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Agrobacterium tumefaciens gene transfer to Casuarina glauca, a tropical nitrogen-fixing tree

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Abstract

Transgenic calli of the tropical tree Casuarina glauca were produced using Agrobacterium tumefaciens-mediated gene transfer. Hypocotyls, cotyledons and epicotyls were excised from 30–60-day old Casuarina seedlings and cocultivated with Agrobacterium strain C58C1(pGV2260) containing the binary vector BIN19GUSINT. Transformed calli were selected on nutrient medium supplemented with 0.5 μM NAA, 2.5 μM BA and 50 mg/l kanamycin. Some of the factors influencing T-DNA transfer to C. glauca explants were studied. Optimal transformation rates were obtained when explants from 45-day old seedlings were cocultivated for 3 days in the presence of 25 μM acetosyringone. Transgenic buds differentiated on 10% of the calli grown on transformed epicotyls. Evidence for genetic transformation was obtained by β-glucuronidase assay, PCR and Southern hybridization.

Keywords: Casuarina glauca; Agrobacterium tumefaciens; Genetic transformation; β-Glucuronidase; Transgenic callus

1. Introduction

Casuarina glauca is a tropical tree of the Casuarinaceae family which includes about 96 species of woody plants primarily native to the southern hemisphere, mostly Australia and the Indo-Pacific region. The plant family Casuarinaceae are typical angiosperms characterized by a distinctive foliage consisting of deciduous, jointed needle-like branchlets with reduced scale-like leaves organized in whorls. The shape of the branchlet reduces the area that is exposed to the air and thereby helps decrease water loss by evapo-transpiration; the leaf structure is a valuable adaptation to survival in arid climates [1,2].

Their symbiotic relationships with soil micro-organisms enable Casuarinaceae to grow in nutrient deficient soils. One of the more important associations is with the filamentous soil actino-mycete Frankia, which fixes nitrogen in Casuarina root nodules [3]. Other microorganisms, mycorrhizas, also invade the roots and contribute to the increase of phosphorus uptake [2]. As a consequence, Casuarina trees are often found on seashores and extreme habitats. The current uses for Casuarinaceae in tropical and sub-tropical

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areas with poor forest resources include sand stabilization, soil rehabilitation, fuel wood and timber production, shelterbelts, animal fodder, and field crop protection [2,4,5].

Although considerable attention has been given to the *Casuarinaceae* because of their potential for revegetation under arid and desert conditions, very little has been done to develop classical or molecular genetic improvement programs for this species. Only one species of the *Casuarinaceae* family, *Allocasuarina verticillata* Lam., has been transformed using the wild-type *Agrobacterium rhizogenes* A4 (agropine type) and 2659 (cucumopine type) strains. Transgenic plants regenerated from hairy roots exhibited an abnormal phenotype characterized by reduced apical dominance, short internodes and extensive ageotropic root systems due to the transfer of the hormone over-producing genes from *A. rhizogenes* [6]. So far, there are no reports on genetic transformation of any *Casuarinaceae* using *A. tumefaciens*.

In this paper, we report the use of a disarmed strain of *A. tumefaciens* to obtain transgenic calli of *Casuarina glauca*. Some of the factors influencing the transfer of an intron-containing β-glucuronidase gene from the strain C58C1(pGV2260; pBIN19GUSINT) [7] to *C. glauca* are presented.

2. Materials and methods

2.1. Plant material and growth media

C. glauca seeds were kindly provided by Dr. El Lakany from the Desert Development Center (Saddat City, Egypt). Chemical scarification was carried out by soaking the seeds in concentrated H_2SO_4 (95%) for 2 min and subsequently rinsing with tap water for 30 min. The scarified seeds were then disinfected with 5% Domestos for 20 min, rinsed 3 times with sterile distilled water, and germinated in Petri dishes on H medium solidified with 8 g/l Difco bacto-agar. This H medium contained 109 mg/l K_2SO_4, 123 mg/l $MgSO_4.7H_2O$, 29 mg/l $Ca(H_2PO_4)2.H_2O$ and 86 mg/l $CaSO_4.2H_2O$ as macroelements, 7 mg/l H_3BO_3, 4.52 mg/l $MnCl_2.4H_2O$, 0.55 mg/l $ZnSO_4.7H_2O$, 0.2 mg/l $CuSO_4.5H_2O$, 0.0625 mg/l $Na_2MoO_4.2H_2O$, pH 5.6. The plantlets were grown for 1–2 months at 28°C with a 16 h photoperiod (50 $\mu E/m^2/s$).

Explants were grown on a basal medium (MSC) containing half-strength Murashige and Skoog (MS) [8] salts, Nitsch and Nitsch vitamins [9], 20 g/l sucrose, solidified with 0.7% Difco bacto-agar. All media were adjusted to pH 5.6 with KOH and autoclaved at 103 kPa for 20 min at 120°C. The hormones used in this study were 6-benzylaminopurine (BA) and α-naphthaleneacetic acid (NAA); they were added to the media before autoclaving. Cefotaxime (Roussel), kanamycin monosulfate (Sigma) and acetosyringone (3',5'dimethoxy-4'-hydroxyacetophenone; Aldrich) were filter-sterilised and added to the media after autoclaving.

