Plant Growth Regulation **44:** 187–197, 2004.
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A biolistic approach towards producing transgenic *Pinus patula* embryonal suspensor masses

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Received 18 August 2003; accepted in revised form 11 October 2004

Key words: *Bar* gene, Conifer, Genetic transformation, GUS reporter gene, Particle bombardment, Somatic embryogenesis

Abstract

Pinus patula Schiede et Deppe is one of the most important softwood species for pulp production by the South African forestry industry. This study was aimed at developing a protocol for the genetic transformation of *Pinus patula* embryogenic tissue. This was achieved via the introduction of a *bar*-GUS cassette under the control of the *ubiquitin* promoter, through biolistic transfer. A stepwise selection was obtained using MSG3 maintenance medium supplemented with BASTA® herbicide at 1 mg l^{-1} followed by 3 mg l^{-1} active ingredient. Transgene delivery was more efficient when the medium was supplemented with 0.25 M sorbitol. Expression of positive histochemical GUS activity in embryonal heads was observed. Positive PCR analysis of both GUS and *bar* transgenes (40% and 47% transformation efficiency, respectively) indicated successful genetic modification of *P. patula* embryonal suspensor masses by the pAHC25 plasmid. This indicates that *P. patula* is amenable to gene transfer.

Abbreviations: ABA – abscisic acid; BA – N^6-benzyladenine; *bar* – the bialaphos resistance gene; DMSO – dimethyl sulphoxide; EDTA – ethylenediamene tetraacetic acid; ESM – embryonal suspensor mass; GUS – β-glucuronidase; MSG3 – maintenance medium (Becwar et al. 1990); PCR – polymerase chain reaction; PEG – polyethelene glycol; TAE – Tris–acetic acid–EDTA buffer; *Taq* – *Thermus aquaticus* enzyme; *uidA* – gene encoding β-glucuronidase from *Escherichia coli*; X-glcA – 5-bromo-4-chloro-3-indolyl β-D-glucuronide

Introduction

South Africa relies on forest plantations of exotic species to help meet its demand for wood and forest products. *Pinus patula*, introduced from Mexico, is now the most important softwood species in the South African forest industry. This is reflected by 375,000 ha of South Africa's afforested area being planted with this species. This represents 25% of the total forestry land-base

(FOA 2002). The efficiency of industrial development is dependent on innovation rather than the exploitation of natural resources due to their often limiting nature. Rainfall, in particular, is very limiting in South Africa and only about 7% of the country receives sufficient rainfall (800 mm/annum or more) for intensive forestry (Denison and Kietzka 1993). The integration of biotechnology would help to address many problems encountered in commercial forestry, such as plantation site constraints, pests and diseases and the requirement for improved product quality. Somatic embryogenesis has given major impetus to clonal propagation and is an excellent source of target tissue for genetic transformation studies. Target tissue is optimum when it is juvenile and actively dividing (Sanford et al. 1993). The benefits of applying biotechnology to tree culture include the speed and accuracy with which improved genotypes can be secured without disturbing existing gene arrangements and overcoming interspecific barriers, both of which are distinct advantages over classical breeding (Grierson and Covey 1984; Charity et al. 2002).

Methods of transgenic plantlet recovery in conifers are far from optimal (Clapham et al. 1995) as many transiently transformed cells are not receptive to stable integration or regeneration. Alternatively, if the appropriate cells are stably transformed they do not continue to divide after transformation (McCabe and Christou 1993). Loss of embryogenic potential due to selective agents (Robertson et al. 1992) and transgene silencing (Matzke et al. 2000) are further factors that complicate progress in plant gene transfer. Traditionally conifers have been inherently recalcitrant to *Agrobacterium* transformation and like other forest tree species have lagged behind when compared with genetic engineering of agricultural crops (Walter et al. 1998; Walter et al. 1999). The advent of particle transfer resulted in biolistics being commonly used for transfer in conifers and it is routinely used in many laboratories, although a low transformation frequency abounds. Few conifers have yielded regenerated plants after transformation by microparticle bombardment. These include *Picea glauca* (Ellis et al. 1993), *Picea mariana* (Charest et al. 1996), *Pinus radiata* (Walter et al. 1998), *Larix laricina* (Klimaszewska et al. 1997), *Picea abies* (Walter et al. 1999), *Betula pendula* (Valjakka et al. 2000) and *Pinus pinaster* (Trontin et al. 2002). Biolistic transformations, in contrast to *Agrobacterium*, have resulted in fragmented or multicopy integration events of the transgene (Meyer 1995; Walter et al. 1998), which may lead to transgene silencing (Kumpatla et al. 1997).

