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Stable *Agrobacterium*-mediated transformation of embryogenic tissues from *Pinus pinaster* Portuguese genotypes

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Abstract Protocols for genetic transformation of maritime pine (*Pinus pinaster* Sol. ex Aiton) embryogenic tissues were developed using the *Agrobacterium* C58pMP90/pPCV6NFGUS. This is the first report of *Agrobacterium*-mediated T-DNA integration in *P. pinaster* confirmed by Southern blot analysis. The omission of casein hydrolysate from culture medium during cocultivation and subsequent subculture was crucial to control *Agrobacterium* growth. Two different transformation protocols were compared: (1) bacterial drops were spread over embryogenic clumps; (2) a mixture of bacterial and embryogenic cell suspensions was plated on filter paper. The highest frequency of transformation (22 independent transformed lines per g fresh weight, for embryogenic clone 31/668/00) was obtained with *Protocol 2*. The same basic procedure allowed transformation of embryogenic cell suspensions, which was dependent on subculture age. From 52 hygromycin-resistant independent lines obtained, 47 showed stable *uid*A gene expression and were PCR-positive for *uid*A gene and 42 for *hpt* gene. No residual *Agrobacterium* was detected in the transformed lines. Transgene integration was achieved using both protocols, as confirmed by Southern hybridization. From 38 (90%) transformed lines successfully cryopreserved and recovered, 71% regrown replicates have maintained the frequency of cell aggregates and early-formed embryos with *uid*A expression. Maturation of 44 transformed lines gave rise to 3 mature somatic embryos, each one coming from a different transformed line. Our results show the high potential of *Protocol 2* for application to different culture systems.

Keywords Conifer · Cryopreservation · Embryogenic cell suspensions · *gus* expression · Maritime pine · Somatic embryogenesis

Abbreviations

AS	Acetosyringone
BAP	6-Benzylaminopurine
CH	Casein hydrolysate

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(2,4-D) 2,4-Dichlorophenoxyacetic acid
SE Somatic embryogenesis

Introduction

Tree improvement is one of the most important tools for facing the increasing world demand for forest products (McNutt and Rennel 1997). In Portugal, the low productivity of *Pinus pinaster* forest has led to a chronic deficit of pinewood (Direcção Geral das Florestas 2002). Genetic transformation, when associated with conventional breeding, may provide a powerful tool for rapidly increasing yield, wood quality or to allow gene functional analysis. *Agrobacterium*-mediated gene transfer has been considered advantageous over particle bombardment since usually it does not lead to the integration of high transgene copy number, which may cause gene silencing (*reviewed by* Hansen and Wright 1999; Gelvin 2003). *Agrobacterium*-based system has been widely applied to different tissues of several pine species (Levée et al. 1999; Wenck et al. 1999; López et al. 2000; Trontin et al. 2002; Tereso et al. 2003; Tang et al. 2001; Tang and Newton 2004; Grant et al. 2004), but transformation remains highly dependent on the genotype. Somatic embryogenesis (SE) from immature zygotic embryos has been the most commonly used method for regeneration of transformed conifer plants (Levée et al. 1997 1999; Wenck et al. 1999; Klimaszewska et al. 2001 2003). In *P. pinaster*, SE was previously achieved (Bercetche and Pâques 1995; Lelu et al. 1999; Miguel et al. 2004) and used in the genetic transformation of French genotypes (Trontin et al. 2002).

In our previous work, two different *Agrobacterium*-mediated transformation procedures have been tested in Portuguese *P. pinaster* embryogenic clones, based on the protocols described for other conifers by Levée et al. (1997, 1999). However, the use of both transformation procedures has led to *Agrobacterium* overgrowth, not allowing the recovery of proliferating embryogenic tissues after cocultivation (Tereso et al. 2003).

The aim of this study was to establish protocols for the genetic transformation of embryogenic tissues and cell suspensions from Portuguese *P. pinaster* genotypes. The effect of cryopreservation of transformed lines on *uid*A gene expression was also addressed. The removal of casein hydrolysate (CH) from the culture medium was crucial for the successful recovery of transformed tissues.

Material and methods

Plant material and tissue culture conditions

Embryogenic cultures of *P. pinaster* were initiated from immature zygotic embryos as previously described (Miguel et al. 2004) from plus trees in Mata do Escaroupim, Portugal. Embryogenic tissues were initiated and maintained by 2-week subcultures on IM6 medium (Miguel et al. 2004) consisting of DCR basal medium with 13.6 μM 2,4-D and 4.4 μM 6-benzylaminopurine (BAP) supplemented with 500 mg l^{-1} casein hydrolysate and 1460 mg l^{-1} L-glutamine. The same medium was used for the cocultivation with *Agrobacterium* and for isolation of regrown tissues in the subsequent subculture. Cultures were maintained in a growth chamber at 22 ± 2°C in darkness.

