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Agrobacterium tumefaciens-mediated transformation of *Pinus pinea* L. cotyledons: an assessment of factors influencing the efficiency of *uidA* gene transfer

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Abstract This study is the first report of a protocol for transfer and expression of foreign chimeric genes into cotyledons excised from *Pinus pinea* L. embryos. *Agrobacterium tumefaciens* EHA105 harbouring the plasmid p35SGUSint was more infective than LBA4404 or C58 GV3850, as determined by the percentage of cotyledons showing *uidA* expression. Factors which significantly affected the T-DNA transfer included: (1) preinduction and concentration of bacteria, (2) days of coculture and (3) the wounding procedure applied. More efficient transfer of the *uidA* gene was achieved growing the bacteria in YEP medium at pH 7, infecting the cotyledons according to the sonication-assisted *Agrobacterium*-mediated transformation procedure with a bacterial density of 1 ($OD_{600\,nm}$) for 5 min, and coculture for 72 h. Using this protocol, 49.7% of the cotyledons showed a diffuse blue staining 7 days after infection. However, all were necrotic 30 days after inoculation. Since a decrease in bacterial density to 0.01 allowed the recovery of about 4% of cotyledons forming buds 1 month after inoculation, we conclude that the high mortality associated with the infection may be related to the hypersensitive response of the plant to bacterial infection.

Key words *Agrobacterium tumefaciens* · Hypersensitive response · *Pinus pinea* · Transformation · Sonication-assisted *Agrobacterium*-mediated transformation

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Abbreviations *AS* Acetosyringone · *GUS* β-Glucuronidase · *HR* Hypersensitive response · *SAAT* Sonication-assisted *Agrobacterium*-mediated transformation

Introduction

Conifers of the genus *Pinus* are economically important forest tree species planted worldwide, with an important role in soil conservation, CO_2 assimilation, wood production and as an energy source. Some species are used in landscape architecture, and have fruit characteristics. *Pinus pinea* L. is an important species of this latter group, growing in the Mediterranean area, and is highly regarded in Spain and Italy, among other countries, for its edible seeds.

Recombinant DNA technology is a powerful tool for the introduction of foreign genes into long-lived perennials and for fundamental studies of gene expression. Difficulties associated with traditional breeding technologies are minimised, and the time necessary to produce genetic changes in woody species is shortened (Cheliak and Rogers 1990). At present, particle bombardment appears to be the best technique for gene transfer into conifers, even though the transformation frequency remains low. To date, *Picea glauca* (Ellis et al. 1993), *Picea mariana* (Charest et al. 1996) and *Pinus radiata* (Walter et al. 1998) are the only conifers from which transgenic plants have been regenerated after microparticle bombardment. Although this methodology is very useful for molecular genetic studies (Birch 1997; Duchesne and Charest 1991; Jefferson 1987; Potrykus 1990), it often suffers from the problems associated with direct DNA transfer methods, which may result in the integration of multiple copies of the transgene, possibly leading to gene silencing (Kumpatla et al. 1997; Sanford 1990).

Agrobacterium tumefaciens-mediated transformation has significant advantages over direct DNA delivery, such as the introduction of one or a few copies

of genes into the plant genome, high co-expression of introduced genes, and less fragmentation of the transgene (Hadi et al. 1996). In addition, *Agrobacterium* is a much more efficient transformation tool in compatible plant species compared to the particle gun protocol (Bidney et al. 1992). However, many hosts are either transformed inefficiently or not at all by *Agrobacterium*. Thus far, the numerous efforts to apply the technique to coniferous species have had limited success. However, successful transfer of foreign genes into conifers by *Agrobacterium* spp. and the regeneration of transgenic plants has been achieved with *Larix decidua* (Huang et al. 1991; Shin et al. 1994) and *L. kaempferi* × *L. decidua* (Levée et al. 1997).