Historically *Agrobacterium*-mediated transformations were unable to overcome species recalcitrance. However, improvements in tissue culture regimes, regeneration protocols and vectors with promoters of plant origin vs. bacterial origin which are easily recognized by the plant transcription machinery (Birch 1997) are some of the factors enabling researchers to return to using genetic manipulation via *Agrobacterium*. This method of transformation has further advantages such as high co-expression of introduced genes, less fragmentation of the transferred gene and the introduction of a single or few copies of the transgene (Hadi et al. 1996) preferentially into transcriptionally active sites of the host genome.

Prolific literature exists on transformation studies in this genus, many of which used biolistic methods, focussing on species such as *P. banksiana* (McAfee et al. 1993; Hay et al. 1994), *P. contorta* (Hay et al. 1994; Yibrah et al. 1996), *P. pinea* (Sul and Korban 1998), *P. radiata* (Walter et al. 1994; Charity et al. 2002), *P. sylvestris* (Magnussen et al. 1994; Aronen et al. 1996) and *P. taeda* (Stomp et al. 1991; Tang et al. 2001), to name a few. To our knowledge, there are no published reports for *P. patula* in the literature. Thus, the aims of this study were to develop a biolistic regime for *Pinus patula* embryonal suspensor masses (ESM) through the introduction of the pAHC25 plasmid which contains the selectable herbicide resistance *bar* gene and the *uidA* reporter gene. A successful gene transfer protocol is a prerequisite for molecular physiology studies towards furthering our understanding of mechanisms regulating plant growth and development. An efficient and reliable transformation method is also an important tool for future applications in forestry. These include: alteration of tree form and performance, abiotic stress tolerance, insect and herbicide resistance, molecular improvement through virus resistance, the analysis and manipulation of the flowering

pathway to achieve accelerated flowering for the benefit of reproduction in conifers; the genetic manipulation of lignin biosynthesis pathways to either decrease the lignin content or change the characteristics of lignin (Walter et al. 1998, Tang and Newton 2003), and the potential introduction of disease resistance to pitch canker fungus, as *P. patula* is highly susceptible to this pathogen (Viljoen et al. 1995). With such transformation tools at hand, the roles of specific genes, promoters and enzymes related to tree physiology and metabolic processes can be investigated, as reviewed by Tang and Newton (2003). We report on the successful delivery and expression of both the *uidA* and *bar* genes into *P. patula* ESM.

Materials and methods

Plant material and culture conditions

Green female cones from four selected *P. patula* families were provided by SAPPI Forests Research, South Africa. Selection of families is based primarily on their growth performance in the field. The cone collection period spanned from December 1999 to the end of January 2000. The cones were harvested from a 10-year-old clonal seed orchard and kept in brown paper bags at 4 °C until seeds were placed in culture (within 4 days of collection). After a brief wash under running water, the cones were surface decontaminated by immersion into 75% ethanol [v/v] for 5 min. They were then transferred to a solution of 1.3% NaOCl [w/v] for 20 min, before being rinsed several times with sterile distilled water. Both solutions contained a few drops of Tween® 20 (Merck, South Africa), with an active ingredient of polyethylene sorbitan mononlaurate, as a surfactant. The seeds were excised aseptically and the seed coats removed. The female gametophytes, containing the immature embryos, were placed on MSG3 initiation medium (Becwar et al. 1990), a modified Murashige and Skoog (1962) medium, supplemented with 30 g l^{-1} sucrose, which encouraged the induction of ESM (Figure 1A) as shown by Jones et al. (1993). Other medium supplements included: 1.5 g l^{-1} L-glutamine (filter-sterilized twice with 0.22 μm Millipore aqueous filters using Sartorius® (Sartorius AG, Germany) filter units prior to addition to the medium); 0.1 g l^{-1} myo-inositol, 2 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 1.0 mg l^{-1} benzyladenine (BA). Media were solidified with 3 g l^{-1} Gelrite® (Labretoria, South Africa). The pH was adjusted to 5.8 with 1 N HCl or 1 N KOH prior to autoclaving at 110 °C, 1.21 kPa for 20 min.