Embryogenic cell suspensions were established using the protocol described by Bercetche and Pâques (1995). Cultures were maintained with shaking at 100 rpm in a growth chamber at 22 ± 2°C in darkness.

The term ''transformed line'' was used to describe a portion of embryogenic tissue deriving from a single transformation event. The term ''embryogenic clone'' refers to an embryogenic culture initiated from a single genotype. Each embryogenic clone (*x/y/z*) is identified by the number of the mother-tree from which the corresponding cone was collected (*x*), a number corresponding to the seed or genotype (*y*), followed by the last two numbers of the year when it was initiated (*z*).

Agrobacterium strain and culture conditions

The disarmed *A. tumefaciens* strain C58/pMP90 (Koncz and Shell 1986) containing the binary vector pPCV6NFGUS (Mathur et al. 1998) was used in the transformation experiments. This

plasmid carries the hygromycin phosphotransferase gene (*hpt*) which confers hygromycin resistance and the *uid*A gene, under the control of the 35S cauliflower mosaic virus (CaMV) promoter and terminators, with the IV2 intron from potato *ST–LS1* gene.

Bacteria were grown in MYA liquid medium (Tefler and Casse-Delbart 1987) containing 50 μg ml^{-1} rifampicin, 20 μg ml^{-1} gentamycin sulphate and 100 μg ml^{-1} carbenicillin (antibiotics from Duchefa Biochemie), on a shaker at 28°C for 16 h. The bacterial cells were thereafter pelleted by centrifugation and ressuspended in liquid proliferation medium to an optical density ($OD_{600\ nm}$) of 0.6.

Transformation Protocol 1

In this procedure, adapted from the method described by Levée et al. (1997), 30 μl of the bacterial suspension were directly applied on embryogenic clumps with 500 mg FW from the embryogenic clones 31/668/00, 54/232/00, 68/455/00, 82/758/00, 31/19/00 and 82/755/00 that showed good maturation ability and growth rate. In clone 31/668/00, the addition of 100 μM acetosyringone (AS) to the bacterial suspension just before inoculation was tested. The clumps were cocultivated on semi-solid medium without CH for 36 h at 28°C, in darkness. To eliminate *Agrobacterium*, cocultivated embryogenic tissues were transferred to semi-solid proliferation medium without CH supplemented with 400 mg l^{-1} timentin (Duchefa Biochemie). After 7–10 days, the growing embryogenic clumps were transferred to semi-solid proliferation medium supplemented with 10 mg l^{-1} hygromycin B (Duchefa Biochemie). The putatively transformed hygromycin-resistant tissues were isolated and maintained for at least six subcultures in the presence of timentin.

Transformation Protocol 2

This method was adapted from the protocol described by Levée et al. (1999). Embryogenic clumps were harvested from 7-days old cultures and suspended in liquid proliferation medium without CH. Afterwards, an equal volume of *A. tumefaciens* suspension ($OD_{600\ nm}$ = 0.6) was mixed with tissue suspension and shaked at 100 rpm for 1 h. Three ml of this mixture (500 mg tissue FW) were thereafter spread on filter paper (Whatman n.2, 5.5 cm diameter) placed over towel papers to drain the excess liquid. The filter paper was then placed over semi-solid proliferation medium without CH and cocultivation was conducted in the same conditions described for *Protocol 1*. The filter paper with embryogenic tissue was then placed over towel papers and washed with 400 mg l^{-1} timentin without CH before transfer to semi-solid proliferation medium supplemented with 400 mg l^{-1} timentin without CH. After 7–10 days, the filter paper was transferred to selection medium, followed by the procedure described for *Protocol 1*. Embryogenic cell suspensions from 4- and 7-day-old liquid cultures of embryogenic clone 31/668/00 were submitted to *Protocol 2*, testing the presence of 100 μM AS. *Protocol 1* was repeated in parallel with *Protocol 2* using the embryogenic clones 31/668/00, 32/82/01 and 32/242/01. Controls for *Protocols 1* and *2* were treated in the same way but excluding *Agrobacterium* infection. A total of three replicates per embryogenic clone and experiment were used.