2.2. Agrobacterium strains

C. glauca was transformed using *A. tumefaciens* strains C58C1(pGV2260; pBIN19GUSINT) [7] and LBA4404(pAL4404; pBIN19GUSINT) [10]. These strains contained the binary vector pBINGUSINT based on Bin19 [7]; in this plasmid, the reporter gene *uidA* contains a plant intron that restricts the β-glucuronidase expression to plant cells; *uidA* is controlled by the CaMV 35S promoter and the polyadenylation signal of the nopaline synthase gene. Before cocultivation of the bacteria with the explants, *A. tumefaciens* strains were grown overnight at 28°C in Ag medium (0.5 g/l K_2HPO_4, 0.2 g/l $MgSO_4.7H_2O$, 0.1 g/l NaCl, 5 g/l mannitol, 1 g/l yeast extract, 1 g/l casaminoacid, pH 7.2) with the appropriate antibiotics (50 mg/l kanamycin, 10 mg/l rifampicin or 25 mg/l streptomycin).

2.3. Transformation procedure

Transformation experiments were performed in a growth chamber at 28°C with a 16 h photoperiod (50 $\mu E/m^2/s$). Hypocotyls, cotyledons and epicotyls were excised with a scalpel from 30–60-day old aseptically grown *C. glauca* seedlings, rinsed in MSC medium and dipped for 1 h with an overnight culture of *A. tumefaciens* diluted 10-fold in MSC medium ($OD_{600\ nm}$ = 0.1 corresponding to $\sim 10^8$ bacteria/ml). After blot drying between sterile filter papers, explants were placed on solidified callus induction medium consisting of MSC medium supplemented with 0.1 mg/l NAA and 0.5 mg/l BA (the cotyledons were placed adax-

ial side up). After 3 days of cocultivation in the growth chamber, the explants were rinsed for 1 h in sterile MSC medium with vigorous stirring, blotted dry and transferred onto MSC medium containing 0.1 mg/l NAA, 0.5 mg/l BA, 250 mg/l cefotaxime (Claforan, Roussel) and 50 mg/l kanamycin.

Calli emerging from the cut ends of explants were excised about 40 days after cocultivation and transferred every 3 weeks onto the same selection medium. Control explants were incubated without bacteria and either placed on selection medium to confirm the absence of callus tissue growth without *A. tumefaciens*, or on cocultivation medium without antibiotics to confirm the potential for callus formation.

2.4. GUS assays

These assays were carried out essentially as described by Jefferson [11]. For in vitro staining, callus tissue was incubated overnight at 30°C with 1 mM X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid; Biosynth AG), fixed in FAA (10% formaldehyde, 5% glacial acetic acid, 42.5% ethanol), and cleared in 70% ethanol prior to microscopic examination. Non-transformed *C. glauca* tissues were included as negative controls with each set of assays.

β-Glucuronidase activity was quantified in the callus extracts using 4-methylumbelliferyl β-D-glucuronide (MUG) (Clontech) as a substrate, according to the procedures described by Jefferson [11]. Fluorescence was measured using an SFM25 spectrofluorimeter (Kontron), and protein concentration was determined using the methods described by Bradford [12]. The β-glucuronidase activity was expressed as μmoles of 4-methylumbelliferone (4-MU) produced per min per mg of protein. The endogenous β-glucuronidase activity was determined in non-transformed calli of *C. glauca*.

2.5. Microprojectile bombardment of Casuarina glauca tissues

The particle gun employed in the experiments was a gun powder device similar to the one described by Li et al. [13]. Freshly prepared tungsten particles (0.8 μm, Sylvania) were used in

all the experiments: a suspension containing 300 mg of microprojectiles in 5 ml of absolute ethanol was sonicated with a microtip for 10 min (Sonifier Branson, output 3), washed 3 times in sterile distilled water, and resuspended in distilled water at a concentration of 60 mg/ml. The tungsten suspension was homogenized by vortexing before loading 5 μl onto a polyethylene microprojectile. The sample chamber was evacuated to 28 inches of mercury during bombardment.

Just before bombardment, cotyledons, hypocotyls and epicotyls ($\sim$1 cm long) were prepared from 30–45-day old *C. glauca* seedlings. The plant targets were placed on the middle of a filter paper disc (Whatman no. 1) that was put in the centre of agar plates (5 cm) containing MSC medium with 12 g/l bacto-agar. The target tissues were placed 6 cm below the macroprojectile stopping plate, and a 0.5 mm mesh stainless steel screen was positioned 3 cm above the tissues to help disperse the tungsten particles. The plates were bombarded once. Each treatment contained 25 explants (cotyledons were placed adaxial side up). The experiments were repeated 3 times.

After bombardment, the explants were washed for 10 min in MSC medium to remove the contaminating gun powder, and subjected to the *A. tumefaciens* transformation procedure described above. Controls included cotyledons, hypocotyls and epicotyls which were not subjected to bombardment.

2.6. PCR analysis

DNA for polymerase chain reaction was isolated using the method described in Bousquet et al. [14]. Reaction mixtures (25 μl) contained 100 ng of template DNA, 20 μM dNTPs, 0.1 μM of each primer, 4% formamide (Fluka-Puriss), 0.36 units of Taq polymerase (Appligene). Reactions were run on a Perkin Elmer thermocycler for 35 cycles at 94°C (1 min), 55°C (1 min) and 72°C (2 min) using oligonucleotide primers with a length of 21 bases. Primers 5'GAATGGTGATTACCGA-CGAAA3' and 5'CCAGTCGAGCATCTCTTC-AGC3' at, respectively positions 736–756 and 1320–1300 from the GUS gene [15] were expected to amplify a 574 bp fragment of the *uidA* gene. *VirD1* primers designed to amplify a region out-

side the T-DNA were used as control of transformation event; *virD1* primers 5'ATGTCGCAAG-GACGTAAGCCCA3' and 5'GGAGTCTTTC-AGCATGCAA3' correspond to nucleotide positions 1827–1848 and 2246–2264 [16] and enable the amplification of a 0.45 kb fragment in the virulence region of *Agrobacterium*. Two reactions were performed on each transgenic callus tested. To ensure that reagents were not contaminated, control DNA from non-transformed calli were included in the experiments. PCR products were separated by agarose gel electrophoresis and visualized with ethidium bromide.