Following embryogenic induction, maintenance of all tissue used in this study was achieved using MSG3 medium where sucrose was substituted with 30 g l^{-1} maltose. Tissue was kept in the dark at 25 °C and subcultured every 2 weeks to maintain the ESM in a proliferative state.

Single clonal lines from three of the four families, genotypes designated 1, 2, and 3, respectively were selected for final transformation experiments on the basis of the relative ease with which they proliferated in culture. Genotype 4 consistently showed poor or slow tissue growth throughout the study and was eliminated.

All of the remaining lines were initiated into suspension culture, which was also used for bulking up of tissue before bombardment. The tissue was maintained on solid MSG3 medium in the dark at 25 °C. Embryogenic suspension cultures were initiated by inoculating approximately 200 mg of friable tissue into 50 ml liquid MSG3 medium. The flasks were stoppered with a sterile cotton wool bung and covered with aluminum foil. The cultures were placed on a rotary shaker at 120 rpm in the dark at 25 °C for 10 days, or until polar embryos were observed.

Regeneration method I

After the final phase of selection, filter paper discs supporting bombarded material were transferred to 240 maturation medium (Pullman and Webb 1994) supplemented with 0.1 g l^{-1} myo-inositol, 0.5 g l^{-1} casein hydrolysate and containing either 0%, 5% or 7.5% polyethylene glycol (PEG) 8000 [w/v] (Sigma, Germany). Prior to autoclaving, the basal medium was pH corrected to 5.7 and solidified with 3 g l^{-1} Gelrite®. The PEG component, L-glutamine (4.5 g l^{-1}) and abscisic acid (ABA) (10 mg l^{-1}) were prepared separately and the pH adjusted prior to autoclaving. A 60% maltose [w/v] solution was also prepared separately and the pH adjusted to 5.7. After autoclaving the PEG and maltose

