Plant Cell Reports (1998) 18: 198–202

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Evaluation of microprojectile-mediated DNA delivery and reporter genes for genetic transformation of the Mediterranean cypress (*Cupressus sempervirens* L.)

Received: 18 November 1996/ Revision received: 19 February 1997/ Accepted: 20 November 1997

Abstract Embryonal-suspensor tissue (EST) of Mediterranean cypress (*Cupressus sempervirens* L.) was tested for microprojectile-DNA delivery (by the PDS-1000/He device) for different subculture periods (9, 15, and 21 days) using the plasmid vectors pRT99GUS [containing the β-glucuronidase (GUS) and neomycin phosphotransferase (NPT II) genes, and the CaMV 35S promoter], pBI426 (with a GUS::NPT II fusion gene under the control of a duplicated 35S RNA promoter), and pCGUδ0 (containing the GUS gene with the ubiquitin intron, under the control of the sunflower ubiquitin promoter). The relative strengths of the promoters as determined by GUS assays were sunflower ubiquitin>35S-35S-AMVE>35S. The highest expression level was observed when 15-day-subcultured EST was bombarded with the pCGUδ0 gene construct, which also showed high activity of the chloramphenicol acetyltransferase and NPT II genes. Green fluorescent areas were observed on EST when bombarded with the p35S-GFP plasmid, carrying the gene for the green fluorescent protein from the bioluminescent jellyfish *Aequorea victoria*.

Key words Particle bombardment · Cypress · Gymnosperm · DNA transformation

Communicated by A. M. Boudet

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Abbreviations *CAT* Chloramphenicol acetyltransferase · *EST* Embryonal-suspensor tissue · *GFP* Green fluorescent protein · *GUS* β-Glucuronidase · *4-MU* 4-Methylumbelliferone · *MUG* 4-Methylumbelliferyl β-D-glucuronide · *NPT II* Neomycin phosphotransferase

Introduction

In the Mediterranean area, the Mediterranean cypress (*Cupressus sempervirens* L.) is one of the few species with characteristics of high drought hardiness and suitability for afforestation on difficult terrains such as calcareous, clayey or rocky soils. In Italy, it is also an important ornamental tree, largely cultivated in parks, along boulevards, and at religious sites (Pozzana 1991). In 1970, a large-scale breeding program was initiated in Italy to select canker-tolerant clones, as the disease, caused by the fungus *Seiridium cardinale*, is at present a factor strongly limiting cypress planting. However, as the long reproductive cycles of conifers render conventional breeding techniques highly time consuming, the development of tissue culture and genetic transformation technologies could significantly aid cypress breeding. Previous reports have demonstrated the induction of highly embryogenic and organogenic tissue from excised embryos of cypress (Lambardi et al. 1994, 1995). These advances open the door to the use of tissue culture for faster propagation of superior and disease-tolerant genotypes and to genetic engineering of important silvicultural traits.

With conifers, substantial progress has been made in gene transfer technologies for gene expression studies and for recovery of transgenic trees. Most recent work has used microprojectile-mediated DNA delivery to obtain transient gene expression in various conifer tissues such as embryonal masses, somatic embryos, zygotic embryos, seedlings, needles, flower parts, pollen, shoot buds, and developing wood (e.g., Goldfarb et al. 1991; Ellis et al. 1991; Stomp et al. 1991; Loopstra et al. 1992; Charest et al. 1993a; Duchesne et al. 1993; Hay et al. 1994; Aronen

et al. 1995). Using this technology transgenic trees have been sucessfully obtained with white spruce (*Picea glauca*; Ellis et al. 1993), black spruce (*P. mariana*; Charest et al. 1996), and tamarack (*L. laricina*; Klimaszewska et al. 1996). *Agrobacterium* mediated DNA delivery was used to recover transgenic trees of *Larix decidua* (Huang et al. 1991) and *L. leptoeuropaea* (Levée et al. 1995); however, tissue cultures of conifers have generally been recalcitrant to transformation with *Agrobacterium* disarmed Ti plasmids.

The present study evaluated the suitability of 1-year-old embryogenic tissues of Mediterranean cypress as targets for the delivery of chimeric marker genes through microprojectile bombardment. The variables of the delivery method were optimized using the β-glucuronidase (GUS) gene and three other marker genes [(neomycin phosphotransferase (NPT II), chloramphenicol acetyltransferase (CAT), and the green fluorescent protein (GFP) from jellyfish genes] were tested for their effectiveness. This work provides basic information for further studies of gene expression and recovery of transgenic trees of *C. sempervirens* L.

