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Stable Transformation and Recovery of Transgenic Plants by Particle Bombardment in *Pinus wallichiana* A.B. Jacks (Himalayan Blue Pine)

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Abstract: *Pinus wallichiana* A. B. Jacks is an important Himalayan pine species predominant in the Northern Himalayan range. We used *in vitro* embryogenic tissue of mature zygotic embryos as target material for genetic transformation. Regeneration of embryogenic tissue before particle bombardment and long selection period, combined with selective agent after bombardment, led to the production of transformed plantlets. Present results indicate that high levels of transient foreign gene expression can be achieved in pines. The procedure reported here is very simple, efficient and reproducible and is applicable across diverse genotypes of Himalayan blue pine. Expression of positive histochemical GUS activity (39%) in the bombarded embryogenic tissue was observed. PCR analysis of *bar* transgenes (52%) transformation efficiency indicated successful genetic modifications of *P. wallichiana* embryogenic tissue by the pAHC25 plasmid, where 50% of the selected plants showed gene integration and expression.

Key words: Genetic transformation, himalayan blue pine, particle bombardment

INTRODUCTION

Genetic transformation is an important tool for forest tree improvement and to investigate the function of genes (Wan and Lemaux, 1994; Tang *et al.*, 2001; Perl *et al.*, 1992). To meet the needs of both functional genomics and tree improvement, a simple, inexpensive, rapid and efficient transformation system successful with a range of pines is ideally required. The availability of an *in vitro* regeneration system is a prerequisite for efficient genetic transformation for most plants. Particle bombardment is based on the acceleration of high velocity coated with DNA into intact plant cells (Klein *et al.*, 1987; Russell *et al.*, 1992). Particle bombardment is usually the best and easy method for delivering DNA to cells for assays of promoter activity by transient expression (Potrykus, 1991). Biolistic transformations, in contrast to *Agrobacterium* (Malabadi and Nataraja, 2003), have resulted in fragmented or multicopy integration events of the transgene (Meyer, 1995; Walter *et al.*, 1998; Nigro *et al.*, 2004), which may lead to transgene silencing (Kumpatla *et al.*, 1997). Particle bombardment has been used for the genetic transformation of several conifer species, including *Pinus kesiya* (Malabadi and Nataraja, 2006), *Picea abies* (Robertson *et al.*, 1992; Haggman *et al.*, 1997), *Picea glauca* (Bommineni *et al.*, 1993), *Picea mariana*

(Charest *et al.*, 1996; Tian *et al.*, 2000), *Larix larcina* (Klimaszewska *et al.*, 1997), *Pinus radiata* (Walter *et al.*, 1998), *Pinus sylvestris* (Haggman *et al.*, 1997; Aronen *et al.*, 2003), *P. monticola* and *Pinus griffithii* (Tang and Newton, 2003), *P. patula* (Nigro *et al.*, 2004), *Picea abies* (Walter *et al.*, 1999) and *Pinus roxburghii* (Parasharami *et al.*, 2006). To our knowledge, there are no published reports for *P. wallichiana* in the literature. The present communication describes the development of an efficient transformation protocol for *P. wallichiana* embryogenic tissue through the introduction of the pAHC25 plasmid, which contains the selectable herbicide resistance *bar* gene and the *uidA* reporter gene. The usefulness of this system for increased production of transgenic plants of *P. wallichiana* is reported for the first time.

MATERIALS AND METHODS

Plant material: *Pinus wallichiana* A. B. Jacks seeds of a genotype (PW145, PW21 and PW106) of open pollinated trees were procured from the Arunachal Pradesh Forest Department, Itanagar, India. Seeds were surface cleansed with 1% Citramide for 2 min and washed thoroughly with sterilized distilled water for three times. Seeds were further treated with sodium hypochlorite solution (4-5% available chlorine) for 2 min, rinsed 5 times with sterile double distilled water and treated with 6% hydrogen peroxide

for 24 h. Prior to dissection of embryos, seeds were surface decontaminated sequentially with 0.1% HgCl₂ for 2 min, immersed in 70% ethanol for 3 min and finally rinsed thoroughly 5 times with sterile distilled water (Malabadi *et al.*, 2002, 2003, 2005).