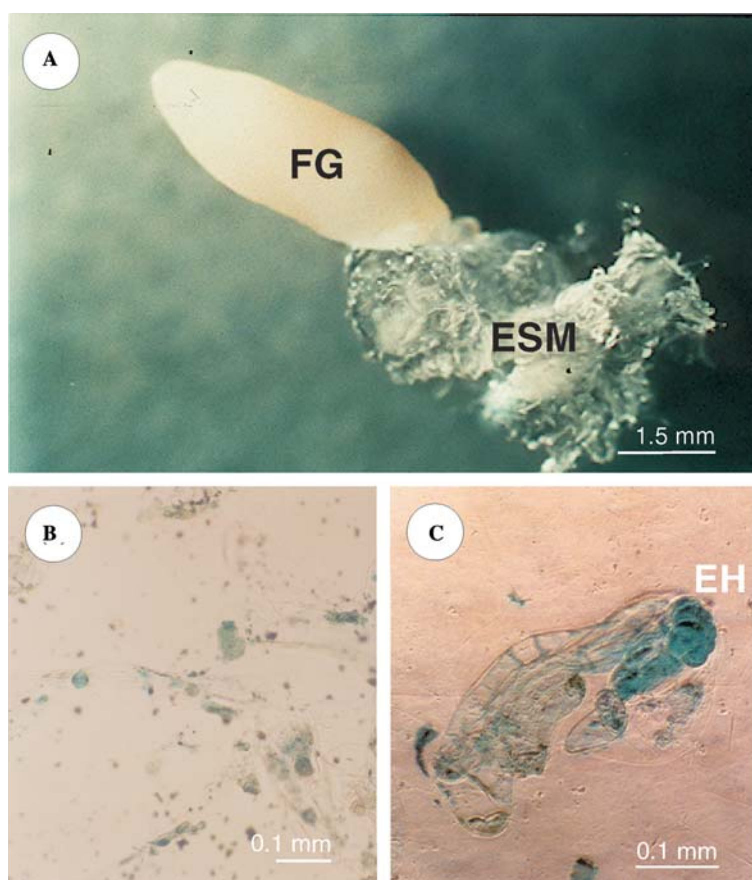

Figure 1. (A) The female gametophyte (FG), extracted from immature seed, had the seed coat removed and was placed on initiation medium for the proliferation of translucent ESM. (B) Plasmolysis of embryonal cells after microparticle blast as an effect of particle bombardment on GUS stained target tissue before inclusion of the pretreatment step. (C) Histochemical analysis of bombarded material using the GUS assay. A single embryo indicates the successful uptake of the GUS construct in DNA-packed embryonal head (EH), as apex of proembryo is stained blue.

solutions and the ABA and L-glutamine solutions were added to the cooled media. The media were poured into 65 mm sterile plastic petri dishes.

Regeneration method II

Due to poor embryo maturation observed in bombarded cultures, the method of regeneration was modified. After the selection regime, proliferating bombarded tissue, was introduced into suspension culture by collecting newly growing peripheral ESM (0.1 g ESM/ml liquid MSG3 medium) from several dishes of the same clonal line. This suspension was shaken vigorously for a few seconds to ensure embryo singulation. Using a sterile Sartorius filter unit, 1 ml aliquots were then filtered onto Whatman No. 1 filter paper. The filter paper discs supporting the embryogenic tissue were placed onto solid 240 maturation medium (Pullman and Webb 1994) supplemented with 10 mg l^{-1} ABA, 60 g l^{-1} maltose, and PEG was omitted. The concentration of gelling agent was increased to 9 g l^{-1} as gel strength was shown by Klimaszewska and Smith (1997) to impact on embryo maturation. Bombarded ESM material was regenerated and matured through to stage 3, as described by Hakman and Von Arnold (1988). Somatic embryos further matured to exhibit a hypocotyl and cotyledonary development.

Gene construct for transformation

The plasmid construct pAHC25 (Christensen and Quail 1996), donated by Dr B. Jénés (Agricultural Biotechnology Centre in Gödöllö, Hungary) was used in this transformation study. This vector consisted of both the selectable marker, *bar*, which encodes for phosphinothricin acetyltransferase (De Block et al. 1987) and the GUS reporter gene encoding β-glucuronidase (Jefferson et al. 1987) each fused between the *Zea ubiquitin* promoter and the *nos* terminator. An eukaryotic intron sequence has also been inserted between the *bar* gene and its promoter, ensuring that bialaphos resistance and β-glucuronidase activity can only be expressed by transgenic plant material and not by residual bacterial contaminants. The Ubi-BAR chimaeric gene provides selection for transformants resistant to BASTA® herbicide (De Block et al. 1987).

Treatment of tissue

Using tissue derived from suspension culture, ESM's from each genotype, were filtered in 1.5 ml aliquots onto Whatman No. 1 filter paper supports and placed onto MSG3 solid medium supplemented with 0.25 M sorbitol as an osmoticum (Walter et al. 1999) or no sorbitol (untreated) and both treatments left on the laminar flow bench overnight. The target tissues (liquid medium-derived cultures) were bombarded after 0, 5, 10 and 14 days growth on solid medium and subcultured onto selection medium the following day. A stepwise selection regime was implemented, consisting of the inclusion of 1 mg l^{-1} followed by 3 mg l^{-1} BASTA® bioactive ingredient (glufosinate ammonium) in the medium at each subculture. BASTA® is a registered product of AgrEvo South Africa (Pty) Ltd that is water-soluble and contains an active ingredient of glufosinate ammonium at 200 g l^{-1}.

