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Short Communication

Optimizing DNA Delivery into Stone Pine Embryogenic Lines

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Abstract

A standardized protocol for *Agrobacterium*-mediated transformation of *Pinus pinea* (stone pine) embryogenic callus is presented after testing the effect of factors such as amount of initial tissue, infection period, bacterial dilution, acetosyringone concentration, plasmid constructions, and *Agrobacterium* strains. Transient *GUS* (beta-glucuronidase) gene expression was used to monitor T-DNA delivery into targeted cells. The most efficient concentrations of the selective agents (kanamycin and phosphinothricin) have been also determined. Significant genotypic variations in response to transformation were observed between the two embryogenic lines tested. Infection of 12 g calli with *Agrobacterium* for 5 min, including 1 minute vacuum, followed by co-cultivation for 3 days was found to be optimum for maximum transformation efficiency. Among constructions, AGL1 strain harboring pTAB16 plasmid at an OD of 0.8, with 200μM acetosyringone significantly increased DNA delivery into the cells. Selection of transformed tissue required 1 mg phosphinothricin or 5 mg/l kanamycin. The optimized protocol would be useful for *Agrobacterium*-mediated genetic transformation of stone pine for genetic and agronomical studies.

ABBREVIATIONS

AS: Acetosyringone; GUS/*gus*A: Betaglucuronidase; Kan: Kanamycin; MLV: Modified Litvay Medium; MS: Murashige and Skoog medium; NOS: Nopaline Synthase Promoter; *npt*II: N*eomycin Phosphotransferase*; OD: Optical Density at 600nm; PPT: Phosphinotricin; SE: Somatic Embryogenesis

INTRODUCTION

Pinus pinea L. (stone pine) is an economically important forest species of the Mediterranean region, where it has been widely cultivated due mainly to the high economic value of its seed crops [1]. Besides its agronomical value, the species is also used for ecological and ornamental purposes. Stone pine is characterized by a very low genetic variation and high adaptative plasticity [2]. Thus, the species is well adapted to the high temperatures and drought characteristics of Mediterranean climates; also it is less sensitive to diseases and pests than other Mediterranean pines, particularly to the pine wilt nematode *Bursaphelenchus xylophilus* [3]. In spite of these environmental adaptations, seed production vary annually mainly due to water shortage [4]. Recently, several studies on gene expression and epigenetic variability under drought stress have been described [2,5]. Further research on assessments of gene function requires the generation of mutants or transgenic plants with altered gene expression [6]. Protocols for *Agrobacterium*-mediated transformation from stone pine isolated cotyledons have been described [7]. However, the advantages of somatic embryogenesis (SE), particularly maintenance of regeneration potential by cryopreservation while the testing of clones is in progress [8], prompted to develop SE protocols for this species firstly described by Carneros et al. [9]. In the present work the conditions for an efficient DNA delivery into stone pine embryogenic lines are reported.

MATERIALS AND METHODS

Plant material and tissue culture

Pinus pinea embryogenic lines (1F11 and 7F11) used in this study were generated as described in [9] and provided by Dr. Mariano Toribio (Instituto Madrileño de Investigación y Desarrollo Rural, Agrario y Alimentario, IMIDRA, Spain). Embryogenic lines were maintained by subcultures to fresh mLV medium [10] every two weeks. Embryogenic calli, grown for 4 days after their transference to fresh medium, were used in all experiments.

Bacterial strains and plasmids

Agrobacterium tumefaciens AGL1 [11], EHA105 [12], C58 [13], and GV3101 [14], strains and plasmid constructions pABC, pBIN35SGUSINT, pBINUbiGUSINT and pTAB16 were used in the experiments. To facilitate transient expression assays, all constructions included *GUS* (*gus*A) gene driven by the CaMV35S

promoter, unless otherwise stated. This gene is not expressed in *A. tumefaciens* due to the insertion of an intron plant in the protein-coding region. The pABC plasmid [15] carries the *Atwbc*19 gene that confers tolerance to kanamycin (Kan), driven by the CaMV35S promoter (gift of Dr. Neal Stewart, University of Tennessee; USA). The pBIN35SGUSINT plasmid contains the *neomycin phosphotransferase* (*npt*II) gene, controlled by the *nos* (nopaline synthase) promoter. This gene confers Kan resistance [16]. The pBINUbiGUSINT plasmid contains the *npt*II gene driven by the *nos* promoter, but the *gus*A gene is under the control of the *ubi*I maize polyubiquitin promoter [7]. The pTAB16 plasmid includes the *bar* gene, which confers phosphinothricin (PPT) tolerance, driven by the CaMV35S promoter [11].