Transgene expression before and after cryopreservation

Expression of *uid*A was analysed by the histochemical β-glucuronidase (GUS) assay according to Jefferson (1987). Hygromycin-resistant embryogenic clones, somatic embryos, and organs of somatic seedling (cotyledons, needles and root tip) were incubated in the GUS buffer for 16 h at 37°C. Needles were then cleared in 70% (v/v) ethanol for 2–3 days. Embryogenic tissues from 42 hygromycin-resistant independent lines were analysed by the GUS assay before and after cryopreservation using the cryopreservation procedure detailed in Marum et al. (2004). Before cryopreservation, 30 mg from each of seven clumps of the same transformation event were collected in the same 1.5 ml Eppendorf tube and suspended in X-Gluc solution. The number of GUS-negative and GUS-positive cell aggregates and early formed embryos was scored. Regrown embryogenic tissues from the cryopreserved

transformed lines were cultivated on selection medium until seven clumps with 500 mg each could be isolated. The GUS assay was then performed as described above.

PCR and RT-PCR analyses

For the polymerase chain reaction (PCR) analysis, genomic DNA was isolated from embryogenic tissues of transformed lines using the DNeasy Plant Minikit (Qiagen, KJ Venlo, The Netherlands) following the manufacturer's instructions. Three combinations of primers were used for PCR amplification of fragments from genes *uid*A, *hpt* and *vir*BG (Table 1). PCR reactions were performed in a UNO-thermoblock (Biometra, Goettingen, Germany) apparatus. Each reaction mixture contained 100 ng of genomic DNA, 1× PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 μM of each primer and 0.03 U μl^{-1} Taq DNA polymerase (Invitrogen, Carlsbad, California, USA). The PCR conditions were 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 62°C (*uid*A), 60°C (*vir*BG) or 56°C (*hpt*) for 1 min and 72°C for 1 min and a final extension step at 72°C for 5 min.

Southern blot analysis

For Southern analysis, genomic DNA was isolated from 1 g frozen tissue, following a protocol of Dellaporta et al. (1983) with minor modifications. Genomic DNA (20 μg) was digested overnight with either *Eco*RI, *Hin*dIII or *Eco* RI/*Hin*dIII (Roche, Basel, Switzerland) at 150 U/20 μg DNA. DNA fragments were denatured in 0.5 M NaOH with 1.5 M NaCl, neutralised in 1 M Tris with 1.5 M NaCl pH 7.4, and transferred onto a positively charged nylon membrane (Schleicher and Schuell BioScience, Keene, NH, USA) using 20× buffer SSC. The probe was obtained through digestion of DNA plasmid pPCV6NFHygGUSINT with *Xba*I (Roche) 16 U/3 μg. The fragments were labelled with [α-^{32}P] dCTP 10 μCi/μl (Amersham, Uppsala, Sweden) using the High Prime kit (Roche) following the manufacturer's instructions. Hybridisation was carried out following standard procedures previously described (Sambrook et al. 2001). After hybridisation at 65°C, the membrane was washed twice in 2× SSC plus 0.1% (w/v) SDS at 65°C for 30 min, once in 0.2× SSC plus 0.1% (w/v) SDS at 65°C for 15 min and exposed to X-Omat AR Scientific Imaging Film (Kodak) for 2 days at −80°C.

Maturation of somatic embryos and conversion to plants

For maturation, embryogenic tissue clumps of each of the transformed lines obtained were isolated from a 7-day-old culture and dispersed in liquid proliferation medium without plant growth regulators (PGRs) by using a micropipette with cut tip and washed twice for 20 min. The embryogenic tissues (1 g) were ressuspended in 10 ml of sterilized distilled water in a centrifuge tube, from which 1-ml aliquots were spread on filter paper (Whatman no.2, 5.5 cm diameter) placed over towel papers to drain the excess liquid. The filter paper was then placed over semi-solid maturation medium based on DCR (Gupta and Durzan 1985) supplemented with 6% (w/v) sucrose (Duchefa Biochemie), 80 μM filter sterilized (±)-*cis,trans*-abcissic acid (ABA) (Precision Biochemicals, Vancouver, Canada), with or without 10 mg l^{-1} hygromycin and solidified with 9 g l^{-1} gelrite. The cultures were maintained in a growth chamber at 22 ± 2°C in the dark for four

Table 1 Primers used for the PCR amplification of fragments from genes *uid*A, *hpt* and *vir*BG