DNA coating of microparticles

One hundred mg of 1.5 μm tungsten microparticles (ELAK Ltd, Hungary) were sterilized by overnight incubation in 2 ml 70% ethanol [v/v]. The particles were briefly spun down at 2400 × *g*. The ethanol was removed and the microparticles were washed twice with 2 ml sterile dH_2O. The sterile particles were stored in sterile 50% glycerol [v/v] solution at −20 °C. Macroparticles were stored in 100% ethanol overnight, placed onto an autoclaved Petri dish and left to air dry. Plasmid DNA was isolated as described by Li et al. (1995) and then coated onto the tungsten particles using the Perl et al. (1992) method to obtain a concentration of 4 μg DNA mg^{-1} tungsten particles.

Particle bombardment

All experiments were performed using a gene gun (Genebooster™, ELAK Ltd, Hungary) with a nitrogen-driven biolistic delivery system. The filtered tissue was bombarded with 10 μl of DNA-coated particles at 40 bar gas pressure per shot, and − 0.40 bar vacuum in the Genebooster™ chamber. The microcarrier travel distance was 70 mm from the stopping plate to the target tissue.

GUS assay

Random samples of bombarded material were histochemically stained with 0.3% 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-glcA) [w/v] buffer (X-glcA (Sigma), 5 mM K-ferrocyanide, 5 mM K-ferricyanide, 0.005% Triton X-100 [v/v], 100 mM Na-phosphate buffer [0.5 M NaH2PO4, 0.5 M Na2HPO4.2H2O], [pH 7], dissolved in methanol) (Jefferson 1987) at pH 7.0 for 6 h to overnight at 37 °C and then viewed under a photomicroscope (Wild Heerbrugg M400, Switzerland).

DNA extraction

Genomic *P. patula* DNA for PCR amplification was extracted after bombarded material had undergone selection at 3 mg l^{-1} BASTA® bioactive ingredient (approximately 3 weeks after particle bombardment) by grinding 0.1 g ESM with liquid nitrogen to a fine powder, using a pestle and mortar. The cellular powder was transferred, to sterile 1.5 ml microfuge tubes in which 500 μl urea extraction buffer (7 M urea crystals, 5 M NaCl,

1 M Tris/Cl [pH 8.0], 0.5 M EDTA, 20% Sarkosyl® [v/v] (British Drug House (BDH), England) was placed and then vortexed for 10 sec. A ratio of 1:1 phenol to chloroform was added to the cell extract and shaken on a tabletop shaker at 120 rpm for 1 h at room temperature. After centrifugation (15 min at 15,000 × *g*), the supernatant was then transferred to fresh microfuge tubes. The nucleic acids were precipitated with a tenth volume 4.4 M ammonium acetate and an equal volume, ice-cold isopropanol, mixed well by inversion and placed at −20 °C for 15 min to precipitate the DNA. Nucleic acids were collected by 15 min centrifugation at 15,000 × *g* and subsequently purified using 70% ethanol, washed with 100% ethanol prior to air-drying for 3–5 min on a laminar flow bench. Isolated genomic DNA was stored in 20 μl ultra pure water (BDH, England) at −20 °C until further use.

PCR-mediated gene detection

The GUS primer set 5′-GGTGGGAAAG CGCGTTACAAG-3′/5′-GTTTACGCGTTGCT TCCGCCA-3′ was used to amplify the *uidA* gene and yielded a fragment of 1.2 kb after electrophoresis on 0.8% agarose [w/v]. A GUS amplification cocktail, modified from the method described by Hare (1998), consisted of a 50 μl reaction with 100 ng genomic template DNA, 1.25 units of *Taq* DNA polymerase (Roche Biochemicals, South Africa), 0.5 μM of each primer, 10 mM of each dNTP: dATP, dTTP, dCTP and dGTP, and 5 μl PCR buffer (Roche Biochemicals, South Africa). To enhance the efficiency of the PCR, 10% dimethyl sulphoxide (DMSO) [v/v] was also included in the reaction mix (Winship 1989). The PCR contents were mixed well and all samples were overlaid with an equal volume of paraffin oil prior to undergoing 36 amplification cycles (Hybaid Thermal Reactor, Hybaid Ltd, England). The PCR was initiated with a denaturation step of 94 °C for 1 min at the beginning of the cycling regime. This was then followed by 35 cycles each comprising of a 94 °C denaturing temperature (30 s), a 60 °C annealing step (30 s) and a 72 °C extension step (45 s). The final stage employed the same denaturation and annealing conditions as described above but the last primer extension step was increased to 5 min.