Bacterial strains were cultured in LB (Luria Broth) medium for 16 hours at 28°C in a horizontal orbital shaker (200 rpm), with appropriate antibiotics to reach the desired optical density (OD). LB medium for AGL1 strain cultures also contained 0.4 g/l of $MgSO_4$. The antibiotics used were rifampicin (50 µg/ml) for selection of AGL1 strain, rifampicin (50 µg/ml) and tetracycline (5 µg/ml) for the C58 strain, nalidixic acid (30 µg/ml) for the EHA105 strain, gentamicin (25 µg/ml) and tetracycline (5 µg/ml) for the GV3101 strain, tetracycline (5 µg/ml) for the pTAB16 construction and Kan (50 µg/ml) for the pBIN35SGUSINT, pBINUbiGUSINT and pABC constructions.

Transformation experiments

Unless otherwise stated, the bacterial suspensions were centrifuged (3000 rpm, 20 min) and the precipitate resuspended in MS liquid medium [17] to reach the desired OD_{600} and 100 µM acetosyringone (AS) was added. For infection, equal volumes of bacterial suspension and tissue (6 g/50 ml mLV) were mixed in a 125 ml kitasato flask and 1 min vacuum infiltration was applied. Ten minutes later, the mix was recovered on 55 mm Ø Whatman no. 2 filter paper and placed on absorbent paper to drain excess liquid. The filter paper was placed on semi-solid mLV medium without casein hydrolysate for a 3 days co-culture period at 25 ± 2 ºC in darkness.

To optimize *Pinus pinea* transformation conditions, the effect of different parameters on DNA delivery were sequentially assayed. These included initial amount of calli (6 or 12 g/50 ml of mLV); infection period (5 or 10 min); bacterial dilution (OD_{600} 0.3 or 0.8); AS concentration (100 vs. 200 µM); plasmids (pABC, pBIN35SGUSINT, pBINUbiGUSINT and pTAB16) and *Agrobacterium* strains (AGL1, EHA105, GV3101 and C58). Four replications were prepared for each experiment and transient *GUS* expression was determined after 3 days coculture.

GUS histological assay

A histological assay for *GUS* transient expression was performed 3 days after co-culture. Filter paper with infected calli was deposited into 60 mm Ø empty plates, with 2 mL of the reagent mix described by Jefferson et al. [18] containing 2 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc), and incubated at 37ºC for 16 h in the dark. Then, each plate was examined microscopically and the number of foci (areas of blue cell staining) was recorded.

Sensitivity to selection agents

The concentrations of two selective agents (Kan and PPT) that inhibited growth of untransformed tissue were determined. To this end, 0.5 g of each embryogenic line was suspended in 5 ml mLV liquid medium. The mix was softly shacked and poured on a Buchner funnel with a 70 mm Ø Whatman filter paper. After draining, aided by a final vacuum pulse, the filter paper with embryogenic cells was transferred to petri dishes containing 25 ml of semisolid mLV medium with PPT (0, 2, 4 or 6 mg/l) or Kan (0, 5,10, 20 or 30 mg/l). Glutamine and casein hydrolysate were excluded from PTT-supplemented media. Three replicates were performed per treatment and calli fresh weight was determined after two weeks in culture.

Statistical analysis

Significance of the different treatment effects on transient *GUS* expression was determined using analysis of variance (ANOVA). Percentage data were subjected to arcsine transformation prior to statistical analysis. Variation among treatments means was analyzed using the Tukey´s procedure [19]. All statistical analyses were performed using SPSS Statistics 20.0.0.

RESULTS AND DISCUSSION

Effect of initial tissue amount

Six or 12 g of the 1F11 embryogenic line were infected with AGL1-pTAB16 (OD_{600} 0.8). The infection of 12 g tissue produced ten times more transient *GUS* expression than 6 g tissue (291.0 vs. 27.3 blue foci per plate, respectively $p \leq 0.05$). Similar rates (10 mg/50mL of embryogenic cells) were successfully used to transform other pine species [20-22]. Based on these results, 12 g/50 ml mLV of callus was selected for subsequent experiments.