Cotyledonary explants of *P. radiata*, recently used by Holland et al. (1997) for the development of an *A. tumefaciens* transformation protocol, are good explants for plant transformation because regeneration via adventitious bud formation minimises the risks of chimeras and somaclonal variation (Tzfira et al. 1997). However, only 16–24% of the infected cotyledons showed β-glucuronidase (GUS) expression 4 days after infection (Holland et al. 1997). In our previous experiments, we observed that *P. pinea* cotyledons dissected from embryos, and for which there are protocols describing the formation of adventitious buds and shoot elongation (García-Ferriz et al. 1994; González et al. 1998), are explants susceptible to A281 (Sciaky et al. 1978), a wild-type agropine strain of *A. tumefaciens* (J. M. Humara, R. J. Ordas, unpublished results).

This study investigated transformation of stone pine cotyledons using the gene transfer system mediated by *A. tumefaciens*. Various studies indicate that monocotyledons and other recalcitrant species can be transformed using *A. tumefaciens* by manipulating various factors such as explant tissues, inoculation and coculture conditions. Furthermore, the *A. tumefaciens* strain and the combination of strain and plasmid may contribute to transformation success (Cheng et al. 1997). This study is the first report of the development and optimisation of a protocol for transfer of foreign chimeric genes in stone pine cotyledons using the binary plasmid p35SGUSint. The systematic analysis and application of several factors, such as *A. tumefaciens* strain, preinduction of the bacteria, bacterial concentration, days of coculture and wounding procedure significantly improved the transformation efficiency of this species.

Materials and methods

Plant material and tissue culture

Embryos from 1-year-old stone pine (*P. pinea* L.) seeds were used. Seeds obtained from selected open-pollinated trees in natural stands were provided by the Servicio de Material Genético of the Ministerio de Medio Ambiente (Spain). After removal of the seed coat, megagametophytes were surface sterilised by immersion in 7% H_2O_2 for 45 min, followed by three rinses in sterile double-distilled water. Megagametophytes were then imbibed in moistened sterile paper for 48 h at 4 °C to facilitate dissection of the embryos.

All plant cultures were maintained in a growth chamber at 26 ± 2 °C with a 16-h photoperiod under a photosynthetic photon flux of 80 ± 5 μmol m^{-2} s^{-1} provided by cool white light fluorescent tubes (TLD 58 W/33, Philips, France).

Bacterial strains

Three *A. tumefaciens* strains of different opine groups were used in all experiments: EHA105 (Hood et al. 1993), LBA4404 (Clontech) and C58 GV3850 (Zambryski et al. 1983), harbouring the binary vector p35SGUSint (Vancanneyt et al. 1990). This plasmid (14 kb) contains the *uidA* gene fused to the CaMV35S promoter, and the *nptII* gene controlled by the *nos* promoter. The *uidA* gene has the PIV2 intron of the gene ST-L1 from potato within its coding sequence, preventing its expression in *Agrobacterium* (Vancanneyt et al. 1990).

Preinduction and culture of bacteria

Single colonies of every *A. tumefaciens* strain were grown in different media according to the infection protocol used. In this study we tested two protocols for the transformation of cotyledons. The first (A) included a preinduction with acetosyringone (AS) at a concentration of 100 μM, and was a modification of Ritchie et al. (1993) originally developed for young maize tissues. The other protocol does not included AS and is an adaptation of that used successfully for the transfer of the *uidA* gene into *Pinus nigra* cotyledons by *A. tumefaciens* (López 1997).

Protocol A. A. tumefaciens vir gene preinduction was accomplished by obtaining single colonies from antibiotic-containing AB-sucrose minimal medium (Chilton et al. 1974) and amplifying the colony overnight in 5 ml of the same medium plus kanamycin (Sigma) 100 μg ml^{-1} and rifampicin (Sigma) 10 μg ml^{-1}. Subsequently, bacteria were collected by centrifugation, and resuspended in 25 ml of a preinduction medium without antibiotics consisting of AB salts, 10 g l^{-1} of glucose, 20 mm MES, 100 μm AS (Aldrich), pH 5.5. *vir* genes were induced at 28 °C for 24 h with shaking (250 rpm) before plant tissue inoculation.

Protocol B. A. tumefaciens was grown in 10 ml of liquid YEP medium (An et al. 1988) supplemented with adequate antibiotics on a rotary shaker for 12 h at 28 °C. An aliquot of this culture was diluted 1:10 in YEP medium without antibiotics, and for grown 5 h prior to tissue inoculation.