Materials and methods

Embryogenic tissue

For this study, embryogenic tissue of cypress, induced from suspensor cells of immature embryos (EST), was used (Fig. 1A). During May 1994, cones were collected from 19-year-old grafted canker-resistant trees in an experimental plantation near Florence, Italy. Seeds were removed and decontaminated, and embryos were excised, as previously described (Lambardi et al. 1995). The whole embryos were plated on DCR medium (Gupta and Durzan 1985), containing 100 mg/l L-glutamine, 500 mg/l casein hydrolysate, 200 mg/l myo-inositol, 30 g/l sucrose and 7 g/l Difco Bacto-agar. In the induction phase, 10 µM 2,4-dichlorophenoxyacetic acid was supplemented as growth regulator. Cultures were incubated at 23°C in the dark. For the maintenance of the EST, several lines were transferred to hormone-free DCR medium, supplemented with 0.5% activated charcoal, and subcultured every 21 days. For microprojectile DNA delivery, 1-year-old EST lines were transferred to fresh media 9, 15, or 21 days before bombardment.

DNA preparation and microprojectile DNA delivery

Three plasmid vectors were compared in different experiments to evaluate their efficiency for EST transformation: (1) plasmid pBI426 (6.3 kb), containing a GUS::NPT II fusion gene (Datla et al. 1991) expression cassette (approximately 3.5 kb), linked to the double 35S promoter with the alfalfa mosaic virus enhancer, (2) plasmid pRT99GUS (6.71 kb), containing the GUS and NPT II genes separately, both flanked by the promoter and terminator sequences of the CaMV 35S gene (Töpfer et al. 1988), and (3) plasmid pCGUδ0 (6.8 kb), containing the sunflower ubiquitin promoter fused to the GUS gene with a ubiquitin intron (Binet et al. 1991). A fourth plasmid vector, the p35S-GFP, was utilized in a specific experiment with cypress EST. This plasmid carries the gene for the GFP from the bioluminescent jellyfish *Aequorea victoria* (Chalfie et al. 1994). Bombarded samples expressing the gene emit a green light when illuminated with blue (450–490 nm) light, and can thereby be used to follow gene expression in vivo. The modified GFP (Haseloff and Amos 1995) sequence contains a mutated coding region between the *Nde*I and *Acc*I sites in the gene, which modifies the codon usage (but not the amino acid sequence), and was designed to alter the 5′ splice site and to lower the A:T content around the cryptic intron. The mGFP4 expression cassette was taken out of the pBIN35SmGFP4 vector (J. Haseloff, personal communication) as a *Hin*dIII-*Eco*RI fragment, in which the sequence is driven by a 35S promoter and flanked by a NOS terminator at the 3′ end of the gene, and cloned into the pBluescript II vector (Stratagene) (Tian et al. 1997).

Plasmid DNAs were coated onto gold particles (1.6 µm diameter) using the $CaCl_2$ precipitation methods developed by Klein et al. (1989). DNA delivery was carried out using the Biolistic particle delivery system PDS-1000/He (DuPont, Wilmington, Del.) following the manufacturer's recommendations and using 1100-psi rupture disks. EST, collected after the different subculture periods, was weighed under aseptic conditions and spread (200 mg per plate) on the surface of a Fisherbrand P5 filter paper (diameter 5.5 mm, medium porosity), placed in the center of a 9-cm-diameter petri dish containing a gelled (0.7% Difco Bacto-agar) DCR medium. For each bombardment, 1 µg of plasmid DNA was used. The plates were placed 12.5 cm from the stopping net, under a vacuum of 800 mmHg.

Histological GUS assay

After EST bombardment, the plates were placed at 25°C in darkness for 48 h. The filter disks supporting the bombarded samples were then placed on top of another disk soaked in 600 µl of GUS reaction buffer – modified according to the method of Jefferson et al. (1987) [10 mM Na_2HPO_4, 10 mM NaH_2PO_4, pH 7.0, 10 mM $K_3(Fe(CN)_6)$, 10 mM $K_4(Fe(CN)_6) \cdot 3H_2O$, 10 mM Na_2EDTA, 0.1% Triton X-100, 1.5 mM X-glucuronide] – in a 9-cm-diameter petri dish. The sealed dishes were incubated at 37°C in darkness for 24 h. Assessment of GUS gene expression was based on the number of expression units (i.e., the number of islands of cells showing blue coloration) per 200 mg of sample tissue (Duchesne and Charest 1991).