2.7. DNA extraction and Southern analysis

DNA was extracted from 1 g of transgenic and control calli according to a protocol derived from Rogers and Bendich [17] (M. Phelep, 1992, Thesis, Ecole Nationale de Génie Rural, des Eaux et Forêts, Paris, France). Tissue was frozen in liquid nitrogen, pulverized with a mortar and pestle, homogenized in 10 ml of nucleic extraction buffer (100 mM Tris–HCl, pH 7.5, 0.7 M NaCl, 20 mM Na_2EDTA, pH 8, 1% CTAB, 0.1 M DDC, and 5 mM DTT) at 65°C. Tubes were incubated for 40 min at 65°C. After 10 min at room temperature, samples were extracted twice with chloroform, and the aqueous phase was precipitated with two volumes of ethanol. The DNA pellet was resuspended in 500 μl of sterile distilled water and purified on two successive cesium chloride gradients [18].

Isolated DNA (10 μg) was cleaved with *Bam*HI or *Hin*dIII, according to Biolabs recommendations, and electrophoresed in a 0.8% agarose gel. DNA was extracted from *A. tumefaciens* C58C1-(pGV2260; pBIN19GUSINT) according to the Ooms et al. [19] procedure; 2 μg were digested by *Bam*HI or *Hin*dIII and used as a positive control for hybridization with the GUS probe. The DNA was denatured and neutralized [18], then transferred overnight to a Nylon filter (Tropilon-plus, Tropix). DNA sequence used as a probe was the 574 bp fragment of the *uidA* gene obtained by PCR as described above. The probe was labelled with ^{32}P-dCTP using an oligolabelling kit (Pharmacia). Hybridization was performed at 42°C in a hybrid-

ization buffer containing 50% formamide (Fluka-Puriss), 7% SDS, 0.25 M Na_2HPO_4, 2 mM Na_2EDTA, 100 μg/ml Heparine, pH 7.2. Filters were rinsed twice with 0.2 × SSC, 1% SDS, 0.1% NaPyrophosphate at room temperature and 3 times in the same solution at 65°C for 20 min. The filter was then exposed to X-ray film at −70°C, with two intensifying screens for 1–7 days.

3. Results

3.1. Callus development and regeneration ability of Casuarina glauca

One of the requirements for plant transformation using *A. tumefaciens* is the ability to regenerate plants from transformed callus. Calli were grown from hypocotyls, cotyledons and epicotyls (1 cm long) excised from 30–45-day old seedlings of *C. glauca*; the callus induction medium consisted of MSC supplemented with 0.5 μM NAA and 2.5 μM BA. Using these conditions, callogenesis was observed on 40% of the epicotyls, 92% of the hypocotyls and 94% of the cotyledons. After 2 months, numerous buds (20–50) differentiated on the calli induced from epicotyls (41%), hypocotyls (14%) and cotyledons (17%). About 2 ± 1 out of 10 buds elongated into shoots which grew to an average height of 6–10 mm within 3 months; rooting of the elongated shoots was induced by a 3-day treatment in the presence of 10 μM indole-3-butyric acid (IBA), followed by a culture on nutrient medium without growth regulator (data not shown).

3.2. Tolerance of C. glauca to kanamycin and cefotaxime

In order to determine the effective concentration for antibiotic selection, sensitivity of *C. glauca* explants to kanamycin was established prior to transformation experiments. Various concentrations of kanamycin (10, 25, 50, 100 and 200 mg/l) were added to the callus induction medium. A total inhibition of callus growth was observed for the explants at antibiotic concentrations higher than 25 mg/l (data not shown); concentrations higher than 100 mg/l caused immediate browning

of the explants. Therefore, for selection of trans-
genic calli, transformed explants were subcultured
on nutrient medium containing 50 mg/l
kanamycin.

Interference of cefotaxime with callus growth
and shoot regeneration ability was also in-
vestigated. No change in growth was detectable
when cotyledons, epicotyls or hypocotyls from *C.
glauca* were cultured on MSC medium containing
growth regulators and 250 mg/l cefotaxime (data
not shown). This concentration of cefotaxime was
chosen to inhibit *Agrobacterium* growth after
cocultivation.

3.3. Growth of kanamycin resistant calli after A. tumefaciens transformation

Initial experiments of transformation were
based on a modified version of the procedure of
Horsch et al. [20] developed for *Nicotiana ta-
bacum*; they were performed as described in the
Materials and methods. *C. glauca* cotyledons,
hypocotyls and epicotyls were cocultivated for
3 days with the strain C58C1(pGV2260; pBIN-
19GUSINT), in the absence of acetosyringone,
and grown on callus induction medium containing
kanamycin and cefotaxime. Three weeks after
cocultivation, calli emerging from the wounded
edges of the explants were observed (Fig. 1A); few
calli appeared on areas where the tissues had been
damaged during handling. Within 2 months, on
the 100 plants tested, 1–3 calli grew in the presence
of 50 mg/l kanamycin on 13% of the hypocotyls,
19% of the cotyledons and 26% of the epicotyls,
whereas antibiotics completely inhibited the
growth of the control explants. Kanamycin resis-
tant calli were excised from *Casuarina* explants
when they were about 2 mm in diameter, and sub-

Fig. 1. Kanamycin resistant calli growing on *Casuarina* explants transformed by C58C1(pGV2260; pBIN19GUSINT). (A) Kanamycin resistant calli emerging from a cotyledon. (B) β-Glucuronidase-expressing calli emerging from an epicotyl. The transformed explants were grown for 3 weeks on nutrient medium containing 0.5 μM NAA, 2.5 μM BA, 50 mg/l kanamycin and 250 mg/l cefotaxime. Bar: 0.5 mm.

cultured every 3 weeks on selective medium; continued callus growth was observed for more than a year.