Subsequently, for both protocols, bacteria were diluted to twice the final absorbance ($OD_{600\ nm}$) with half-strength liquid LP medium modified by Aitken-Christie et al. (1988), supplemented with 30 g l^{-1} of sucrose and 4.44 μm benzyladenine (1/2LP1 medium), pH 5.5 or 5.8 (protocols A or B, respectively). AS to a concentration of 100 μM was added only in protocol A.

Inoculation of *P. pinea* cotyledons with *A. tumefaciens*

After imbibition on moistened sterile paper for 48 h at 2–4 °C, stone pine embryos were aseptically removed from megagametophytes, and cotyledons were excised. Explants were transferred to Petri dishes containing 10 ml of 1/2LP1 medium pH 5.5 or 5.8 (protocols A or B, respectively), as well as AS to a concentration of 100 μM for protocol A. Prior to inoculation, cotyledons were wounded by scraping with a scalpel to promote the release of *vir* inducers to the medium.

Explants were inoculated by adding 10 ml of the bacterial suspension. Bacteria used for the susceptibility assay were cultured according to protocol A, and the bacterial concentration adjusted to 1 ($OD_{600\ nm}$) before inoculation. In contrast, bacteria for the optimisation experiments were grown using both protocols, and the bacterial concentrations were 0.01, 0.1, 0.25, 0.5 and 1 ($OD_{600\ nm}$), depending on the assay. The inoculation was

conducted at 28 °C for 30 (protocol A) or 5 (protocol B) minutes on a rotary shaker (50 rpm). Then, the explants were blotted on sterile filter paper to remove the excess liquid, and cocultured 48 or 72 h in the dark at 25 ± 2 °C, on Petri dishes containing 15 ml of solid 1/2LP1 medium, pH 5.5 or 5.8. Finally, the explants were transferred to baby food jars containing 15 ml of solid 1/2LP1 medium pH 5.8 supplemented with cefotaxime (Claforan, Roussell S.A.) and vancomycin (Diatracín, Dista S.A.), both at a concentration of 250 μg ml^{-1} to eliminate *Agrobacteria*. The explants were subcultured 15 days later, and transferred to elongation medium (1/2LPC0, same medium but without benzyladenine), supplemented with activated charcoal 5 g l^{-1} and antibiotics (pH 5.8), 30 days after inoculation.

Wounding procedures

The effect of different wounding methods on the efficiency of *uidA* gene transfer 7 days after inoculation was studied. Intact or scraped cotyledons, as well as particle-gun-wounded and ultrasound-wounded cotyledons were tested. Ultrasound-wounded cotyledons were infected according to the sonication-assisted *Agrobacterium*-mediated transformation technique (SAAT; Trick and Finer 1997).

Microprojectile bombardment creates wounds due to the impact of particles over much of the surface area of the cotyledon, producing sites possibly receptive to *Agrobacterium* infection. Particle gun wounding was performed by arranging 60–70 cotyledons around the perimeter of a 2-cm-diameter circle in Petri plates containing 15 ml of solid 1/2LP1 pH 5.8, and bombarding once using the Biolistic PDS-1000/He system (Bio Rad, Hercules, Calif.), as previously described by Humara et al. (1998). A vacuum of 85 kPa was used, and approximately 500 μg of gold particles with a mean diameter of 1 μm (Bio Rad) were delivered. The gap distance from the rupture disc to the macrocarrier (1 cm), the macrocarrier travel distance (6 mm), the helium pressure (6.2 MPa) and the travel distance between the stopping screen and the target tissue (6 cm), were kept constant. Particles were prepared according to Russell (1993), but TE buffer without DNA was used. Cotyledons bombarded were transferred to Petri dishes containing 10 ml of liquid 1/2LP1 medium pH 5.8 before inoculation. The rest of the infection was done as described above (protocol B).