The *bar* gene was successfully amplified as described by Vickers et al. (1996) using Expand™ High Fidelity *Taq* DNA polymerase (Roche Biochemicals, South Africa) with the *bar* primer 5′-ATATCCGAGCGCCTCGTGCATGCG-3′ (custom synthesized by Roche Products, South Africa), designed for use with pAHC25 construct by Wan and Lemaux (1994), and yielded a 0.34 kb fragment if template was present. The *bar* gene products were analysed on a 1.5% agarose [w/v] gel (0.04 M Tris–Acetate, 0.002 M EDTA, pH 8.5) in TAE buffer after PCR.

Results and discussion

In this study, pretreatment with sorbitol was observed to maintain tissue integrity during the biolistic process. Microscopic analysis, after particle transfer, showed cell-burst in treatments lacking the osmotic treatment (Figure 1B), while a marked, positive effect was observed on culture cell integrity by inclusion of an osmoticum in the medium prior to biolistic transfer Figure 1C). Osmotic enhancement was suggested to have resulted from plasmolysis of the cells that may have reduced cell damage by preventing or making protoplasm extrusion less likely from bombarded cells (Vain et al. 1993; Li et al. 1994) and may have improved particle penetration itself (Sanford et al. 1993). The inclusion of 0.25 M sorbitol had the most beneficial effects on Day 0 of Line 1, and on Day 14 of Line 2 (Table 2), which was the only treatment to produce harvestable embryos suitable for plant regeneration.

After 10 days of subculture, embryogenic tissue growth was completely inhibited at 4 mg l^{-1} of glufosinate ammonium, the lowest concentration capable of inhibiting the growth of ESM on MSG3 maintenance medium. Decreasing amounts of new embryogenic tissue growth was observed at 1 mg l^{-1}, 2 mg l^{-1} and 3 mg l^{-1} BASTA® bioactive ingredient, respectively. However, in order to reduce toxicity to regenerating or recovering bombarded ESM all selection media contained a maximum of 3 mg l^{-1} BASTA® active ingredient.

The mode of peripheral ESM sampling from solid maintenance medium (MSG3) for embryo

Table 1. Survival (%) of liquid-derived cultures on selection and maturation media following bombardment with an optimized protocol.

	Bombardment day		0			5			10			14		
Genotype	Procedure followed and medium used		Control*	Untreated‡	Sorbitol†	Control	Untreated	Sorbitol	Control	Untreated	Sorbitol	Control	Untreated	Sorbitol
1	Selection medium	1 mgl^{-1}	100	100	100	100	100	100	100	100	100	100	100	100
	Selection medium	3 mgl^{-1}	70[a]	50	90.9	90	70	100	100	100	100	100	100	100
	Maturation medium	240	70	50	72.7	60[a, b]	70[a, b]	87.5[b]	50	60	12.5	100	100	100
	Subculture	240[c]	50[d]	10[d]	54.5[d]	20[d]	30[d]	37.5[d]	20[d]	20[d]	0	100[d]	90[d]	90[d]
2	Selection medium	1 mgl^{-1}	100	100	100	88.9	100	100	100	100	100	100	100	100
	Selection medium	3 mgl^{-1}	90	50	80[1]	88.9	100	100	100[e]	100[e]	100	100	90	90
	Maturation medium	240	20	20	20	11.1	25	11.1	0	0	0	100	90	90
	Subculture	240[c]	0[e, b]	0[e, b]	0[e, b]	0[e, b]	0[e, b]	0[e, b]	-	-	-	70[d]	70[d]	80[d]
3	Selection medium	1 mgl^{-1}	100	100	100	100	100	100	100	100	100	100	100	100
	Selection medium	3 mgl^{-1}	100	100	100	100	100	100	100	100	100	100	80	90
	Maturation medium	240	100	100	90.9	40	60	66.7	100	40	50	100	80	90
	Subculture	240[c]	50[f]	70[f]	54.5	20	20	33.3[d]	60	0	30[d]	40[g]	60	40