Gene	Primer combinations	Amplified fragment (bp)
*uid*A	5′-CCCGGCAATAACATACGGCGT-3′ 5′-CCTGTAGAAACCCCAACCCGT-3′	366
hpt	5′-AATAGCTGCGCCGATGGTTTCTACA-3′ 5′-AACATCGCCTCGCTCCAGTCAATG-3′	515
*vir*BG	5′-GCGGTGAGACAATAGGCG-3′ 5′-GAACTGCTTGCTGTCGGC-3′	490

subcultures of 3 weeks each. The mature somatic embryos were isolated and either submitted to the GUS assay or placed on ABA-free maturation medium containing 0.4% (w/v) gelrite in a 92×16 mm Petri dish at 4°C in the dark, until hypocotyl elongation occurred. Somatic embryos were then transferred to 1/4 DCR germination medium supplemented with 10 g l^{-1} activated charcoal (Duchefa Biochemie), 2% (w/v) sucrose and 4 g l^{-1} gelrite. In the first week of germination, cultures were kept in darkness, then transferred to diffuse light in the second week and thereafter to 16-h photoperiod under a light intensity of 35 μmol m^{-2} s^{-1} provided by cool-white fluorescent lamps (Philips TLD 36W/84) in a growth chamber at 22 ± 2°C. Plantlets showing elongated radicle and hypocotyl were then transferred to Magenta culture vessels (Sigma) containing 1/4 DCR germination medium supplemented with sucrose 2% (w/v) and 4 g l^{-1} gelrite, for further development. Cotyledons, needles and root tips were then collected for the GUS assay and PCR analysis.

Results and discussion

Tissue culture and selection of putative transformed tissue

There was no relevant reduction in the tissue growth rate caused by the absence of CH from tissue proliferation medium in both subcultures for the three embryogenic clones tested (data not shown). After cocultivation, no bacterial growth was observed around the inoculated embryogenic clumps (*Protocol 1*) and only a slight growth occurred around the edges of the filter paper (*Protocol 2*), opposite to what happened in media containing CH, where *Agrobacterium* overgrowth was observed. The strategy of CH removal to control *Agrobacterium* overgrowth had already been followed for *Abies alba* and *A. nordmanniana* embryogenic tissues (A. Rahmat, personal communication) and in our work with *P. pinaster* it was crucial for the recovery of embryogenic tissue growth after cocultivation.

The antibiotic timentin 400 mg l^{-1} was required for at least six subcultures to ensure complete *Agrobacterium* elimination, being afterwards removed from the semi-solid proliferation medium. After the first subculture on selection medium, putatively transformed lines could be individualised either from the original clumps (*Protocol 1* (Fig. 1a) or from the filter paper (*Protocol 2* (Fig. 1b), in a total of 52 hygromycin-resistant lines from four different genotypes. Stable hygromycin-resistant lines produced enough tissue that could be used in the GUS assay, whereas the non-transformed tissues turned brown and died within 1–3 weeks of selection depending on the embryogenic clone.

Expression of β-glucuronidase gene and transformation efficiency

The expression of β-glucuronidase gene (*uid*A) was analysed in the putatively transformed lines by the GUS assay. In the present experiments with *P. pinaster*, about 90% of the independent hygromycin-resistant lines were also GUS-positive (47 out of 52). The five GUS-negative selected clumps were not analysed by PCR, meaning that *uid*A could be present although with an expression level below the detection threshold of the GUS assay and, therefore, transformation efficiency could have been underestimated. Activity of GUS enzyme was not detected in control tissues. *Protocol 1* was repeated in parallel with *Protocol 2*, using the embryogenic clone 31/668/00 (established in year 2000) and two other clones, 32/82/01 and 32/242/01 (established in 2001), since tissue age can affect maturation of *P. pinaster* embryogenic tissues. Embryogenic clone 31/668/00 produced transformed line(s) in all the transformation experiments performed—*Protocol 1* with or without AS, repetition of *Protocols 1* and *2*, and the experiment using embryogenic cell suspensions. This clone is probably highly competent for transformation, as also found for an embryogenic clone of *P. mariana* (Klimaszewska et al. 2003).

Most of the transformed lines (23 out of 38) obtained by *Protocols 1* and *2* showed chimerical *uid*A expression—25 to 75% cell aggregates stained blue after the GUS assay (Fig. 1c) (Table 3) and variable GUS intensity in blue cell aggregates. In other conifers, a mixture of

