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TECHNICAL ADVANCE

Genetic transformation of the actinorhizal tree *Allocasuarina verticillata* by *Agrobacterium tumefaciens*

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Summary

We have developed an efficient transformation system for the tropical actinorhizal tree *Allocasuarina verticillata* using *Agrobacterium tumefaciens* mediated gene transfer. Mature zygotic embryos were inoculated with the disarmed strain C58C1 carrying, in the binary vector BIN19, the *nptII* gene, providing kanamycin resistance as a selectable marker, and the reporter gene β-glucuronidase containing an intron. The transformed embryos were cultivated on nutrient medium supplemented with 0.5 μM NAA, 2.5 μM BA, 100 mg l^{-1} kanamycin and 250 mg l^{-1} cefotaxime. After 2 months, a 21% transformation frequency was obtained. Within 6–9 months, transgenic plants were recovered from 70% of the transformed calli. The presence of the transgenes was demonstrated by PCR analysis and by the expression of the β-glucuronidase; integration of the T-DNA was confirmed by Southern hybridization. More than 100 transgenic plants from a total of 23 independent transformation events have been successfully established in soil. The possibility to obtain nitrogen-fixing nodules after inoculation of transgenic *A. verticillata* plants by the actinomycetal strain of *Frankia* Allo2 was established.

Introduction

Allocasuarina verticillata Lam. belongs to the family *Casuarinaceae*, which consists of about 96 species of trees and shrubs native to Malaysia and the Australo-Pacific region. *Casuarinaceae* are multipurpose pioneer tree species and have a wide range of uses, from environmental protection to fuelwood production (Dawson, 1990; National Research Council, 1984). *A. verticillata* has the ability to grow vigorously on poor coastal sands and saline soils. This capacity for rapid growth comes from a symbiotic association with an actinomycete of the genus *Frankia*, which allows *A. verticillata* to fix dinitrogen in root nodules, so-called actinorhizae (Benson and Silvester, 1993).

Actinorhizae are found in at least 20 other angiosperm genera that belong to eight different families. Little is known about the biochemistry and molecular biology of the infection process and nodule formation in actinorhizal plants. Evidence for nodule-specific gene expression in two actinorhizal plants, *Alnus glutinosa* and *Casuarina glauca*, has been recently reported (Gherbi *et al.*, 1996; Goetting-Minesky and Mullin, 1994; Jacobsen-Lyon *et al.*, 1995; Ribeiro *et al.*, 1995). Studying the similarities and differences between these actinorhizal symbioses and the *Rhizobium*–legume symbiosis is of great interest since it could lead to a better understanding of the evolution of symbiotic associations in general.

Transgenic plants are valuable tools in exploring the regulation of plant symbiotic gene expression (de Bruijn *et al.*, 1990). The capacity of actinorhizal trees for transformation has been reviewed by Sederoff (1995). Tumours were obtained after genetic transformation of *Alnus incana* and *Alnus glutinosa* by wild-type *A. tumefaciens* strains (Mackay *et al.*, 1988). Transient expression of the β-glucuronidase gene under the control of the 35S promoter was observed after electroporation of *A. incana* protoplasts (Seguin and Lalonde, 1988). Up to now the only transgenic actinorhizal plant reported in the literature was obtained when *A. verticillata* was transformed by *A. rhizogenes* (Phelep *et al.*, 1991).

This paper reports that *Agrobacterium tumefaciens* can be used successfully to transfer foreign genes from a binary vector into *Allocasuarina verticillata*. Transgenic plants were recovered, and we obtained nitrogen-fixing actinorhizae expressing the β-glucuronidase gene under the control of the 35S promoter.

Results

Tolerance of Allocasuarina verticillata *to selective agents*

Allocasuarina verticillata sensitivity to kanamycin (KM) and cefotaxime (CFX) was established prior to transformation experiments in order to determine the effective concentration for selecting transformed cells and eliminating *Agrobacterium*. In the absence of antibiotics, green compact calli developed after 1 week on all the wounded

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embryos, and bud differentiation was observed within 2–4 weeks on all the calli. After 2 months, shoots with an average length of 2–3 cm were observed. The presence of 50 mg l^{-1} KM inhibited shoot regeneration, but the embryos expanded before bleaching (data not shown). Hence, a concentration of 100 mg l^{-1} was used for selection. Higher kanamycin concentrations caused immediate browning of the embryos.