Effect of infection period

Twelve g of 1F11 and 7F11 embryogenic lines were infected with AGL1-pTAB16 (OD_{600nm} 0.8) for 5 or 10 min. No significant differences were observed between the embryogenic lines, but a 5 minutes infection period was significantly more effective ($p \leq 0.05$) than 10 minutes (232.0 blue foci versus 60.0, respectively). Five min infection also produced the highest transformation rates from stone pine cotyledons [7]. However, shorter period of infection have been used on *Pinus radiata* [20] and *Pinus pinaster* (maritime pine) [23]. Five minutes infection period was selected for subsequent experiments.

Effect of bacterial dilution

The effect of two AGL1-pTAB16 bacterial dilutions (OD_{600} 0.3 and 0.8) was tested on 1F11 and 7F11 lines. An OD_{600} of 0.8 increased GUS expression on both embryogenic lines (average of 173.1 blue foci vs. 25.1, for an OD of 0.8 and 0.3, respectively; $p \leq 0.05$). Similar results were previously reported for cotyledon transformation of the species [7]. On the contrary, bacterial dilution did not affect *GUS* transient expression on *Pinus pinaster* embryogenic lines [23]. An OD of 0.8 was selected for subsequent studies.

Effect of AS concentration

Three different AS concentrations (0, 100 and 200 µM) in the

bacterial dilution medium used for infection of 1F11 and 7F11 embryogenic lines were tested. Irrespective of the embryogenic line, the highest AS concentration favored significantly *GUS* transient expression (average of 462.1, 178.2 and 38.6 blue foci for 200, 100 and 0 µM AS, respectively; $p \leq 0.05$). The interaction between AS concentration and the embryogenic line was also significant, bests results being obtained on 1F11 line and AS 200 µM, where a mean of 647.7 blue foci were observed (Figure 1 & Table 1). In some conifers, adding AS to the coculture medium increases transient expression [21,24-25]. On the contrary, in *Pinus pinaster* AS did not increase *GUS* expression [23,26]. A concentration of 200 µM AS was selected for further studies.

Effect of plasmid constructions

Plasmids pABC, pBIN35SGUSINT, pBINUbiGUSINT and pTAB16 integrated into AGL1 strain were assayed. Regardless of the embryogenic line, pTAB16 plasmid was more effective in terms of transient *GUS* expression especially on line 1F11 (Table 2). The pTAB16 plasmid had not been previously assayed to transform *Pinus* species although the *bar* gene was already used for maritime pine transformation [22].

Effect of bacterial strains

Lines 1F11 and 7F11 were infected with AGL1, EHA105, GV3101 or C58 strains harboring the pBIN35SGUSINT plasmid. Irrespective of the embryogenic line, AGL1 induced the highest levels of GUS expression (average of 323.1 blue foci/plate vs. 6.8 and 0.0 for EHA105 and each of the GV3101 and C58 bacterial strains, respectively; Table 3). The low susceptibility of stone pine embryogenic tissue to EHA105 and C58 infection was not found in other pine species. Thus, although C58 strain was also ineffective to transform stone pine cotyledons, EHA 105 successfully did it [7], and C58 is being routinely used to transform maritime pine [23]. AGL1 was also effective to transform radiata pine cotyledons [27], nevertheless, its effect on embryogenic lines from maritime pine seems to be genotype-dependent [23,28].

Selective agent's sensitivity

In both embryogenic lines, callus growth was significantly reduced by all Kan and PPT concentrations tested. Growth reduction percentages ranged from 40 to 90% (data no shown). Thus, 5-10 mg/l Kan or 1-2 mg/l PPT can be used to select transformed stone pine tissue. Similar concentrations have been used to select another pine species [22,27].

Our results do not differ substantially from those previously reported for other *Pinus* species except for the requirement of higher AS concentration, and for the high infection capability of AGL1 which can be explained by the presence of extra virulent factors in this strain [29]. The variability detected in the mean number of GUS foci among different series of similar experiments, even when the same infection protocol was used, might be due to the metabolic state of the tissue, including its degree of