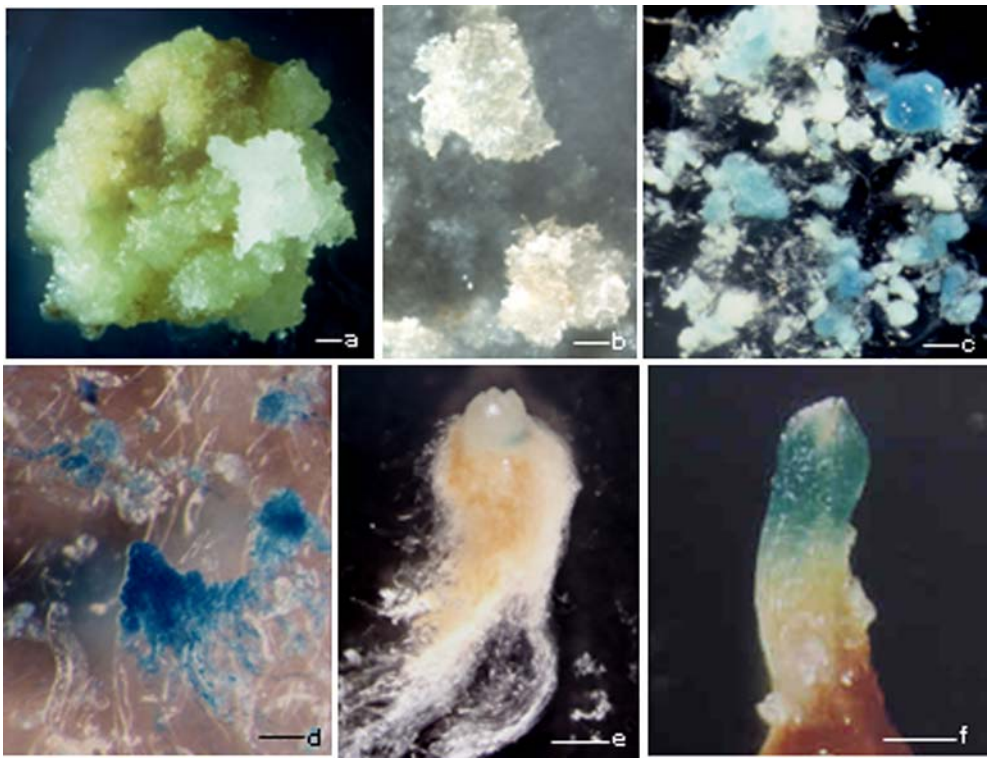

Fig. 1 Different aspects of the *Agrobacterium*-mediated transformation of *P. pinaster*. (**a**) Embryogenic clump on selection medium showing an emerging portion of a hygromycin-resistant tissue (*Protocol 1*). (**b**) Emerging portions of proliferating tissue on filter paper placed on proliferation medium with timentin, 7 days after cocultivation (*Protocol 2*). (**c–f**) Expression of *uid*A after GUS-testing on embryogenic aggregates and early formed embryos (**c, d**), in early cotyledonary somatic embryo at the base of the cotyledons ring (**e**) and in a later cotyledonary somatic embryo in cotyledons and hypocotyls (**f**). *Bars* **a, b, e, f** = 1 mm; **c, d** = 250 μm

GUS-positive and GUS-negative embryogenic aggregates was also observed at least in some transformed lines (Ellis et al. 1993; Walter et al. 1998; Tian et al. 2000). This could be explained by the existence of different proportions of transformed to non-transformed cells. Also, variations in the expression level of *uid*A gene are possible in different transformed lines, which can be explained by phenomena such as the position effect of the insertion (*reviewed by* Matzke and Matzke 1998). Gene expression could also vary due to copy number effect (Cervera et al. 2000). A high copy number may lead to gene silencing (Matzke et al. 1994). Complex configurations of the integrated T-DNA may also affect gene expression (Stam et al. 1997). In numerous *Larix decidua*, *Abies alba* and *A. nordmanniana*, a variable GUS activity was observed as well in lines transformed with the same construct, whereas the portion of GUS positive embryos and expression level increased during maturation. In all these cases, a chimerical nature of the transformed lines could not be found (unpublished data).

The transformation frequency was considered as the number of transformed lines per gram of embryogenic tissue FW. The highest transformation efficiency was obtained in the embryogenic clone 31/668/00 using *Protocol 2* (22 transformed lines/g FW) (Table 2). With *Protocol 2*, higher transformation frequencies were obtained with the embryogenic clone 31/668/00 than with clones 32/82/01 and 32/242/01. Five out of nine *P. pinaster* embryogenic clones tested in transformation could regrow after infection and proved to be transformed. These results suggest that the success of the transformation process in *P. pinaster*

Table 2 Transformation efficiency of three embryogenic clones (average number of transformed (hygromycin-resistant and GUS-positive) lines per g FW from three replicates ± SEM) in *Protocols 1* and *2*; and results from PCR analysis for the presence (+) or absence (−) of genes *uid*A, *hpt* and *vir*BG in 47 putative transformed lines