Cotyledons wounded by SAAT were also tested. Immediately before their excision, cotyledons were transferred to 10 ml of liquid 1/2LP1 medium pH 5.8, where they were mixed with the bacterial inoculum prepared as explained in protocol B. Then, cotyledons and bacteria were placed in an ultrasound machine (Bransonic 12) for 2 min of the 5-min inoculation period.

Controls for each type of wounding procedure were not inoculated to assess the effect on cotyledon survival and morphogenesis.

GUS histological assay

Transient expression of GUS was assayed histochemically 7 days after inoculation by staining the cotyledons with X-GLUC (Clontech), according to Jefferson (1987). Approximately 90–110 cotyledons per experiment were used for the histological assay, and vacuum-infiltrated in the solution for 5 min. Thereafter, the cotyledons were incubated for 2 days at 37 °C in darkness. This incubation period was selected on the basis of previous experiments which indicated that optimal *uidA* gene activity is observed after 2 days of incubation. After staining, explants were cleared in 70% ethanol for 24 h prior to counting the number of GUS foci. Blue spots were viewed under a dissecting microscope (Nikon SMZ-U) and each blue area was considered as one GUS-expressing focus. This is a minimum estimate of the number of discrete areas of cells with activity, because some foci may have resulted from more than one expressing cell.

Statistical analysis

All experiments were performed at least four times with a minimum of 200 explants per experiment. Data are presented as mean ± SE. Qualitative data were analysed statistically using the chi-square test. Quantitative data were analysed using ANOVA and the differences contrasted using Duncan's multiple-range test. All statistical analyses were performed at the 5% level using the SPSS statistical package software.

Results and discussion

Comparison of three *A. tumefaciens* strains

This study demonstrates for the first time the transfer and expression of T-DNA from three different *A. tumefaciens* strains to *P. pinea* cotyledonary cells. Three different opine-type *A. tumefaciens* strains (EHA105, LBA4404 and C58 GV3850) with various chromosomal backgrounds, all harbouring p35SGUSint, were compared in stone pine cotyledons (Table 1). The expression of *uidA* in *P. pinea* cotyledons 7 days after inoculation with the bacteria showed high variability in the number and intensity of GUS foci among cotyledons. This was previously observed in embryogenic lines of *Picea sitchensis* (Drake et al. 1997), tomato cotyledons (Hamza and Chupeau 1993), cuttings of chrysanthemum (De Jong et al. 1994) and *P. nigra* cotyledons (López 1997). In agreement with these latter authors, variability among different series of similar experiments was also observed in *P. pinea* cotyledons, even when the same infection protocol was used.

The most efficient *A. tumefaciens* strain for the transfer of T-DNA to stone pine cotyledons was

Table 1. Effect of different disarmed strains of *Agrobacterium tumefaciens* harbouring the plasmid p35SGUSint on *uidA* expression in *Pinus pinea* cotyledons 7 days after inoculation. Data are presented as the mean ± SE of at least four different experiments. In each column, values with different letters are significantly different ($P \leq 0.05$)

Agrobacterium tumefaciens strain	GUS positive cotyledons(%)	Mean ± SE number of GUS foci/cotyledon	Cotyledons forming buds 30 days after inoculation (%)
EHA105	5.1 a	2.05 ± 0.73 a	42.5 ab
LBA4404	0.4 b	1 ± 0.33 b	48 a
C58 GV3850	0.4 b	1 ± 0.33 b	31 b
Control	0 c	0 c	86.5 c

EHA105, an agropine-type bacteria derived from EHA101 (Hood et al. 1993). Five percent of cotyledons inoculated with EHA105 p35SGUSint showed an average of two GUS foci per cotyledon, while only 0.4% of the explants inoculated with LBA4404 or C58 showed GUS expression (Table 1). EHA105 is a disarmed derivative of A281, a hypervirulent bacterium that has been used successfully to infect various plant species, including *Pinus* species (Holland et al. 1997; Loopstra et al. 1990; Morris et al. 1989; Stomp et al. 1990). In addition, preliminary infections of *P. pinea* cotyledons with an agropine-type *A. tumefaciens* strain, A281, an octopine-type strain, Ach5, and two nopaline-type strains, C58 and 82.139, showed that only strain A281 was able to induce tumours at reasonable frequency (data not shown). Moreover, EHA105 displayed the highest efficiency for the transfer of *uidA* to cotyledonary explants of *P. nigra* (M. López, 1997).