A concentration of 250 mg l^{-1} CFX was sufficient to inhibit *Agrobacterium* C58C1(GV2260; pBinGUSint) growth and had no influence on the frequency of bud and shoot regeneration. At 500 mg l^{-1} CFX, callus growth and shoot elongation were slightly reduced (data not shown). Therefore, antibiotic selection to identify putative transformed *A. verticillata* calli was performed in the presence of 100 mg l^{-1} KM and 250 mg l^{-1} CFX.

Transformation of A. verticillata *mature embryos*

Three weeks after cocultivation with *Agrobacterium* C58C1(GV2260; pBinGUSint), 21% of the transformed embryos exhibited green organogenic calli growing in the presence of kanamycin on the wounded tissues. This low frequency of transformation was due to the poor germination rate of *A. verticillata* seeds; usually less than 30% of the seeds could germinate. Eighty-five to 90% of calli growing on kanamycin were found to express the *uidA* gene, as determined by histochemical analysis (Figure 1b); no indigo blue colour was detected in control calli (data not shown). After 2 months, around 10–20 buds were present on 70% of the putatively transformed calli. Although some variation was observed within calli, resulting from different transformation events, callus growth and bud differentiation was usually slightly slower when antibiotics were present in the regeneration medium. Non-transformed embryos did not produce calli on plates containing antibiotics (data not shown).

Regeneration of kanamycin-resistant shoots

Twenty-three transgenic calli, resulting from three separate transformation experiments, were studied further. After 2 months of culture, they suffered from hyperhydricity with shoots becoming thick, translucent and brittle. The use of 40 g l^{-1} sucrose (instead of 20 g l^{-1}) and 8% Bactoagar (instead of 7%), and the replacement of plastic caps by cotton caps greatly reduced vitrification.

After 2–4 months, kanamycin-resistant calli exhibited an average of 10 shoots approximately 1.5–2 cm tall. These calli were transferred on MS medium containing kanamycin and cefotaxime, but deprived of growth regulators. Three weeks later, shoots had an average length of 5–6 cm.

Rooting of kanamycin resistant shoots and transplantation

Shoots between 4 and 6 cm long were excised from the calli cultured without growth regulators, transferred for 3 days in MS medium containing 25 μM NAA, and rooted in liquid MS medium containing 40 g l^{-1} sucrose. The first roots were observed 3 days after the auxin treatment. After 1 month, 96% of the transgenic shoots and 100% of the control shoots had developed one to three roots with an average length of 3.5 cm. The phenotype of the putatively transformed plants was found to be similar to those of the control plants (Figure 1c).

One month after the rooting treatment, 139 transformed plants regenerated from the 23 independent kanamycin resistant calli were transferred into a glasshouse. Eighty-three percent survived to this transfer (Figure 1d). Some of these plants have been grown for more than 1 year in the glasshouse.

β-glucuronidase gene expression in transgenic A. verticillata *plants*

A histochemical assay for β-glucuronidase activity demonstrated the presence of blue coloured cells in both the stems and the roots of the putatively transformed *A. verticillata* plants (Figure 2). A constitutive *uidA* expression was observed in shoots (Figure 2a), whereas in roots, reporter gene activity was found to be stronger in the vascular tissue and meristematic region. The root tips stained more intensely than any other region of the roots (Figure 2c). Young lateral roots were characterized by a low expression of the *uidA* gene (Figure 2d). Quantitative data and detailed cytological analysis of the transgenic *A. verticillata* trees expressing the CaMV 35S promoter will be published elsewhere (Franche, Diouf and Duhoux, manuscript in preparation). β-glucuronidase expression was never detectable in stems or roots (Figure 2b–e) of control plants, thus indicating that *A. verticillata* had no or very low background of GUS activity.