Embryogenic clone	AS 100 μm	Replicate	No. hyg^R GUS$^+$ lines/500 mg FW		Transformation efficiency		No. transformed lines PCR +/−		
			Prot. 1	*Prot. 2*	*Prot. 1*	*Prot. 2*	*uid*A	*hpt*	*vir*BG
31/668/00	−	1	1	N.T.	0.67	N.T.	1+	1+	1−
		2	0						
		3	0						
31/668/00	+	1	1	N.T.	0.67	N.T.	1+	1−	1−
		2	0						
		3	0						
54/232/00	+	1	0	N.T.	0.67	N.T.	1+	1+	1−
		2	1						
		3	0						
31/668/00	−	1	1	17	1.33	24.67	39+	35+	39−
		2	1	11					
		3	0	9					
32/82/01	−	1	0	0	0.67	1.33	3+	3+	3−
		2	1	1					
		3	0	1					
32/242/01	−	1	0	1	0.00	1.33	2+	2+	2−
		2	0	0					
		3	0	1					

No. hyg^R GUS$^+$ lines: number of transformed lines; *Prot.*: *Protocol*; N.T.: not tested

Portuguese embryogenic clones is genotype-dependent. This was also reported for French *P. pinaster* embryogenic clones (Trontin et al. 2002) with four out of six lines transformed, and for *L. kaempferi* × *L. decidua* (Levée et al. 1997) with four out of seven lines transformed with variable efficiencies.

The addition of AS in *Protocols 1* and *2* to improve the transformation frequency was tested but without any beneficial effect. In some conifers, an increased transformation efficiency of embryogenic tissues by adding AS has been reported, such as in *P. strobus* (100 μM, Levée et al. 1999), *P. abies* and *P. taeda* (25–50 μM, Wenck et al. 1999) and *P. glauca* (50 μM, Le et al. 2001), but in *L. kaempferi* × *L. decidua* (100 μM, Levée et al. 1997) and French genotypes of *P. pinaster* (100 μM, Trontin et al. 2002), no improvement could be achieved.

For the embryogenic cell suspensions, the preculture duration was critical for the success of transformation. Transformed cultures were recovered only from 4- but not from 7-day-old cultures. The fourth day in a liquid medium subculture possibly corresponds to a high level of cell division which promotes the transformation event. In the presence of 100 μM AS during cocultivation of tissue and bacterial suspensions, no hygromycin-resistant lines were obtained, indicating that in the tested conditions, this compound did not improve transformation. In *P. glauca* embryogenic tissues, higher transformation efficiency was also obtained for cell suspensions with a short subculture period (5-day-old) but in this case, in the presence of AS.

Effect of cryopreservation on *uid*A gene expression in embryogenic tissues

The effect of cryopreservation on the tissue regrowth rate and *uid*A expression of 42 out of 47 independent *uid*A positive lines obtained is reported in Table 3. Transformed lines were considered to have regrown after cryopreservation when at least one of three replicates produced at least 1 g FW of embryogenic tissue. About 90% (38 out of 42) of the cryopreserved transformed lines were able to grow thereafter, indicating that genetic transformation did not affect the success of the cryopreservation process

Table 3 Effect of the cryopreservation on *uid*A expression of 38 individual GUS/*uid*A-positive (transformed) lines

Transformed line	GUS$^+$ cell aggregates before cryo. (%)	Replicates	% GUS$^+$ cell aggregates after cryo. (%)	Transformed line	GUS$^+$ cell aggregates before cryo. (%)	Replicates	% GUS$^+$ cell aggregates after cryo. (%)
B1C1	25–75	1	25–75	L21	25–75	1	<25
		2	25–75			2	<25
		3	25–75			3	>75
B2C1	25–75	1	25–75	L22	25–75	1	25–75
		2	25–75			2	25–75
		3	<25			3	25–75
B2C2	25–75	1	<25	L23	25–75	1	25–75
		2	25–75			2	25–75
		3	<25			3	25–75
L2	25–75	1	>75	L24	25–75	1	25–75
		2	>75			2	25–75
		3	>75			3	25–75
L3	25–75	1	25–75	L25	>75	1	>75
		2	25–75			2	>75
		3	25–75			3	>75
L4	25–75	1	25–75	L26	>75	1	>75
		2	25–75			2	>75
		3	25–75			3	>75
L5	>75	1	>75	L27	25–75	1	0
		2	*			2	0
		3	>75			3	0
L6	25–75	1	25–75	L28	>75	1	25–75
		2	25–75			2	25–75
		3	<25			3	25–75
L7	>75	1	>75	L29	>75	1	*
		2	>75			2	*
		3	>75			3	>75
L8	25–75	1	25–75	L30	>75	1	>75
		2	<25			2	>75
		3	<25			3	>75
L9	25–75	1	25–75	L33	<25	1	<25
		2	25–75			2	25–75
		3	25–75			3	<25
L10	25–75	1	25–75	L35	<25	1	>75
		2	25–75			2	>75
		3	25–75			3	>75
L11	25–75	1	25–75	L37	25–75	1	25–75
		2	25–75			2	*
		3	<25			3	*
L12	>75	1	>75	L38	<25	1	<25
		2	>75			2	>75
		3	>75			3	<25
L13	25–75	1	25–75	L39	25–75	1	<25
		2	25–75			2	25–75
		3	25–75			3	25–75
L15	25–75	1	25–75	L40	25–75	1	>75
		2	<25			2	>75
		3	25–75			3	>75
L18	25–75	1	25–75	L41	<25	1	<25
		2	25–75			2	<25
		3	>75			3	<25
L19	<25	1	0	L43	>75	1	>75
		2	25–75			2	>75
		3	<25			3	*