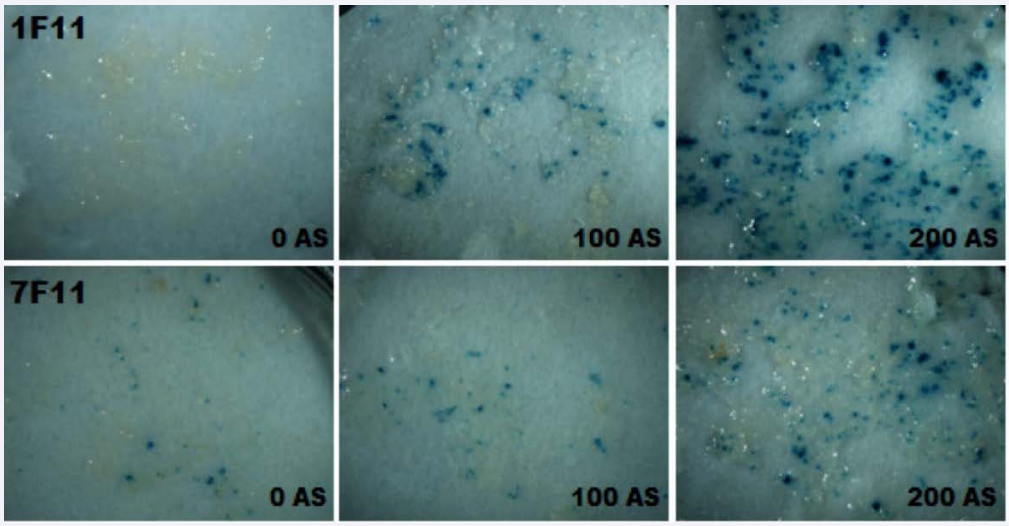

Figure 1 Effect of AS concentrations (0, 100 and 200 µM) on GUS expression on two stone pine embryogenic lines 1F11 (up) and 7F11 (bottom).
Abbreviations: AS: Acetosyringone; GUS: gusA expression

Table 1: Effect of AS concentration on *GUS* expression in 1F11 and F11 embryogenic lines of *Pinus pinea*. Data are the mean ± SE of at least four measurements. Values with different letters are significantly different according to Tukey´s test ($p \leq 0.05$).

	Mean number of GUS foci/plate		
	AS concentration (µM)		
Embryogenic line	0	100	200
1F11	0.0 ± 0.0 b	270.7 ± 116.9 b	646.7 ± 64.9 a
7F11	77.2 ± 49.9 b	85.7 ± 49.8 b	277.5 ± 75.1 b

Abbreviations: AS: Acetosyringone; GUS; *gus*A expression; SE: Standard Error

Table 2: Effect of different plasmid constructions, integrated in AGL1 strain, on *GUS* expression in 1F11 and F11 embryogenic lines of *Pinus pinea*. Data are the mean ± SE of at least four measurements. Values with different letters are significantly different according to Tukey´s test (p≤0.05).

	Mean number of GUS foci/plate			
	Plasmid constructions			
Embryogenic line	pABC	pBIN35SGUSINT	pBINUbiGUSINT	pTAB16
1F11	112.3 ± 36.7 b	157.8 ± 28.8 b	161.5 ± 38.4 b	606.5 ± 58.4 a
7F11	31.5 ± 26.5 b	74.0 ± 28.7 b	16.8 ± 7.1 b	252.3 ± 28.2 ab

Table 3: Effect of bacterial strains, harboring the pBIN35SGUSINT plasmid, on *GUS* expression in 1F11 and F11 embryogenic lines of *Pinus pinea*. Data are the mean ± SE of at least four measurements. [z]Effect of bacterial strain. For each column, values with different letters are significantly different according to Tukey´s test (p≤0.05).

	Mean number of GUS foci/plate			
	Bacterial strains			
Embryogenic line	AGL1	EHA105	C58	GV3101
1F11	332.5 ± 31.8	11.5 ± 5.9	0.0 ± 0.0	0.0 ± 0.0
7F11	313.7 ± 49.2	2.2 ± 2.2	0.0 ± 0.0	0.0 ± 0.0
Mean[z]	323.1 ± 27.3 a	6.8 ± 3.4 b	0.0 ± 0.0 b	0.0 ± 0.0 b

phenolization, which may vary within the time that the two trials were performed, affecting differently to the interaction between *A. tumefaciens* and plant cells [21,30].

CONCLUSION

Here we present for the first time a protocol for DNA transfer into *Pinus pinea* embryogenic lines that includes: a) The use of AGL1 strain harboring the pTAB16 plasmid grown at a bacterial OD_{600} of 0.8; b) the centrifugation and resuspension in MS liquid medium with 200 µM AS; c) five minutes infection with an initial one minute vacuum; d) the regeneration of transgenic lines, after 3 days co-culture, on selection medium with 1 mg/l PPT. In plasmids with *npt*II gene, the selection medium must include 5 mg/l Kan. This protocol opens up significant possibilities for genetic improvement of *Pinus pinea* selected lines.

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Author contributions

J.S; and I.A. conceived and designed the experiments; MB and JM performed the experiments; MB and IA analyzed the data; MB, JS and IA wrote the paper.

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