Molecular analysis of regenerated plants

A PCR analysis was used to detect the presence of T-DNA in 20 transgenic *A. verticillata* plants; data on four of them are shown in Figure 3. Primers to the *nptII* and *uidA* genes gave the expected 736 bp (*nptII*) and 574 bp (*uidA*) PCR bands (Figure 3a and b, lanes 4–7). In addition, we conducted parallel PCR analyses with primers specific for the *virD1* gene of *Agrobacterium tumefaciens*. Because these sequences are present on the resident Ti plasmid, but are not transferred to the plant, they provide a control for the presence of contaminating vector bacteria (Hamill *et al.*, 1991). DNA from putatively transformed plant tissue ampli-

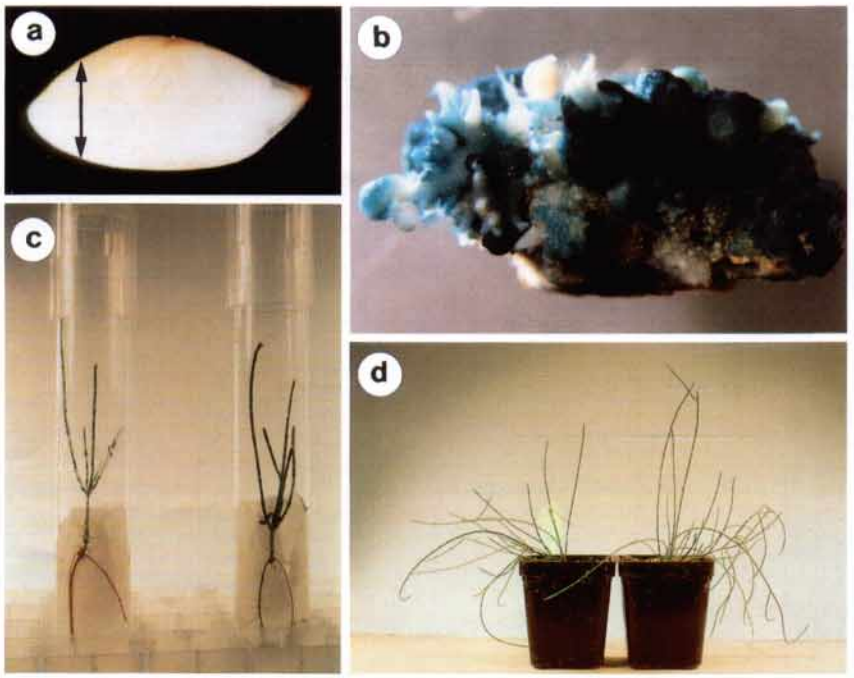

Figure 1. Regeneration of transgenic *A. verticillata* plants after genetic transformation by *A. tumefaciens* C58C1(GV2260; pBinGUSint).
(a) Mature zygotic embryo of *A. verticillata*. The black arrow indicates the site of wounding on the cotyledons.
(b) β-glucuronidase expression in a 1-month-old callus growing on kanamycin.
(c) Non-transformed (left) and transgenic (right) rooted *A. verticillata* plants.
(d) Transgenic plants in a glasshouse.