Fluorometric GUS assay

After bombardment, EST (from a 9-day subculture period) was grown at 25°C in darkness for 48 h, then transferred to 1.5-ml microfuge tubes containing 5 mg of Al_2O_3 and 200 µl of GUS extraction buffer modified according to the method of Jefferson (1987). Fluorescence levels were quantified using a TKO-100 minifluorometer (Hoeffer Scientific Instruments, San Francisco, Calif.) calibrated with 4-methylumbelliferone (4-MU) standard solutions. Data were expressed in pmol 4-MU/min per milligram of extracted protein as determined by Bradford assay (Bio-Rad, Canada).

NPT II and CAT assays

The assays were performed on 9-day-old cultures of cypress EST bombarded with the pCGUδ0 plasmid. In a single experiment, 35 plates were prepared as described above, and utilized as follows: 20 samples were bombarded and used for the assays (10 for each one), 5 samples were bombarded and used to carry out a comparative GUS histological assay, 10 samples were not bombarded and served as control. Both the NPT II, and the CAT ELISA assays were performed according to the manufacturer's recommendation (5 Prime → 3 Prime, USA).

GFP assay

For this assay, five plates of 9-day-subcultured EST were prepared as described above and bombarded with the p35S-GFP plasmid. Following bombardment, GFP gene expression was observed at different times using a Zeiss epifluorescence microscope. The following filter combination was used: excitation filter 450–490 nm, dichroic mirror 510 nm, barrier filter LP 520 nm. The light source was provided by a HBO 50 W high-pressure mercury bulb and the photographs were taken using Kodachrome 25 film.