3.4. Analysis of β-glucuronidase gene expression in transgenic calli

β-Glucuronidase expression was determined by histochemical assays and fluorometric analysis [11]. Out of 50 kanamycin resistant calli tested, 43 (86%) exhibited blue sectors. The staining pattern of the calli ranged from virtually all the cells turning blue to only a few blue cells; dark blue and pale blue colouring were observed (Fig. 1B). Untransformed C. glauca calli had no detectable GUS activity when analyzed with X-gluc (data not shown).

GUS activity in the transgenic calli varied from 1.5 to 628 μmol/min/mg protein, with an average activity of 243.5 $\pm$ 187 μmol 4-MU/min/mg protein, whereas endogenous β-glucuronidase activity in C. glauca was 0.27 $\pm$ 0.15 pmol 4-MU/min/mg protein. Seven calli growing on kanamycin and negatively stained with X-gluc did not show any significant β-glucuronidase activity when tested by fluorometry, suggesting that either the β-glucuronidase gene had been deleted during T-DNA transfer, or that these calli had escaped to antibiotic selection.

3.5. PCR and Southern blot analyses of transformed calli

PCR was used to demonstrate whether T-DNA was present in six C. glauca calli growing with kanamycin. Five putatively transformed calli were tested in PCR reactions using primers to the uidA reporter gene. A band of 574 bp corresponding to the relevant sequence of the uidA gene was reproducibly found in the transgenic calli (Fig. 2A, lanes 3–7) and not in the non-transformed sample (Fig. 2A, lane 2). To demonstrate that the sequence amplified with the uidA primers was not due to an Agrobacterium contamination, additional PCR reactions were set up using the virD1 primers. The fragment of 0.45 kb corresponding to the sequence of the virD1 gene (Fig. 2B, lane 8) was not detected in the putatively transformed calli (Fig. 2B, lanes 3–7); this result indicates that the kanamycin resistant calli do not contain a significant amount of residual Agrobacterium.

Southern blot analysis was performed to provide additional molecular evidence for the incorporation of foreign DNA into the C. glauca genome. Purification of Casuarina genomic DNA on cesium chloride gradients was deemed necessary to achieve complete digestion by the restriction enzymes. Due to the low yield of DNA recovery, Southern blot analyses were performed on
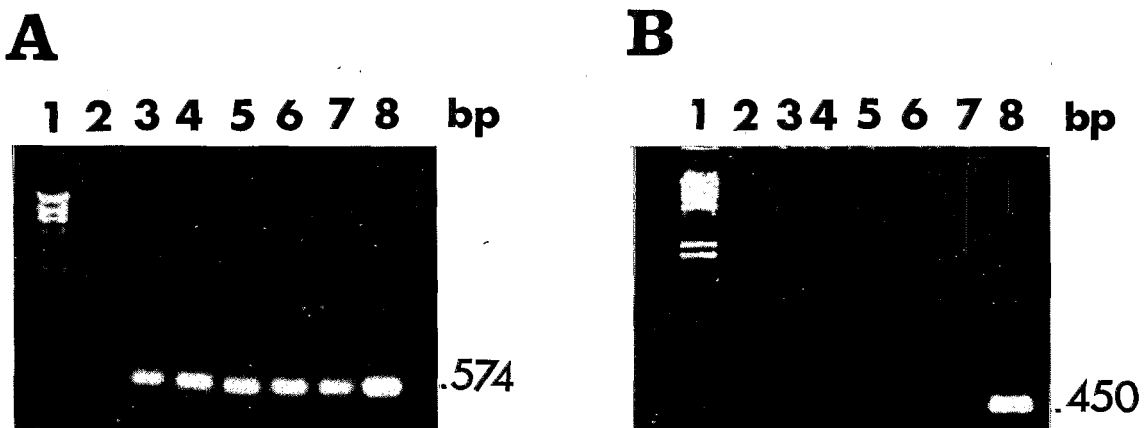