Thirty days after the infection, which is the end of the bud induction period, a remarkable decrease in the survival rate of the infected cotyledons was observed. Thus, although 86% of control cotyledons were alive and formed buds, only 31–48% of the cotyledons infected with *A. tumefaciens* survived (Table 1). Consequently, EHA105 was chosen as a model system to optimise the transformation parameters for *P. pinea* cotyledons.

Evaluation of factors affecting T-DNA transfer

Various factors influencing the efficiency of T-DNA delivery were evaluated using *A. tumefaciens* EHA105 p35SGUSint. These factors included two protocols for the preinduction of *A. tumefaciens*, the cell density for inoculation ($OD_{600\,nm}$ of 0.25, 0.5 and 1), and the coculture period (2 and 3 days); all were tested independently.

uidA expression was assessed histochemically 7 days after inoculation of cotyledons with 12 different treatments (Table 2). The intensity of GUS staining and also the number of GUS foci appeared to be quite variable among cotyledons (data not shown). GUS foci were well defined, corresponding probably to one or a collection of small individual spots which showed a faint blue colour (Fig. 1A). GUS foci were presented across the entire cotyledonary surface, mainly in the area of excision of the cotyledon from the embryo, or associated with wounded zones. Some of the foci were very small and hardly visible.

Table 2. Effect of preinduction of bacteria, bacterial density, and coculture time on *A. tumefaciens*-mediated *uidA* gene transfer efficiency in *P. pinea* cotyledons, measured 7 days after inoculation. Data are presented as mean ± SE number of at least four different experiments. Values with different letters are significantly different ($P \leq 0.05$)

Protocol	Bacterial concentration ($OD_{600\,nm}$)	Days of coculture	Treatment no.	Mean ± SE total number of GUS foci per treatment
A	0.25	2	1	Not detected
		3	2	0.02 ± 0.01 a
	0.5	2	3	0.05 ± 0.04 ab
		3	4	0.1 ± 0.05 a
	1	2	5	0.02 ± 0.02 ab
		3	6	0.16 ± 0.03 a
B	0.25	2	7	0.28 ± 0.08 ab
		3	8	0.46 ± 0.09 bcd
	0.5	2	9	0.4 ± 0.15 cd
		3	10	0.95 ± 0.14 ef
	1	2	11	0.7 ± 0.13 cf
		3	12	1.19 ± 0.14 e

Statistical analysis revealed interactions between the infection protocol (A or B) and the bacterial density (1, 0.5 or 0.25) or the coculture period (2 or 3 days), but not between the densities and the days of coculture.

When a modification of the protocol published by Ritchie et al. (1993) for maize was used, we did not find significantly differences for any of the treatments