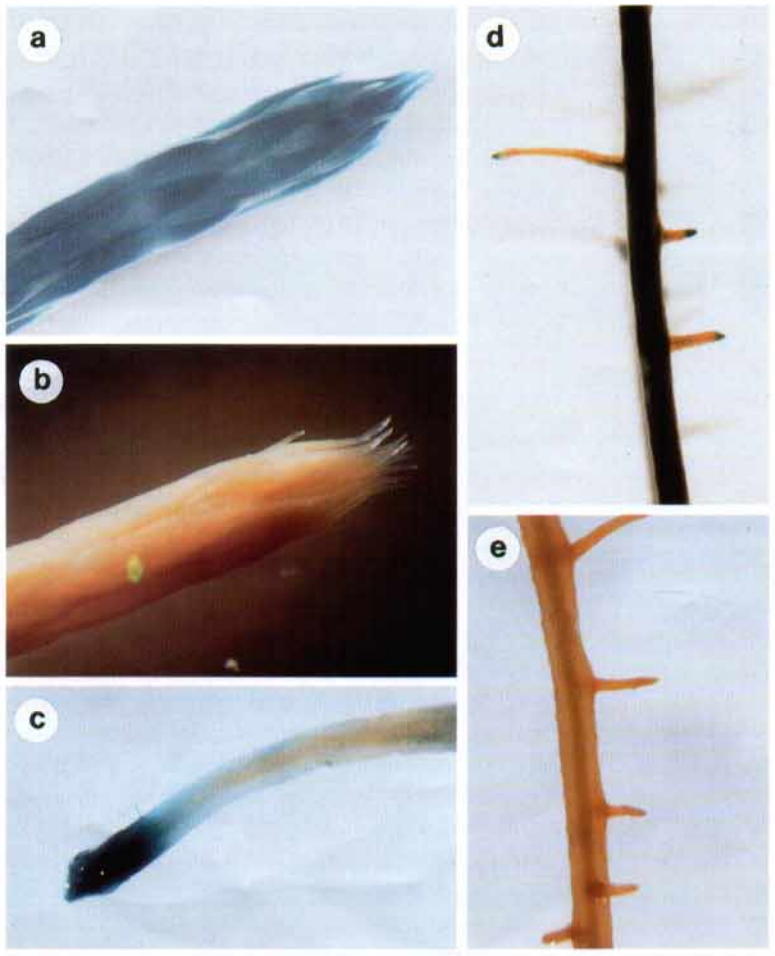

Figure 2. Histochemical staining for β-glucuronidase activity in transformed *A. verticillata* plants.
(a) Transformed shoot.
(b) Non-transformed shoot from a control plant.
(c) Transformed root tip.
(d) Transformed root.
(e) Non-transformed root from a control plant.

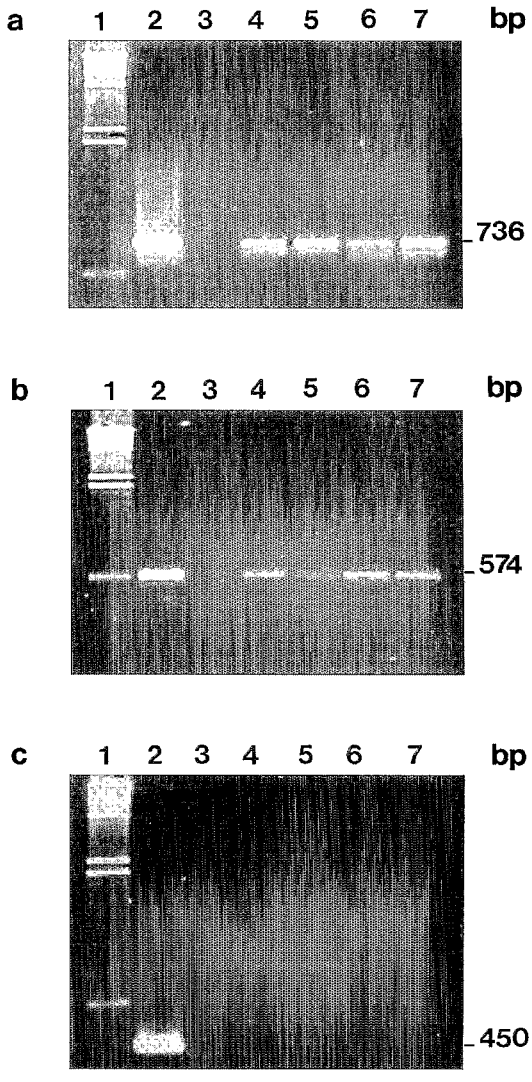

Figure 3. PCR analysis of *A. verticillata* plants transformed by *A. tumefaciens* C58C1(pGV2260; pBinGUSint). DNAs were primed with oligonucleotides specific to the *nptII* (a) and *uidA* (b) genes or to the *virD1* gene of *A. tumefaciens* (c).
(1) *Hin*dIII digested λ DNA.
(2) Positive control C58C1(pGV2260; pBinGUSint).
(3) Untransformed control plant.
(4)–(7) Putatively transformed plants growing on kanamycin.

fied with the *vir* region primers never gave rise to an amplification product (Figure 3c, lanes 4–7). This result indicates that the fragments amplified with the *nptII* and *uidA* primers have not been caused by continuous *Agrobacterium* contamination.