Fig. 2. PCR analysis of C. glauca calli transformed by A. tumefaciens C58C1(pGV2260; pBIN19GUSINT). DNAs were primed with oligonucleotides specific to the uidA reporter gene (A) or to the virD1 gene of Agrobacterium tumefaciens (B). 1: HindIII digested λ DNA. 2: untransformed control callus. 3–7: putatively transformed calli growing on kanamycin. 8: positive control C58C1(pGV2260; pBIN19GUSINT).
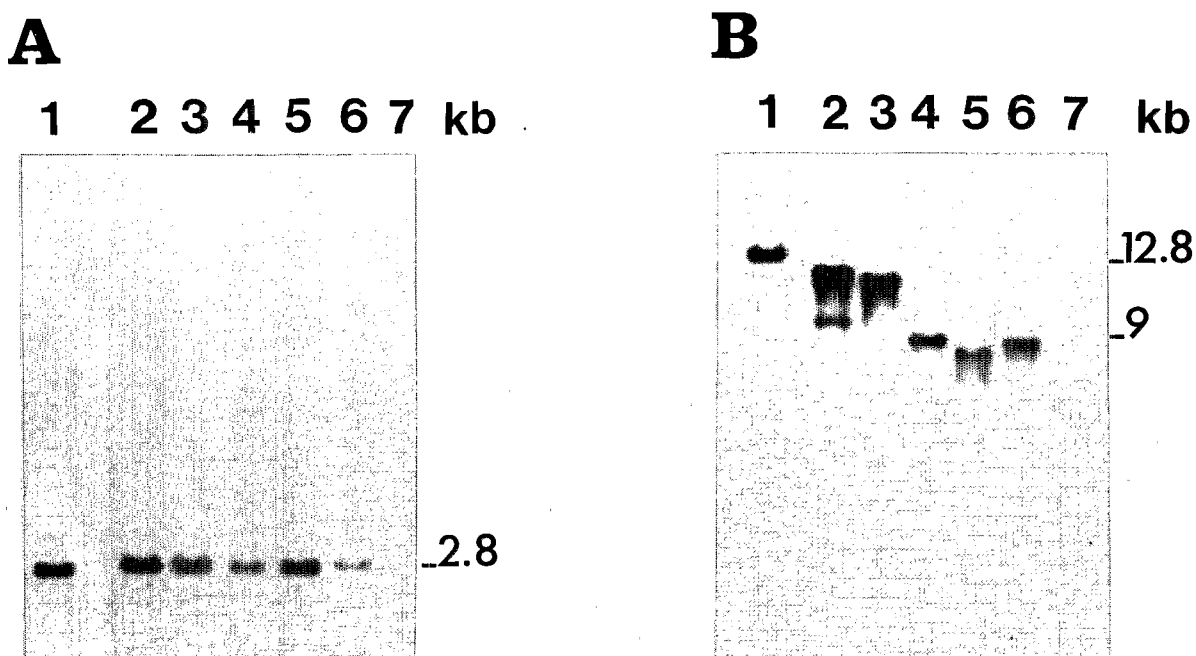
A

B

Fig. 3. Southern blot analysis of three transformed *Casuarina* calli. Genomic DNA were digested by *Hin*dIII (A) or *Bam*HI (B). 1: DNA from *A. tumefaciens* C58C1(pGV2260; pBIN19GUSINT). 2–6: genomic DNA from five putatively transformed *Casuarina* calli. 7: genomic DNA from a non-transformed *Casuarina* callus. DNA· were hybridized to a 574 bp fragment containing the β-glucuronidase coding region.

only five calli growing in the presence of 50 mg/l kanamycin, and on one non-transformed callus. DNA from *Agrobacterium* and from *Casuarina* calli were digested by *Hin*dIII and *Bam*HI; the Southern blots were hybridized with the 574 bp fragment containing the GUS gene. The enzyme *Hin*dIII generates within the vector pBINGU-SINT a 2.8 kb fragment (Fig. 3A, lane 1) containing the *uidA* gene sequence with the 35S promoter and terminator [7]. Hybridization of the GUS probe to the *Hin*dIII-digested genomic DNA demonstrated the presence of full-length copies of the *uidA* gene (Fig. 3A, lanes 2–6) in the five transgenic calli. A unique *Bam*HI site is located 2.4 kb to the right of the left border on the plasmid pBINGUSINT [21]. After digestion with *Bam*HI and hybridization to the GUS probe, a 12.8 kb *Bam*HI fragment corresponding to the linearized vector pBIN19GUSINT is obtained (Fig. 3B, lane 1). Using DNA from transformed calli, the *uidA* probe hybridized to variable-sized *Bam*HI bands

ranging from about 12 to 8.5 kb (Fig. 3B, lanes 2–6). This pattern of hybridization is consistent with the integration of one (Fig. 3B, lanes 3–7) or two copies (Fig. 3B, lane 2) of the T-DNA into *Casuarina glauca*. No hybridization was found in non-transformed control *Casuarina* callus (Fig. 3A and B, lane 1).

3.6. Microprojectile bombardment of the explants prior to A. tumefaciens transformation

The wounded areas of the epicotyls, hypocotyls and cotyledons of *C. glauca* excised with a scalpel are very small: hypocotyls and epicotyls have a diameter of less than 1 mm, and the wounded area of a cotyledon is ~1 mm long. To increase the injured areas and possibly enhance the efficiency of transformation, epicotyls and cotyledons from 30–45-day old *C. glauca* were subjected to bombardment by naked high velocity microprojectiles (tungsten beads of ~1 μm in diameter) prior to

Table 1
Transformation rate of *Casuarina* explants bombarded by high
velocity microprojectiles prior to *A. tumefaciens* treatment

Treatment	Transformation rate (%)		
	Hypocotyls	Cotyledons	Epicotyls
No bombardment	6.6	12.1 ($\pm$3)	35.6 ($\pm$11)
Bombardment	0	10.1 ($\pm$4)	9.2 ($\pm$4.6)

Explants were excised from 100 seedlings. Half of them were
bombarded by naked high velocity tungsten particles before
Agrobacterium transformation. One experiment was performed
with hypocotyls, with 3 repeats using cotyledons and epicotyls.
Standard errors are indicated.

transformation by C58C1(pGV2260; pBIN19GU-
SINT). Although some injury was visible on the
targets (data not shown), there was a lower num-
ber of transformed calli on bombarded explants
than on the untreated controls (Table 1). A 2-fold
reduction of the tungsten concentration (30 in-
stead of 60 mg/ml) or an increase of the distance
between the stopping plate and the target tissues
(8 instead of 6 cm) did not lead to an increase in
transformation efficiency (data not shown).

3.7. Factors influencing transformation

In order to improve the efficiency of transfor-
mation, some factors including the time of
cocultivation, the age of *C. glauca* seedlings, the
addition of acetosyringone during cocultivation,
and the time of contact with *Agrobacterium* were
studied.