* – non-bombarded control; ‡ – bombarded tissue with no pre-treatment; † – bombarded tissue with a sorbitol pre-treatment; [a] – poor growth; [b] – brown; [c] – second subculture on 240 medium; [d] – small clumps of tissue; [e] – no growth; [f] – yellow, v. little new growth; [g] – glassy appearance.

Table 2. Regenerated bombarded ESM after improved regenerating regime (% surviving plates).

	Bombardment day		0			5			10			14		
Genotype	Procedure followed, medium used and number of embryos harvested per treatment tested		Control	Untreated	Sorbitol	Control	Untreated	Sorbitol	Control	Untreated	Sorbitol	Control	Untreated	Sorbitol
1	Maturation medium	240	100	100	100	100	100	100	100	100	-	100	100	90
	Subculture	240	0	0	20	75	66.7	25	0	0	-	80	80	60
	No. embryos harvested	0	0	0	0	0	0	0	0	-	0	0	0	
2	Maturation medium	240	-	-	-	-	-	-	-	-	-	100	100	100
	Subculture	240	-	-	-	-	-	-	-	-	-	100	100	100
	No. embryos harvested	-	-	-	-	-	-	-	-	-			54	
3	Maturation medium	240	-	-	-	-	-	-	-	-	-	-	-	-

- not tested.

singulation in suspension culture for re-establishment of regenerating material on maturation medium could have given great impetus to the regeneration process. This correlates with observations that embryogenic tissue collected from the periphery of the ESM colony was more amenable to maturation than tissue from the inner part or the entire culture when plated onto maturation media (Ramarosandratana et al. 2001).

Embryos from two of the three lines (Lines 1 and 2) chosen for biolistic experiments indicated good regeneration ability and underwent all stages of maturation. Despite the survival of bombarded treatments and the typically prolific nature of Line 3, a possible genotypic response of decreased maturation potential and tissue becoming highly mucilaginous was observed after several months in culture, irrespective of particle transfer (Table 1). Line 2 was most consistent in embryo production and was the only genotype that produced embryos after using an improved regeneration regime (Table 2). As this was one of the most prolific lines, this could be due to an inherently high embryogenic and maturation potential. Plantlets from somatic embryos derived from ESM can be regenerated using the protocol described by Jones and Van Staden (1995) prior to *ex vitro* transfer.

Analysis of transgenic Pinus patula

It has been established that osmoticum-supplemented media increases transient and stable transformation efficiency (Perl et al. 1992; Vain et al. 1993; Walter et al. 1994; Clapham et al. 1995; Walter et al. 1998). Bombarded ESM samples exhibited a range of expression strength of the β-glucuronidase enzyme, although higher magnification revealed that the embryonal heads (Figure 1B) had expressed the transient GUS activity and had turned a turquoise-blue colour.