To provide further physical evidence for the integration of foreign DNA into an *A. verticillata* genome, a Southern blot analysis of *Hin*dIII and *Eco*RI restriction digests of genomic DNA from shoots from five putative transgenic plants was performed with the β-glucuronidase gene serving as a probe. Hybridization was observed in all putatively transformed plants, but not in DNA from the control plant. Transformed plants exhibited a 2.8-kbp

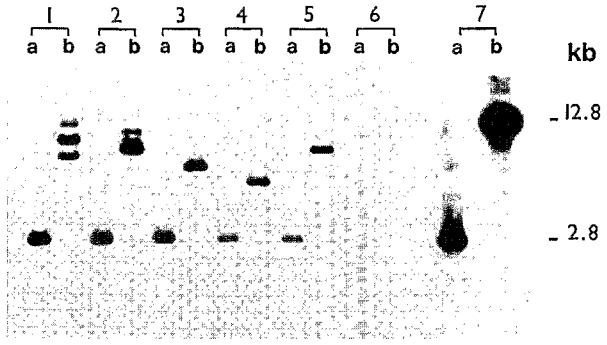

Figure 4. Southern blot analysis of five transformed *A. verticillata* plants. Genomic DNA were digested by *Hin*dIII or *Eco*RI.
(a) *Hin*dIII digests.
(b) *Eco*RI digests.
(1)–(5) Genomic DNA from five putatively transformed *A. verticillata* plants.
(6) Genomic DNA from a non-transformed *A. verticillata* plant.
(7) DNA from *A. tumefaciens* C58C1(pGV2260; pBinGUSint).
DNA were hybridized to a 574-bp fragment containing the β-glucuronidase coding region.

*Hin*dIII hybridizing band (Figure 4, lanes 1a–5a), which is the expected size of the *Hin*dIII fragment of the pBinGUSint containing the *uidA* gene (Figure 4, lane 7a). The unique *Eco*RI site, located 2.4 kbp to the right of the left border on the plasmid pBin19GUSint (Vancanneyt *et al.*, 1990), was used to discriminate integrated and non-integrated forms of the vector DNA. After digestion with *Eco*RI of total plant DNA and hybridization with the *uidA* probe, the Southern blot would be expected to give a single 12.8-kbp fragment for a non-integrated vector molecule (Figure 4, lane 7b). The presence of fragments of less than 12.8 kbp confirms the insertion of the T-DNA into the *A. verticillata* genome (Figure 4, lanes 2b–5b). The *uidA* probe did not bind to untransformed control plant DNA (Figure 4, lanes 6a and 6b).

Nodulation of transgenic plants by Frankia

Twenty-eight transgenic plants regenerated from eight independent calli and 13 control plants regenerated from seven non-transformed calli were transferred to a glasshouse and inoculated by *Frankia* Allo2. Two months after inoculation, 78.6% of the transgenic *A. verticillata* plants had developed one to 13 nodules (3.3 nodules on average), which exhibited one to 34 lobes (Figure 5a). The same number of nodules was observed on 84.6% of the control plants. Ten nodules were sliced with a scalpel into 1 mm fragments to allow the penetration of X-gluc. Indigo-blue crystals were visible in the central cylinder and the cortex of the nodules (Figure 5b and d); no β-glucuronidase activity was visible in the periderm and the endoderm. *Frankia*-infected cells appeared either pale blue or white, suggesting that the reporter gene activity was lower than in the actinomycete free cells. No blue colour was observed when non-transformed nodules were incubated in the