In preliminary experiments, we tried to deter-
mine the transformation efficiency by counting the
blue sectors 5 days after cocultivation. It was
sometimes difficult to correlate the number of ex-
plants expressing GUS after 5 days with the num-
ber of explants that subsequently developed
kanamycin resistant calli. This was probably
because of low transformation efficiency and of
the small size of the wounded areas. We decided it
would be more significant to count the calli that
grew on kanamycin and that expressed the β-glu-
curonidase activity 2 months after cocultivation.

C. glauca was first tested for susceptibility to
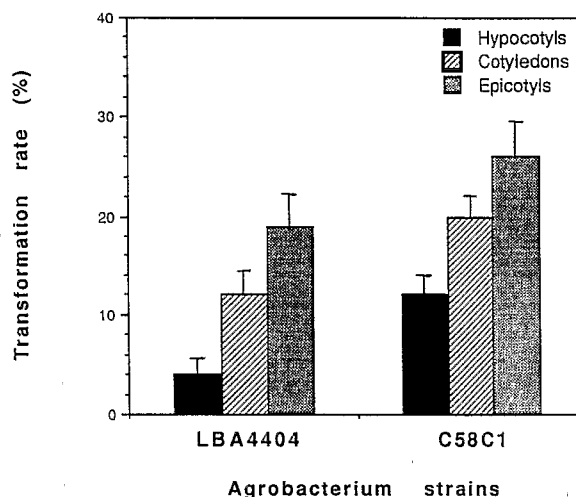another disarmed strain of *A. tumefaciens*, LB44-

Fig. 4. Transformation of *C. glauca* explants by two disarmed
strains of *A. tumefaciens*. Epicotyls, cotyledons and hypocotyls
were cocultured 3 days either with C58C1(pGV2260;
pBIN19GUSINT) or LBA4404(pBIN19GUSINT). The rate of
transformation was determined after 2 months of growth on se-
lective medium. Each treatment included 50 explants and was
repeated 3 times. Standard error bars are shown.

04(pLBA4404; pBIN19GUSINT). Results pre-
sented in Fig. 4 indicated that using LB4404-
(pLBA4404; pBIN19GUSINT), the number of
kanamycin resistant calli expressing the β-glucu-
ronidase activity was about 1.5-2.5 times lower
than the number obtained with the strain
C58C1(pGV2260; pBIN19GUSINT), which was
used in all subsequent experiments.

Acetosyringone, a plant phenolic compound
which affects the level of transcription of the *vir*
genes of *Agrobacterium* [22] was added at a con-
centration of 25 μM to the culture of *Agrobac-
terium* and to the cocultivation plates. As shown in
Table 2, the addition of 25 μM of acetosyringone
led to a 1.5-2-fold increase in *Casuarina* transfor-
mation efficiency.

Different cocultivation times appear to be speci-
fic to different plant species [23]. Explants from
30-day old seedlings were cocultivated for 1, 2, 3
and 7 days with *Agrobacterium*. Fig. 5 shows that
the transformation frequencies were at their maxi-
mum value after 3 days of culture with *Agrobac-
terium*. After 7 days some explants appeared

Table 2
Effect of 25 μM acetosyringone on transformation rate of *Casuarina glauca* hypocotyls, cotyledons and epicotyls after inoculation with C58C1(pGV2260; pBIN19GUSINT)

Acetosyringone μM	Transformation rate (%)		
	Hypocotyls	Cotyledons	Epicotyls
0	12.3 ($\pm$3)	19 ($\pm$2.5)	20 ($\pm$4)
25	20 ($\pm$3.5)	29 ($\pm$5)	40.5 ($\pm$10)

25 μM of acetosyringone were added in the agrobacterial culture and during cocultivation. Explants excised from 50 seedlings were tested for each experiment. Experiments were repeated 3 times. Transformation rate was defined as the percentage of explants developing calli in the presence of kanamycin and expressing the β-glucuronidase activity. Standard errors are indicated.

damaged by the agrobacteria and turned brown; thus reducing the transformation frequency.

The effect of explant age on T-DNA transfer was studied. The hypocotyls, cotyledons and epicotyls were excised from 30, 45, 60 and 80-day old seedlings and transformed using the standard pro-
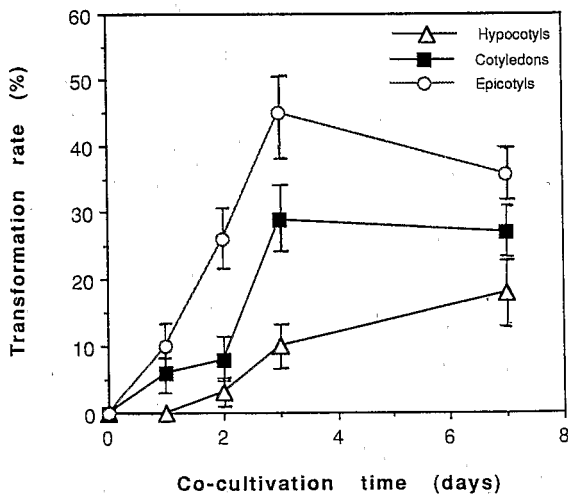