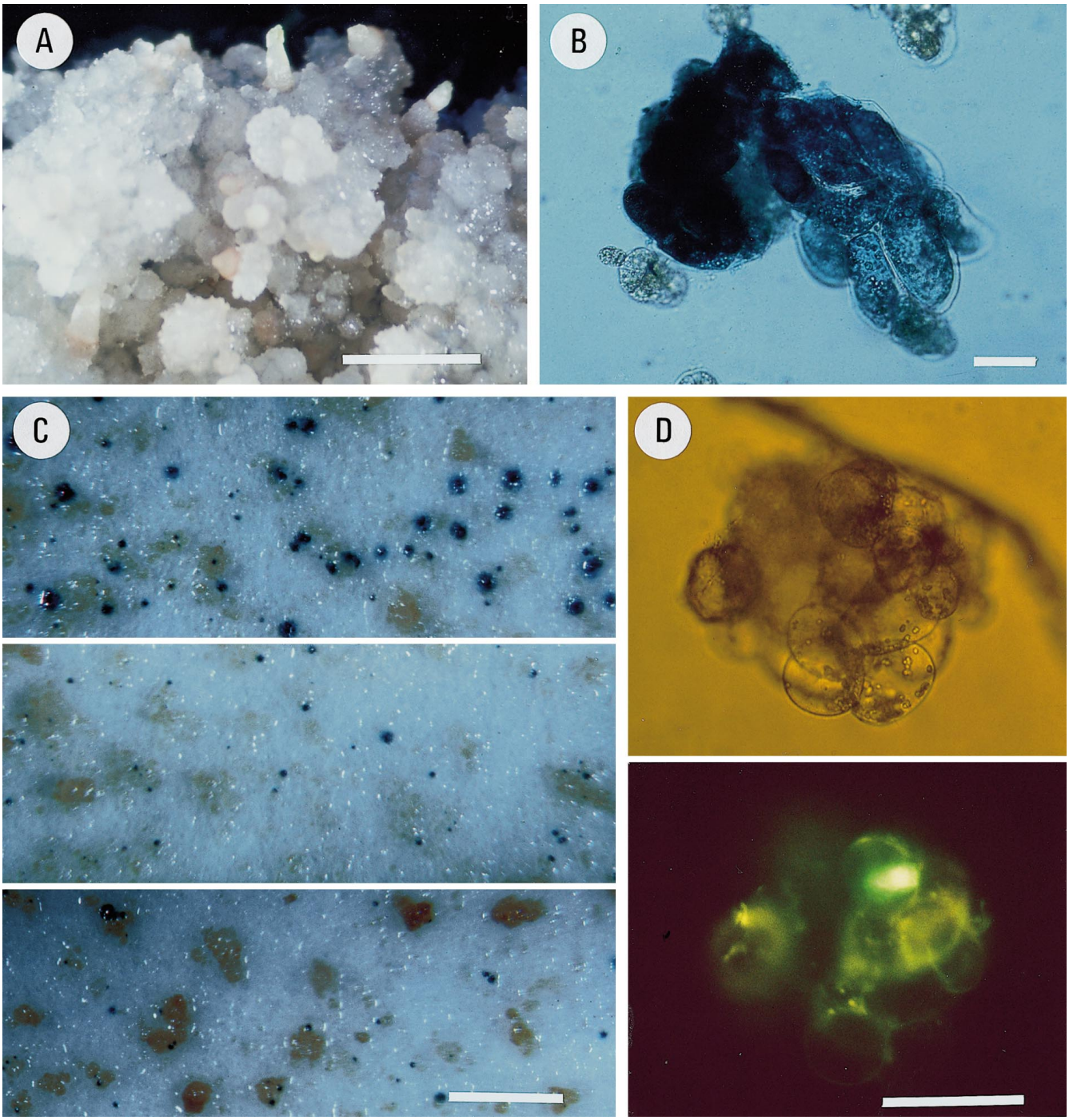

Fig. 1 **A** White, translucent EST of cypress utilized for bombardment. Numerous somatic embryos are forming (*bar* 5 mm). **B** GUS gene expression in a developing pro-embryo bombarded with pCGUδ0 (*bar* 0.1 mm). **C** In cypress, GUS gene expression was strongly influenced by the plasmid vector used. On 15-day-subcultured EST, the number of expression units was pCGUδ0 (*top*)>pBI426 (*center*)>pRT99GUS (*bottom*) (*bar* 10 mm). **D** When EST of cypress (*top*) was bombarded with the p35S-GFP plasmid, carrying the gene for the GFP from the bioluminescent jellyfish *Aequorea victoria*, green fluorescent areas were observed (*bottom*) by an epifluorescence microscope (excitation filter 450–490 nm, dichroic mirror 510 nm, barrier filter LP 520 nm) (*bar* 0.05 mm)

Table 1 GUS activity by histological assay in EST of *Cupressus sempervirens* L. after microprojectile DNA delivery, with different plasmids and subculture periods (number of expression units per 200 mg of sample tissue; means±SE). Means followed by different lower-case letters are significantly different by Tukey's test at $P \leq 0.05$. Plasmid general means are significantly different when followed by different upper-case letters (Tukey's test at $P \leq 0.05$)

Subculture (days)	Plasmid		
	pBI426	pRT99GUS	pCGUδ0
9	166.6 ± 50.6 cd	177.6 ± 30.8 cd	314.3 ± 58.9 bc
15	335.2 ± 27.5 b	81.8 ± 16.4 d	491.8 ± 10.2 a
21	173.6 ± 16.1 cd	71.5 ± 8.5 d	193.2 ± 35.2 bcd
Mean	225.2 A	110.3 B	333.1 C

Statistical analyses

Unless otherwise stated, each experiment was repeated at least twice, with five to six samples per plasmid. Data were subjected to analysis of variance (ANOVA), and significant differences between means were selected by the Tukey's HSD test at $P \leq 0.05$.

Results and discussion

Several reports have been published on gene delivery in conifer embryogenic cultures of larch, spruce, pines, and *Taxus* using microprojectile bombardment (Duchesne and Charest 1991, 1992; Newton et al. 1992; Charest et al. 1993b, 1995; Ellis et al. 1993; Klimaszewska et al. 1996; Luan et al. 1996) in which marker genes such as that for GUS were successfully expressed. These studies served as a basis to develop protocols for the stable genetic transformation of these cultures and subsequent regeneration of transgenic trees. Similarly, microprojectile-DNA delivery using the PDS-1000/He system has been used here with cypress embryogenic cultures. The bombardment parameters, such as type and size of microprojectiles, the method of DNA precipitation, the rupture disks, the position of the samples in the apparatus, and the timing of the gene expression assay, were chosen according to previous work with embryogenic tissue of other conifers (Charest et al. 1993b; Séguin et al. 1996), and remained the same for all the experiments. To obtain data comparable to our previous work, the GUS gene was used initially as a reporter for transient expression. The levels of transient GUS gene expression in EST of cypress were highly variable as reflected by the size of the standard error (Table 1). However, the GUS gene was easily detected using the X-gluc histochemical assay. Clusters of cylindrical and spherical cells were the most frequent components of the observed blue spots. However, GUS gene expression was also histochemically detected on the whole surface of developing somatic proembryos (Fig. 1B). GUS gene expression was never detected from unbombarded samples, utilized as controls.