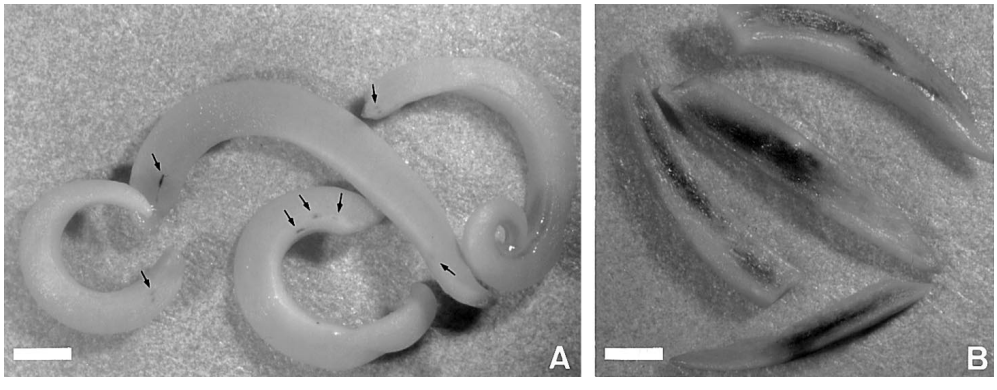

Fig. 1A, B Histochemical assay of GUS in *Pinus pinea* cotyledons 7 days after their inoculation with *Agrobacterium tumefaciens* EHA105 p35SGUSint. **A** Infection treatment no. 12: scraped cotyledons, bacteria grown according to protocol B, and 5 minutes of inoculation with a bacterial density of 1 ($OD_{600\,nm}$). Three days of coculture. *Arrows* indicate GUS foci (*bar*: 1.3 mm). **B** SAAT procedure: cotyledons and bacteria were sonicated for 2 min, left at room temperature for another 3 min before the explants were transferred to coculture medium for 72 h. The bacterial density was 1 ($OD_{600\,nm}$) (*bar*: 1 mm)

Table 3. Effect of different wounding procedures on *A. tumefaciens*-mediated *uidA* gene transfer into *P. pinea* cotyledons, measured 7 days after inoculation. Data are presented as the mean ± SE number of at least four different experiments. Values with different letters are significantly different ($P \leq 0.05$)

Wounding procedure	Number of assayed cotyledons	Percentage of GUS-positive cotyledons	Mean number of GUS foci/cotyledon ± SE
None	338	16.6 a	4.1 ± 0.73 a
Scrape	311	8.4 b	2.57 ± 0.3 a
Bombardment	281	5.3 b	4.26 ± 1.11 a
SAAT	318	27.7 c	Diffuse staining
Control	153	0 d	0 b

performed (treatments 2–6). In contrast, treatments 7–12, in which *A. tumefaciens* was grown according to the procedure of M. López (1997), showed significant differences between treatments. Higher *A. tumefaciens* cell density and longer time of coculture yielded more efficient T-DNA delivery to stone pine cotyledons (Table 2). The protocol that yielded the best levels of GUS expression was the adaptation of the one developed by M. López (1997) for *P. nigra* cotyledons (protocol B). Consequently, it was not necessary to grow the bacteria in AB minimal medium, at acidic pH and with AS to obtain *uidA*-expressing stone pine cotyledonary cells. Overall, the best treatment was number 12: cotyledons inoculated for 5 min with EHA105 p35SGUSint grown according to protocol B, using a bacterial density of 1 ($OD_{600\,nm}$) and a coculture of 3 days (Fig. 1A).

As preinduction with AS is not essential when the phenolic compounds exudated by the wounded tissue are sufficient to activate a *vir* response (Cheng et al. 1997; Hiei et al. 1997, Park et al. 1996), and because in our previous experiments studying *vir* gene induction, we demonstrated the activation of the virulence cascade by exudates of *P. pinea* cotyledons (Humara et al., in press), our results revealing that AS was not necessary had been expected.

Other factors possibly improving transformation frequency are bacterial density and cocultivation time. A coculture of 3 days was adequate for T-DNA transfer (Table 2), whereas longer periods led to an uncontrollable bacterial infection resulting in explant necrosis (data not shown). The reduction of the coculture to 48 h resulted in lower levels of GUS expression. These results are in agreement with Drake et al. (1997), who found a 15-fold increase in the transfer efficiency using a coculture time of 72 h compared to 48 h, as well as with the data of Holland et al. (1997) with *P. radiata* cotyledons. In general, most of the studies show better transfer efficiencies when the coculture time is between 48 and 72 h (Muthukumar et al. 1996; Tzifira et al. 1997).

A. tumefaciens-infected cotyledons showed a decrease in their bud-forming capacity, quite variable among experiments with a particular treatment. Thus, the percentage of cotyledons forming buds 30 days after inoculation varied between 2 and 24% for treatment 12, or between 3–53% for treatment 6. Generally, the average percentage of cotyledons forming buds 1 month after the inoculation was 13–40% (treatments 5 and 12, respectively).