Fig. 5. Effect of cocultivation duration on transformation rate. Explants from 50 seedlings were transformed with C58C1(pGV2260; pBIN19GUSINT) and cocultured in the presence of 25 μM acetosyringone. Transformation rate was determined after 2 months. Experiment was repeated 3 times. Standard error bars are shown.
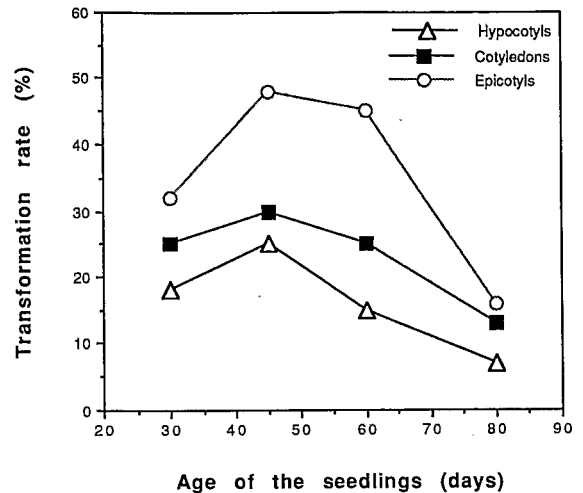

Fig. 6. Influence of explant age on *Casuarina* transformation rate. For each age, explants from 50 seedlings were cocultivated with C58C1(pGV2260; pBIN19GUSINT) in the presence of 25 μM acetosyringone. The number of kanamycin resistant calli expressing β-glucuronidase activity was determined 2 months after transformation.

cedure. The results presented in Fig. 6 show that the best transformation frequency was observed with explants derived from 45-day old seedlings.

Attempts to increase the time of contact (8 or 16 h instead of 1 h) between the explants and the dilution of *Agrobacterium* culture had no significant effect on the T-DNA transfer efficiency (data not shown).

3.8. Bud differentiation on transformed C. glauca calli

Using the callus induction medium containing 0.5 μM NAA and 2.5 μM BA, differentiation of numerous buds was occasionally observed on 10% of the transgenic calli obtained from the epicotyls (Fig. 7A and B); no regeneration occurred on calli induced from cotyledons or hypocotyls. Four months were necessary for bud differentiation on transgenic calli, whereas buds generally appeared on the non-transformed calli within 2 months of culture. The delay observed with the transformed calli may be due to the presence of antibiotics in the nutrient medium.
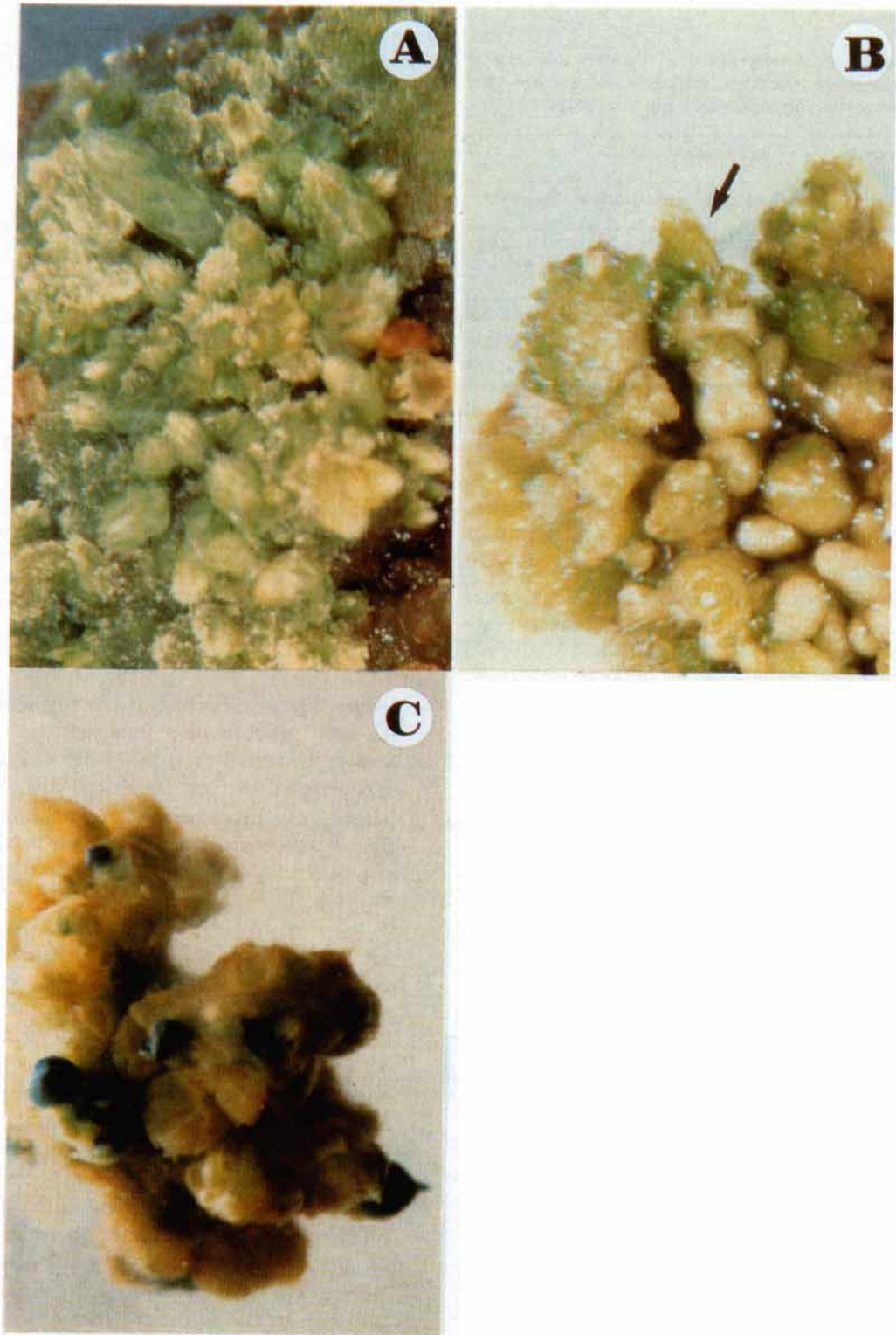
Fig. 7. Caulogenesis and histochemical determination of *uidA* gene expression in transgenic *C. glauca* calli. (A) Buds and shoots growing on a non-transformed control callus on nutrient medium with 0.5 μM NAA and 2.5 μM BA. (B) Buds appearing on a kanamycin resistant callus 4 months after cocultivation; the arrow indicates the presence of a 1 mm shoot. (C) β-Glucuronidase activity in a transformed callus exhibiting buds.