The GUS transgenes (Figure 2A), were resolved on 0.8% agarose [w/v] at 1.2 kb. An estimated 40% transformation efficiency was concluded from the samples tested (14/36 samples). These results indicated that embryogenic tissue of this species was amenable to genetic transformation

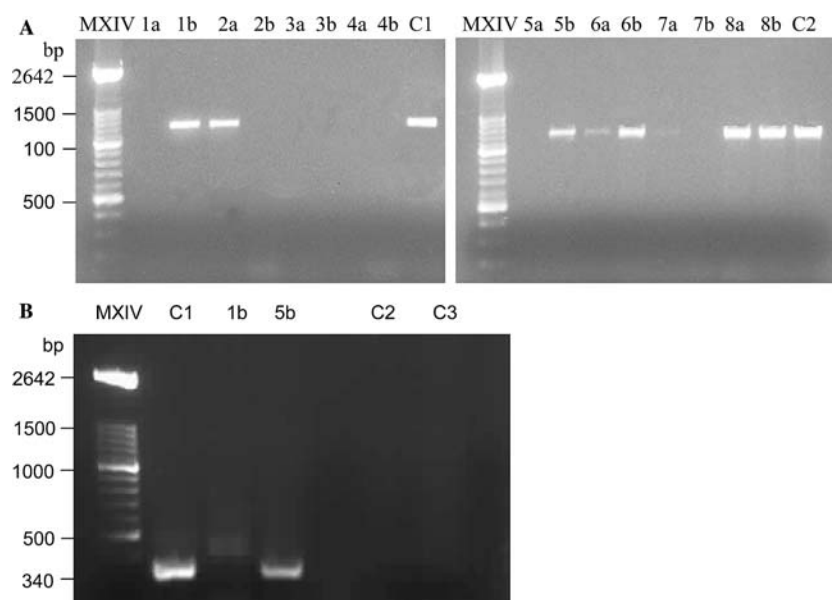

Figure 2. (A) PCR-mediated amplification of GUS products. The DNA contents of the lanes are: Molecular Weight Marker XIV (MXIV) Roche Biochemicals; samples of genomic DNA from bombarded *Pinus patula* tissue; positive controls of pAHC25 are indicated by C1 and C2. (B) PCR-mediated amplification of *bar* products from genomic DNA of transformed *P. patula* ESM. Amplification products obtained from a PCR-regime for *bar* gene products described by Vickers et al. (1996). DNA contents of the lanes are: MXIV; positive control of pAHC25 (C1); genomic samples of bombarded *P. patula* coded as 1b and 5b; negative control (C2), genomic sample of unbombarded *P. patula*; and negative control (C3), no genomic DNA included in reaction.

and the GUS reporter gene could be incorporated into and expressed in the *P. patula* genome. This indicated efficient splicing of the eukaryotic intron.

The smaller *bar* amplicon was resolved at 0.34 kb (Figure 2B) using the PCR regime described by Vickers et al. (1996). Of the 36 samples tested, 17 contained positive *bar* amplicons resulting in higher transformation efficiency (47%) than GUS. Perhaps the smaller gene was easier to incorporate into the genome and was expressed at a higher rate during selection. This indicated that co-integration of both the reporter GUS gene and the herbicide resistant *bar* gene did not always occur.

In conclusion, this study has successfully established a biolistic gene transfer regime. Evidence from PCR analysis and positive GUS analysis indicates that *P. patula* ESM is an appropriate target for genetic manipulations. Additional evidence to conclude physical linkage between the introduced transgenes and the *P. patula* genome is underway. Furthermore, a comprehensive examination into the use of *Agrobacterium* as a vessel for gene transfer is in progress in our laboratories. This would extend the scope of genetic transformation of *P. patula* as with other investigations in a variety of tree species (Tzfira et al. 1996; Holland et al. 1997; Tang et al. 2001; Trontin et al. 2002; Charity et al. 2002; Pappinen et al. 2002). This transformation protocol provides a platform towards the commercial exploitation of transgenic *P. patula*, as well as the use of transgenic studies for the advancement of our fundamental understanding of the molecular physiology of coniferous trees.

Acknowledgements

We thank Dr D. Berger for providing GUS primer sets and Dr B. Jénés who donated the pAHC25 plasmid. The following people are also thanked for technical assistance, Dr P. Hills, Mrs L. Fisher and Miss C. Ford. We are grateful to Sappi Forests Research for providing plant material. The financial assistance of the National Research Foundation (NRF) and Technology and Human Resources for Industry Programme (THRIP) is gratefully acknowledged.

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