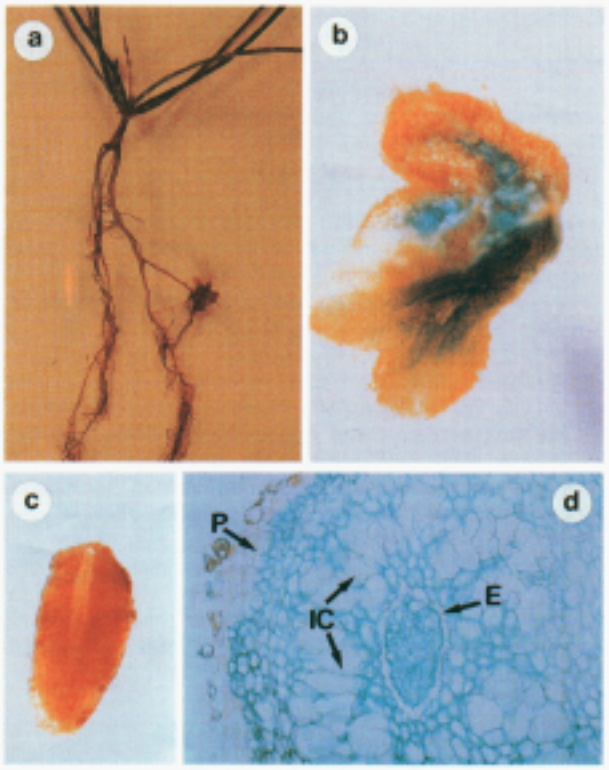

Figure 5. Nodulation of transgenic *A. verticillata* plants.
(a) Transgenic *A. verticillata* plant with root nodules induced by *Frankia* Allo2.
(b), (c), (d) β-glucuronidase expression in transgenic nodule (b, d) and non-transformed nodule lobe (c). Nodule sections were treated with X-gluc and photographed with a binocular (b)–(c) or with a microscope (d). E: endoderm; P: periderm; IC: *Frankia*-infected cells.

presence of X-gluc (Figure 5c), nor when 1 mM X-gluc was added to a pure culture of *Frankia* Allo2 (data not shown).

Two months after inoculation by *Frankia*, acetylene reduction activity was compared in transgenic nodules from three transformed plants and in untransformed nodules from three control plants. Values were similar: 25.1±4.4 mmol of C_2H_4 per g (dry weight) of nodules per hour for the transformed plants and 33.7±11.6 for the control plants.

Discussion

Transgenic *A. verticillata* plants were recovered through the transformation of mature zygotic embryos by *A. tumefaciens* C58C1(GV2260; pBin19GUSint). The regeneration protocol reported in this study is simple since it only requires one medium and produces shoots in 2–4 months. Continuous shoot proliferation was obtained on the transgenic calli, and within 6 months, 10–20 transgenic rooted plants were recovered from each callus growing on kanamycin.

In a previous study, our laboratory reported the genetic transformation of *A. verticillata*, using the wild-type *A. rhizogenes* strains A4 and 2659 (Phelep *et al.*, 1991). Transgenic plants regenerated from hairy roots had an abnormal phenotype characterized by reduced apical dominance of the shoots, and growth of highly branched and ageotropic roots. Conversely, plants obtained after transformation by *A. tumefaciens* displayed no morphological aberrations.

The transformation frequency obtained with the strain C58C1(GV2260; pBin19GUSint) was 21% of the total number of *A. verticillata* embryos. Since only 25–30% of *A. verticillata* seeds could germinate further, the transformation rate of viable embryos must be around 70%. Various factors, such as plant genotype, strain of *Agrobacterium*, time of cocultivation and composition of the cocultivation medium, are known to affect the efficiency of gene transfer by *Agrobacterium* [for a review, see Hooykaas and Schilperoort (1992)]. In this paper, the optimal transformation conditions for T-DNA transfer to *Casuarina glauca*, a related actinorhizal tree, were used on *A. verticillata* (Franche *et al.*, 1994; Le *et al.*, 1996). Therefore, it is likely that the efficiency of *Agrobacterium* gene transfer into *A. verticillata* could be improved by studying some of the transformation factors with this new tree species.

Wounded mature zygotic embryos of *A. verticillata* appear as valuable targets for the T-DNA transfer by *A. tumefaciens* and calli developing on these embryos have a good regeneration ability. Using C58C1(GV2260; pBin19-GUSint) on cotyledons, epicotyls and hypocotyls excised from 30-day old seedlings of *C. glauca*, we previously reported the differentiation of transgenic buds on transformed calli (Le *et al.*, 1996). Nevertheless, shoot growth was never observed, and regeneration of transgenic *C. glauca* trees was not achieved. The use of wounded embryos for transformation/regeneration of *C. glauca* is currently being investigated.

The 35S promoter of cauliflower mosaic virus is the most widely used promoter for plant transformation. Although it has been reported as a constitutive sequence which is essentially expressed in all plant parts, its level of expression has been shown to be partly organ specific and dependent on the cell cycle and the host plant (Nagata *et al.*, 1987; Benfey *et al.*, 1990). In *A. verticillata*, a constitutive expression was observed in the shoots, whereas in roots and nodules, differences in staining suggested a certain degree of tissue-specific expression. Nevertheless, any interpretation of differences in staining should take into account a number of factors, such as cell size, degree of vacuolation, substrate accessibility and the level of transcription (Jefferson, 1987).