Another factor which can be varied to optimise *Agrobacterium*-mediated transformation is the procedure used to improve access of *Agrobacterium* and also to create an area of wounding to induce plant cells to produce phenolic compounds for *vir* gene induction (Table 3). None of the control cotyledons wounded but not inoculated showed any *uidA* gene expression. The highest percentage of GUS expressing cotyledons (27%) was achieved with the sonication-assisted *Agrobacterium*-mediated transformation (SAAT). The explants showed a diffuse GUS expression all over the surface of the cotyledon, making the quantification of the number of foci impossible (Fig. 1B). About 16% of unwounded cotyledons showed an average of 4 GUS foci per cotyledon. Only 8.4% of scraped cotyledons, and 5.3% of cotyledons bombarded with gold particles showed GUS expresssion. It is hypothesised that only wounded cells are susceptible to transformation (Bidney et al. 1992; Shimoda et al. 1990). Potrykus (1991) suggests that only plants with an appropriate wound response develop larger populations of wound-adjacent competent cells for regeneration and transformation. Therefore, although excessive wounding is probably detrimental to stable transformation, the frequency of gene transfer mediated by *A. tumefaciens* can in some species be significantly enhanced by inducing wounds in the target tissue (Bidney et al. 1992). For this reason, we studied three wounding procedures including scraping the cotyledons with a scalpel, bombardment and SAAT. The generation of microwounds in the cotyledons through high-velocity microprojectile bombardment has increased the infection rates in some species (Bidney et al. 1992; Gutiérrez et al. 1997; Tingay et al. 1997). Nevertheless, the analysis of the data (Table 3) showed about 16% of intact cotyledons with GUS expression, a percentage significantly higher than those obtained scraping the cotyledons (8.4%) or wounding them with gold particles (5.3%). In contrast to dicots, and similarly to monocots, conifer tissues consisting of actively dividing cells, such as those in cotyledons, might be transformable in the presence of *vir*-inducing compounds, and a wound response might not be necessary (Hiei et al. 1997). However, using SAAT for cotyledon wounding yielded about 28% GUS-positive explants (Table 3), with a diffuse GUS expression all over the surface of the coty-

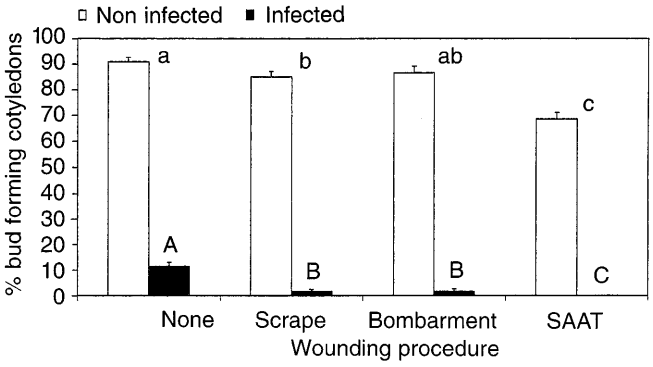

Fig. 2. Percentage of cotyledons forming buds at the end of the bud-forming period. Stone pine cotyledons with different types of wounds were inoculated with *A. tumefaciens* EHA105 p35SGUSint according to treatment 12. Cotyledons that were not infected were used as controls. Data are presented as the mean ± SE of at least four different experiments. Columns with different letters are significantly different ($P \leq 0.05$)

ledon, making quantification of the number of foci impossible (Fig. 1B). Ultrasound may be creating more microwounds than any other wounding procedure (Trick and Finer 1998).

Ultrasonication has been reported to mediate gene uptake in plant protoplasts, suspension cells and intact pieces of tissue (Joersbo and Brunstedt 1992; Sawahel 1996). Our work demonstrates the advantages of combined sonication of stone pine cotyledons and *A. tumefaciens* to achieve high levels of expression of the *uidA* gene (Fig. 1B), similar to reports by Trick and Finer (1997, 1998) for soybean, cowpea, wheat and white spruce, among other species.

One month after inoculation the percentage of surviving cotyledons forming buds was recorded (Fig. 2). Although sonication of pine cotyledons in the SAAT procedure reduced shoot formation compared to scraping or wounding by bombardment, any of these wounding procedures can be used for obtaining transgenic plants. However, inoculation with the bacteria dramatically reduced the survival rate of the explants as assessed 30 days after infection (Fig. 2). None of the SAAT cotyledons were alive at the end of the bud induction period, and only 1% and 2% of the cotyledons scraped or bombarded, respectively, formed buds. The best survival rate (11%) was obtained with cotyledons that only had wounds caused by excision from the embryo and subsequent manipulation.