Calli exhibiting some bud differentiation were tested for β-glucuronidase expression in the presence of the substrate X-gluc. As shown in Fig. 7C, dark blue buds were observed. The heterogeneity in colouring may have resulted from non-penetration of the substrate in the compact callus; it could also be explained by the presence of non-transformed cells which had escaped to antibiotic selection. No blue staining was detected in buds growing on non-transformed calli used as controls (data not shown).

Although continuous bud proliferation was observed for several months on 24 transformed *C. glauca* calli, shoot growth never occurred, and transgenic plants have not been regenerated.

4. Discussion

Using the *A. tumefaciens* binary vector pBIN19 carrying the *nptII* and *uidA*-intron genes [7], we have obtained transgenic calli of *C. glauca* expressing β-glucuronidase activity. The presence of the *uidA* gene was confirmed by PCR analysis, and by Southern blot. Kanamycin resistance appears as a reliable marker in *Casuarina* transformation.

Staining for GUS activity revealed differences in colour intensity. Results were confirmed by fluorometric analysis of the level of β-glucuronidase activity. Similar differences in gene expression have been reported in transgenic calli of other species [24]. Integration in different chromosomal positions, copy number, subsequent rearrangements, physiological or developmental stages of the individual transgenic calli might influence the level of expression of the introduced gene [25]. Methylation of the GUS reporter gene is also known to alter gene expression in some plants [26].

Microprojectile bombardment appears to be an effective method for improving transformation frequencies by *A. tumefaciens* on tobacco leaves and sunflower meristems [27]. The macro and microwounds generated by the accelerated microparticules both increased the percentage of *Agrobacterium*-transformed explants and increased the number of transformed calli per explant [27]. Our negative results could be explained by a number of factors such as excessive injury of the target due to the gas blast and the acoustic shock generated by the particle gun, or toxicity of the tungsten particles and/or of the gun powder [28]. Another interpretation could be that *Agrobacterium* gene transfer does not occur in the cells wounded by the microparticules.

The success of a transformation protocol depends on many factors including the plant genotype, the strain of *Agrobacterium*, the selectable marker, the growth regulator balance prior to and after transformation, and the cocultivation time [23,29]. Attempts to study the factors influencing transformation frequency of *C. glauca* at an early stage were not successful, although the presence of an intron in the *uidA* gene prevented expression of the reporter gene in *A. tumefaciens*. Similar difficulties have been reported during the assessment of factors affecting gene transfer of apple; correlation was not always good between transient *uidA* expression during early transformation steps, and the number of transgenic calli resulting from the stable integration of the T-DNA [30].

The results reported in this paper have shown that the bacterial strain is important for the efficiency of gene transfer into *C. glauca*. Between the two disarmed strains of *A. tumefaciens* tested, C58C1 carrying the Ti plasmid GV2260 [7] was found to be better than LB4404(pAL4404) [10]. With these strains, epicotyls of *C. glauca* appeared to be the best tissues for T-DNA transfer.

The frequency of transformation of *C. glauca* was slightly enhanced by the addition of 25 μM acetosyringone during cocultivation. Acetosyringone is one of the low molecular weight phenolic compounds which are naturally excreted by wounded plant cells and acts as an inducer of the virulence (*vir*) genes of *Agrobacterium* [22]. The effect of acetosyringone on transformation efficiency depends on the plant species or genotype, and the *Agrobacterium* strain. Other signal molecules such as syringaldehyde, caffeic acid and sinapic acid were not tested in this work.

Different cocultivation periods appear to be specific for different plant species. The transformation frequency for *C. glauca* was highest after 3 days of cocultivation. Ability to accept DNA from *A. tumefaciens* was found better for 45-day old seedlings.

The results above indicated that a suitable trans-

formation protocol for *C. glauca* would include: 25 μM of acetosyringone during cocultivation with the strain C58C1(pGV2260), a 3-day cocultivation time, and explants from 30–45-day old seedlings. Under these conditions, 20% of the hypocotyls, 30% of the cotyledons, and 40% of the epicotyls were routinely transformed. Additional factors such as the inoculum density, carbon source and pH during cocultivation, and hormonal composition of the nutrient medium still need to be tested.

Green buds expressing the β-glucuronidase activity were obtained on 12% of the transgenic calli growing on *C. glauca* epicotyls transformed by the strain C58C1(BIN19-GUSINT). Up to now, these buds have failed to develop further. Shoot growth inhibition may be due to the effect of the antibiotics [31]. Experiments are in progress to optimize the conditions for regeneration of *C. glauca* under selection pressure.

Our results provide valuable information for developing an *A. tumefaciens*-based transformation protocol for *C. glauca*. Developing a gene transfer system will have great potential for genetic improvement of *Casuarinaceae* trees by introducing genes responsible for agronomically important traits, i.e. insect resistance genes. Transgenic *Casuarinaceae* trees will also be valuable tools for studying the regulation and molecular organization of tropical tree genes, and more specifically of plant genes involved in the symbiotic process with the actinomycete *Frankia*.

Acknowledgements

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