We established the capability of *A. tumefaciens*-transformed adventitious roots of *A. verticillata* to be nodulated with *Frankia* and to fix nitrogen. Nodulation efficiency was found to be the same for both transformed plants and seed-germinated control plants. On the other hand, we

recently reported that only 40% of transformed hairy roots induced on *C. glauca* by *A. rhizogenes* A4RS could be nodulated by *Frankia,* whereas 100% of the control plants developed nodules (Diouf *et al.*, 1995). This suggested that the *rol* genes and/or the *A. rhizogenes* auxin genes could interfere with the nodulation process by modifying the host plant hormone balance.

Transgenic *A. verticillata* containing chimeric actinorhizin promoters fused to reporter genes will be valuable tools in studying the regulation of actinorhizal gene expression in nodules, roots and shoots. They make it possible to identify *cis*-acting elements in nodule-specific or nodule-enhanced gene induction. They may also help in assigning a defined function to actinorhizins, using antisense experiments, and in studying early signal exchanges between actinorhizal plants and *Frankia.* Furthermore, using a disarmed strain of *A. tumefaciens* for transferring foreign genes into *A. verticillata* paves the way for the introduction of agronomically useful traits into *Casuarinaceae* trees, which play an important role in reforestation and soil reclamation in developing countries.

Experimental procedures

Plant material

Allocasuarina verticillata seeds were collected in Australia and obtained from Versepuy Company (Le Puy-en-Velay, France). Seeds were treated for 2 min by H_2SO_4 and washed for 30 min under running tap water. Then they were disinfected with 5% calcium hypochlorite for 30 min, and washed three times with sterile water. They were left overnight in the dark in sterile water. A wound was then made on the cotyledons with a scalpel (Figure 1a), the seed coats were peeled off and the embryos were transformed by *Agrobacterium tumefaciens* as described below.

Plant culture media

The MS regeneration medium consisted of Murashige and Skoog (1962) macro and micro-elements supplemented with Nitsch and Nitsch (1965) vitamins, 20 g l^{-1} sucrose, pH 5.6, and 0.7% Difco Bactoagar. NAA (0.5 μM) and 2.5 μM BA were added as growth regulators and the medium was then autoclaved for 20 min at 120°C. The explants were cultured under 50 μmol m^{-2} s^{-1} provided by cool fluorescent tubes (daylight Sylvania 36W/GRO) at 26°C with a 16 h photoperiod. Regenerating shoots were subcultured at intervals of 3 weeks.

Tolerance of A. verticillata *to selective agents*

The kanamycin and cefotaxime sensitivity of *A. verticillata* was tested by culturing 50 non-transformed mature zygotic embryos in MS medium containing NAA, BA and varying levels of antibiotics (KM: 0, 25, 50, 100, 200 mg l^{-1}; CFX: 0, 100, 250, 500 mg l^{-1}). After 2 months, callus growth and bud development were scored.

Agrobacterium *strain and transformation procedure*

A. tumefaciens strain C58C1(GV2260) (Vancanneyt *et al.*, 1990), containing the binary vector pBinGUSint which carries the marker genes *uidA* and *nptII,* was used for transformation experiments. The agrobacteria were grown overnight at 28°C in Ag medium (0.5 g l^{-1} K_2HPO_4, 0.2 g l^{-1} $MgSO_4,7H_20$, 0.1 g l^{-1} NaCl, 5 g l^{-1} mannitol, 1 g l^{-1} yeast extract, 1 g l^{-1} casaminoacid, pH 7.2) containing 50 mg l^{-1} KM and 10 mg l^{-1} rifampicin. When used in transformation experiments, the bacterial suspension was diluted to approximately 10^8 cells ml^{-1} in MS medium ($OD_{600\ nm}$=0.1).