Table 3 continued

Transformed line	GUS$^+$ cell aggregates before cryo. (%)	Replicates	% GUS$^+$ cell aggregates after cryo. (%)	Transformed line	GUS$^+$ cell aggregates before cryo. (%)	Replicates	% GUS$^+$ cell aggregates after cryo. (%)
L20	25–75	1	<25	L47	>75	1	>75
		2	<25			2	>75
		3	25–75			3	>75

Cryo.: cryopreservation; * No regrowth; ''Cell aggregates'' included also early formed embryos

and that cryopreservation did not affect the stability of the transgenic line. The percentage of GUS-positive aggregates after cryopreservation was maintained in 71% replicates (74 in 104), it increased in 14% and decreased in 15%, from a total of 38 transformed lines of the embryogenic clones 31/668/00, 32/82/01 and 32/242/01. This result suggests that apparently cryopreservation does not significantly affect *uid*A expression, as reported for another transgene in *Papaver somniferum* (Elleuch et al. 1998). Cryopreservation can be used for long-term storage of transformed *P. pinaster* embryogenic tissues, avoiding the need for continuous subcultures and thus minimizing the risk of somaclonal variation and of loosing the maturation ability with aging.

Molecular analysis of putative transformed lines and regenerated plantlets

The transformed state of the hygromycin-resistant lines obtained by the *Agrobacterium* transformation method was demonstrated by PCR amplification of the expected fragment bands of 366 bp for *uid*A gene (Fig. 2A) and 515 bp for *hpt* gene (Fig. 2B), whereas no amplification was detected in the sample from untransformed tissue. From the 47 lines obtained from the *Protocols 1* and *2* which were hygromycin resistant and GUS-positive, 100% were *uid*A-positive and 89% were *hpt*-positive (Table 2). No amplification of *vir*BG genes could be detected in any of the 47 lines after 6 subcultures on timentin-containing