The cause for this reduction in the number of cotyledons forming buds has not been investigated, but might be related to the hypersensitive response (HR) as part of plant defence against pathogens (E. Hood, personal communication). It is possible that the infection of stone pine cotyledons with *A. tumefaciens* triggers a HR killing most of the cells, and consequently reducing the percentage of live cotyledons forming buds during the bud induction period. This is supported by the observation that a reduction in the bacterial density led to an increase in the number of explants showing regenerative processes. Orlikowska et al. (1995) also found a five- to tenfold reduction in the regeneration efficiency of seedling explants of safflower, probably associated with the HR activated by *A. tumefaciens* infection. They suggest that infection-induced responses in wounded peripheral cells could systemically affect regeneration from adjacent and more distant organogenetic cells.

Based on these results, it was concluded that the detrimental effect on cotyledons related to *A. tumefaciens* infections might be diminished by decreasing the bacterial density. Consequently, five different bacterial densities were tested with sonicated *P. pinea* cotyledons. Results obtained 7 days after inoculation are shown in Table 4. Uninfected control cotyledons did not show *uidA* expression. Higher bacterial density increased the transfer frequency of the marker gene (Tables 2 and 4). This relationship has been observed for several species, such as black poplar (Confalonieri et al. 1994) and Sitka spruce (Drake et al. 1997). Consequently, the best percentage of *uidA*-expressing cotyledons (49%) was found when the bacterial density ($OD_{600\,nm}$) was 1. With this density, and with 0.5 and 0.25, the number of GUS foci per cotyledon could not be counted because some of the cotyledons became completely blue after staining (Fig. 1B). However, a decrease in the blue intensity linked to the reduction of the bacterial density was observed (data not shown). A bacterial density of 0.01 ($OD_{600\,nm}$) yielded 13.4% of cotyledons with *uidA* expression, with an average number of six GUS foci per cotyledon.

Thirty days after tissue inoculation, we analysed the percentage of cotyledons forming buds. Cotyledons exposed to ultrasound for 2 min, but not inoculated with bacteria, formed buds at a frequency of 58%. This

Table 4. Effect of bacterial density on *A. tumefaciens*-mediated *uidA* gene transfer into *P. pinea* cotyledons, measured 7 days after inoculation. Data are presented as the mean ± SE number of at least four different experiments. Values with different letters are significantly different ($P \leq 0.05$)

Bacterial concentration ($OD_{600\,nm}$)	Number of assayed cotyledons	Percentage of GUS-positive cotyledons	Mean ± SE number of GUS foci/cotyledon
1	320	49.7 a	Diffuse staining
0.5	376	27.7 b	Diffuse staining
0.25	337	28.5 b	Diffuse staining
0.1	350	23.1 b	10.2 ± 1.5
0.01	328	13.4 c	6.25 ± 1.03

frequency was reduced to 4% when the explants were inoculated with a bacterial density of 0.01. With higher densities, no cotyledons were alive at the end of the bud-forming period.

Although a high transient expression frequency does not necessarily result in high stable transformation frequencies, the detection of GUS expression in *P. pinea* cotyledons 7 days after inoculation with *A. tumefaciens* EHA105 p35SGUSint indicated that this system may be used to insert foreign genes into this important species. The high mortality associated with the infection might be related to the defence system of the plant, and a reduction in the bacterial density used for inoculation increased slightly the percentage of live cotyledons forming buds 1 month after the inoculation with the bacteria.

Previous data have demonstrated that the CaMV35S promoter is not very efficient in *P. pinea* cells (Humara et al., in press), whereas the *UbB1* promoter from sunflower (Binet et al. 1991) and the maize ubiquitin gene promoter *Ub1* (Christensen et al. 1992) showed higher expression. We have now cloned a new binary vector considering all the data available, which might allow us to improve the efficiency of transformation, and to reduce the stress associated with the infection through a reduction in bacterial density.

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