For each transformation experiment, 150 embryos were scalpel-wounded and immersed for 1 h in the diluted *Agrobacterium* C58C1(GV2260; pBinGUSint) suspension. After blot-drying between sterile filter papers, explants were placed on solidified MS medium containing 25 μM acetosyringone, 0.5 μM NAA and 2.5 μM BAP for 3 days. The *A. verticillata* embryos were then rinsed three times, for 1 h each time, with sterile MS medium containing 250 mg l^{-1} CFX (Claforan, Roussell) to eliminate the *Agrobacteria,* blotted dry on sterile filter paper and placed on the MS regeneration medium containing 100 mg l^{-1} KM and 250 mg l^{-1} CFX. In each transformation experiment, 25 untransformed wounded embryos were placed on MS regeneration medium without antibiotics (control for regeneration) and on MS regeneration medium with antibiotics (control for antibiotic selection).

Selection and regeneration of transformants

Calli emerging from the cut ends of the wounded embryos were excised about 3–6 weeks after cocultivation with *Agrobacterium,* and transferred to glass tubes (2.5 cm in diameter, 20.5 cm in length) for shoot regeneration. Shoots were removed from the callus regularly and transferred to a rooting medium. Regenerated plants were transplanted into pots containing a sterilized 1:1 (v/v) mixture of sand–vermiculite (one plant for every 12-cm-diameter pot) and acclimatized under a plastic cover for 10 days before gradual exposure to growth chamber conditions. Acclimatized plants were transferred to a glasshouse.

Cultivation of Frankia *and nodulation of* A. verticillata

The *Frankia* Allo2 strain used for the inoculation of transgenic *A. verticillata* was grown in a modified BAP medium for 4 days (Benoist *et al.*, 1992). The culture was then washed twice with a 1/4 strength NH_4^+-free Hoagland medium (Hoagland and Arnon, 1938), homogenized through a syringe needle (0.7-mm diameter), and the pellet resuspended in 1/4 Hoagland without NH_4^+. The protein concentration was determined according to the method of Bradford (1976), and the inoculum was diluted so as to contain an amount of *Frankia* corresponding to 50 μg of protein in a volume of 4 ml.

During the 2 weeks preceding the inoculation by *Frankia, A. verticillata* transgenic plants were irrigated twice a week with a NH_4^+-free 1/4 strength Hoagland medium. The inoculum of *Frankia* was then applied into a 3-cm-deep hole, according to the procedure of Selim and Schwenke (1995). One month after the appearance of nodules, acetylene reduction activity (ARA) in the whole root system was assessed according to the method described by Hardy *et al.* (1968).

Assay of β-glucuronidase activity

Expression of the *uidA* gene in transgenic roots and stems was assayed by histochemical staining according to Jefferson (1987).

Non-transformed stem and root of *A. verticillata* plants were used as negative controls.

To allow a better impregnation by the β-glucuronidase substrate, *A. verticillata* transgenic nodules were cut with a scalpel into 1-mm slices, vacuum-infiltrated for 15 min with the 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc) buffer, and left overnight at 37°C in the presence of 5 mM X-gluc. A histochemical analysis of the transgenic nodules was then carried out as reported by Diouf *et al.* (1995).

PCR analysis

DNA for polymerase chain reactions was isolated using the method described by Bousquet *et al.* (1989). The primers used for PCR were: 5'GAATGGTGATTACCGACGAAA3' and 5'CCAGTCGAGC-ATCTCTTCAGC3' for the GUS gene (Jefferson *et al.*, 1986), 5'ATTGCACGCAGGTTCTCCGG3' and 5'AGAACTCGTCAAGAAG-GCGA3' for the *nptII* gene (Mazodier *et al.*, 1985), and 5'ATGT-CGCAAGGACGTAAGCCCA3' and 5'GGAGTCTTTCAGCATGGAG-CAA3' for the *virD1* gene of *A. tumefaciens* (Hamill *et al.*, 1991). These primers were expected to give products of 736 bp for the *nptII* gene, 574 bp for the *uidA* gene and 450 bp for the *virD1* gene. Amplification was performed with *Taq* polymerase in a three-temperature program (94°C for 1 min, 55°C for 1 min and 72°C for 2 min). Control DNA from non-transformed calli was included in the experiments to ensure that reagents were not contaminated.

DNA isolation and Southern blot analysis

DNA isolation and Southern hybridization were carried out as reported by Le *et al.* (1996). DNA sequence used as a probe was the 574-bp fragment of the *uidA* gene obtained by PCR as described above.

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