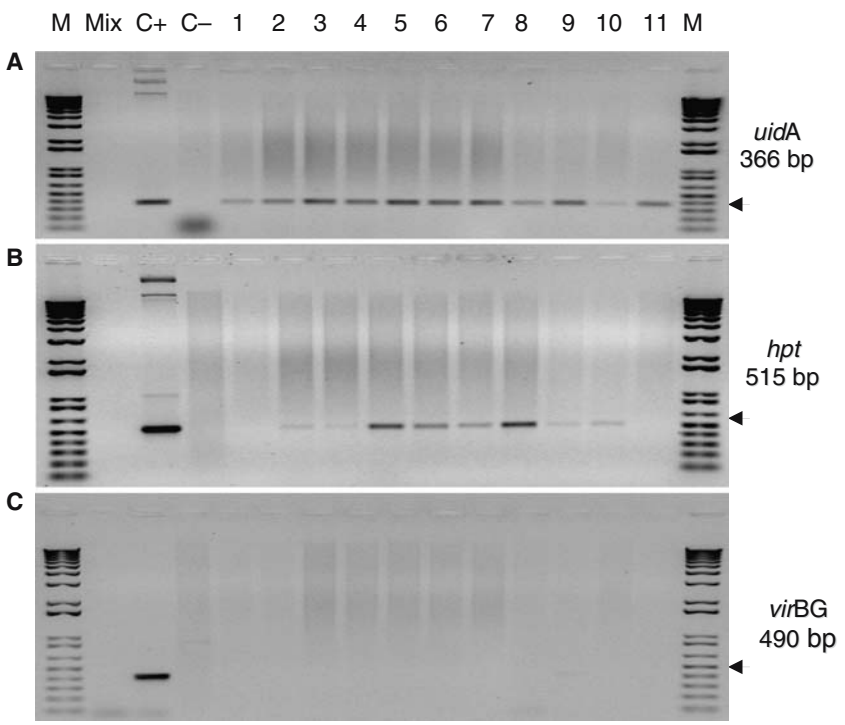

Fig. 2 PCR analysis of genomic DNA from hygromycin-resistant lines obtained from *Protocols 1* or *2*, using primers for the gene fragments **(A)** *uid*A 366 bp, **(B)** *hpt* 515 bp and **(C)** *vir*BG 490 bp. M: 1 kb plus DNA ladder. Mix: PCR mix (internal control). C$^+$: plasmid pPCV6NFHygGUSINT. C$^-$: non-inoculated control of clone 31/668/00. Lanes 1–11: transformed lines L1 to L11

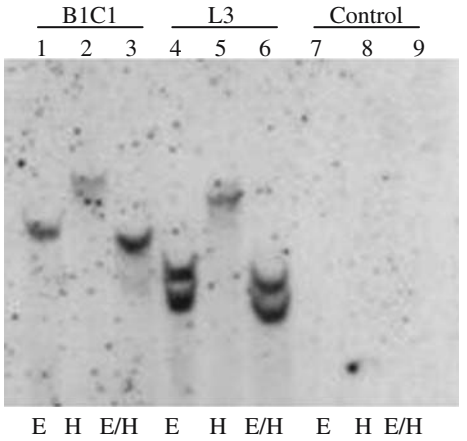

Fig. 3 Southern blot hybridization analysis of genomic DNA from transgenic lines B1C1 obtained by *Protocol 1*—lanes 1 (10 kb), 2 (20 kb), 3 (8 kb); and L3 obtained by *Protocol 2*—lanes 4 (5 + 4 kb), 5 (18 kb), 6 (4.9 + 3.8 kb), using a probe for *uid*A gene. Control—non-transformed tissues (lanes 7, 8, 9)—and B1C1 and L3 resulted from the embryogenic clone 31/668/00. E: *Eco*RI; H: *Hin*dIII; E/H: *Eco*RI plus *Hin*dIII

medium, indicating that the GUS-positive lines were not *Agrobacterium* contaminated (Fig. 2C).

Integration of the T-DNA into the genome of two GUS/PCR-positive lines obtained by the *Protocols 1* (line C1B1) and *2* (line L3) was confirmed by Southern blot analysis (Fig. 3). Genomic DNA was digested with *Eco*RI recognizing sites within the T-DNA (Fig. 3) and *Hin*dIII with no recognition sites within the T-DNA. The transformed line B1C1 contained at least one gene copy and L3 contained at least two copies of the T-DNA inserted in different loci. No hybridisation signal was detected in non-transformed embryogenic tissue (Fig. 3).

Maturation of somatic embryos and conversion to plantlets

From the 44 transformed lines submitted to maturation, only 3 gave rise to mature somatic embryos, even in the absence of hygromycin in maturation medium. These results suggest that maturation ability was partially lost with the extended time in culture, a phenomenon that was also observed in control tissues from the same embryogenic clones used in this work. This was also frequently observed in *P. radiata* embryogenic clones maintained for 12–18 months in culture (Walter et al. 1998). Therefore, transformation and cryopreservation of transformed lines have to be performed as soon as possible after initiation of the embryogenic tissue. During maturation, GUS expression detected by the blue staining could be localised in the whole cell aggregate (Fig. 1d), at the base of cotyledon insertion at an early stage cotyledonary embryo (Fig. 1e) or in cotyledons and hypocotyl in a more developed cotyledonary embryo (Fig. 1f). The three mature somatic embryos obtained from transformed lines were all GUS-positive. However, the needles of the regenerated somatic plantlets were GUS-negative and PCR-negative for genes *uid*A, *hpt* and *vir*BG. These results suggest that transformed embryogenic clones showing ability for plant regeneration were chimeras and plants were regenerated from non-transformed cells.

In conclusion, two transformation protocols for *P. pinaster* embryogenic clones by *Agrobacterium* inoculation of embryogenic tissues were developed. The main important factors for the success of transformation were the efficient control of *Agrobacterium* growth by omission of CH, the genotype and the physiological state of the embryogenic tissue. Foreign gene transfer, integration and expression were successfully achieved. This is the first report of *Agrobacterium*-mediated T-DNA integration in *P. pinaster* confirmed by Southern blot analysis. The transformation and regeneration procedures described in this work are being used at early stages of embryogenic clone establishment to increase the chances of transgene plant recovery.

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