCCA-3′ and 5′-GGAGTC-TTTCAGCATGGAGCAA-3′ allowed amplification of a 0.45-kb sequence from the *virD1* gene (Hamill *et al.* 1991). Amplification was performed with *Taq* polymerase (Apligene) in

a three-temperature program (94° C for 1 min, 55° C for 1 min, and 72° C for 2 min) with 35 cycles.

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