Transient gene expression was strongly influenced by both the age of the subculture at the time of bombardment, and the plasmid vector used (Table 1). EST bombarded 15 days after subculture yielded the highest GUS gene expression with two of the three plasmids tested (491.8 units for pCGUδ0 and 335.2 units for pBI426). No significant difference was observed among the different subculture periods using the pRT99GUS plasmid. These results are consistent and of the same order of magnitude as with other conifer species (Charest et al. 1993b; Klimaszewska et al. 1996). An effect of subculture time on transient gene expression was also observed for larch and black spruce (Duchesne and Charest 1991; Duchesne et al. 1993). Comparing the three vectors [differing in their promoter sequences, based on a single CaMV 35S promoter (pRT99GUS), a double 35S promoter linked to a translational enhancer element from alfalfa mosaic virus (pBI426), and on a ubiquitin promoter from sunflower], the relative levels of gene expression detected histologically were: sunflower ubiquitin>35S-35S-AMVE>35S (Fig. 1C). Indeed, with reference to the means of the three subculture periods, the blue spot number after bombardment with pBI426 and pCGUδ0 was respectively twice and thrice that of the pRT99GUS plasmid.

Table 2 GUS activity by MUG assay (pmol/min per milligram of extracted protein) in EST of *C. sempervirens* L. after microprojectile DNA delivery (means±SEs). Means followed by different letters are significantly different by Tukey's test at $P \leq 0.05$

Plasmid	MUG assay
pBI426	67.9 ± 11.8 a
pRT99GUS	16.8 ± 1.9 a
pCGUδ0	529.1 ± 106.5 b

Differences in transient gene expression yielded by the plasmids were also observed by the methylumbelliferyl β-D-glucuronide (MUG) fluorescent assay (Table 2). With EST from a 9-day subculture, pCGUd0 yielded the highest GUS activity (529 pmol/min per milligram of protein extracted). As with the histological assay, no significant difference was detected between the pRT99GUS and pBI426 plasmids for this specific subculture time. Endogenous fluorescence was not observed in EST of cypress (nonbombarded control).

The plasmid pCGUδ0 carries NPT II and CAT reporter genes whose level of transient expression was also tested following bombardment of EST subcultured for nine days. Both the enzymes were easily detectable (1653±194 pg NPT II protein/mg total protein, and 1751±146 pg CAT protein/mg total protein), showing that the genes were functional in the tested cypress tissue. This is in accordance with other conifer species, e.g., larch (Charest et al. 1991) and spruce (Charest et al. 1996). As another potential marker to screen transformation in cypress, the GFP gene from jellyfish was tested after bombardment into EST. Green fluorescent areas were observed and the intensity of fluorescence ranged from barely visible to bright green (Fig. 1D). This agrees with previous observations on white and black spruce, European larch and white pine (Tian et al. 1996). No background fluorescence was detected in nontransformed EST. Even if the level of detection of GFP in cypress was substantially lower than GUS (data not

shown), that GFP is detectable by a nondestructive method could represent a major advantage over GUS as a transformation marker.

In conclusion, this study demonstrates that the Mediterranean cypress has a similar response to microprojectile bombardment as other conifer species, and that the common reporter genes used for genetic transformation are functional in this species. The plasmid that gave us the highest level of transient gene expression with the GUS gene was pCGUδ0, making it a good candidate to test in stable transformation experiments. To our knowledge, this is the first report of transient gene expression in the *Cupressus* genus, which can be now added to the three other conifer genera successfully used for this type of experiment. The present results are a first step toward gene expression studies and genetic engineering of silvicultural traits in Mediterranean cypress.

Acknowledgements M. Lambardi thanks the National Research Council of Italy for the award of a fellowship (NATO/CNR), which was spent at the Petawawa National Forestry Institute of Chalk River, Canada. We would like to thank J. Haseloff for kindly providing the modified GFP sequence.

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