Culture medium and initiation of embryogenic tissue:

Mature zygotic embryos (Fig. 1A) of 3 genotypes (PW145, PW21 and PW106) were cultured individually on half-strength inorganic salts MSG basal medium (Becwar *et al.*, 1990) containing 2.0 g L⁻¹ Gellan gum (Sigma), 90 mM maltose (Hi-media, Mumbai), 1 g L⁻¹ L-glutamine, 1 g L⁻¹ casein hydrosylate, 0.5 g L⁻¹ meso-inositol, 0.2 g L⁻¹ p-aminobenzoic acid and 0.1g L⁻¹ folic acid. The 24-epibrassinolide was purchased from CID Tech. Research Inc., Mississauga, Ontario, Canada (www.cidtech-research.com/brass.html). The stock solutions of 24-epiBrassinolide were prepared in absolute ethanol. Thus, the medium was supplemented with a range of 24-epibrassinolide (24-epiBL) concentrations (0.1, 0.5, 1, 2, 5, 10 and 15 µM) and 9.0 µM 2, 4-D. The

cultures were raised in 25×145 mm glass culture tubes (Borosil) containing 15 mL of the medium and maintained in dark for 4-6 weeks at 25±3°C. Nutrient medium without 24-epiBrassinolide served as a control. The pH of the medium was adjusted to 5.8 with NaOH or HCl before Gellan gum was added. The medium was then sterilized by autoclaving at 121°C and 1.08 Kg cm⁻² for 15 min. L-glutamine, p-aminobenzoic acid and 24-epibrassinolide were filter sterilized and added to the media after it had cooled to below 50°C.

All the cultures were examined for the presence of embryonal suspensor masses by morphological and cytological observations of callus. The cultures showing white mucilaginous embryogenic tissue were identified and subcultured on the initiation medium (Fig. 1B) for further three weeks for the better development of embryonal suspensor masses. The half-strength (inorganic salts) MSG basal medium (Becwar *et al.*, 1990) supplemented with 9.0 µM 2, 4-D and 2.0 µM 24-epiBrassinolide was used as an initiation medium for this purpose.

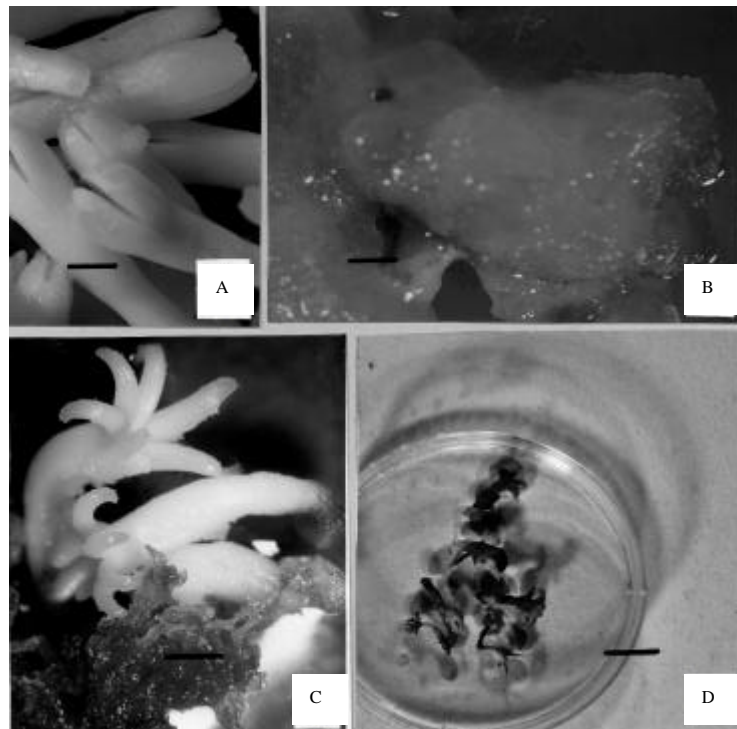


Fig. 1: Recovery of transgenic plants of genotype PW145 of *Pinus wallichiana* after bombardment of embryogenic tissue raised on MSG basal medium supplemented with 24-epiBr A- Group of mature zygotic embryos dissected from seed (scale bar 10 mm = 0.95 mm). B- Re-growth of bombarded white mucilaginous embryogenic callus on initiation medium (scale bar 10 mm = 8 mm). C- Development of advanced cotyledonary somatic embryos on maturation medium after bombardment (10 mm = 9.3 mm). D-Transgenic seedlings on germination medium

Maintenance of embryogenic tissue: The white mucilaginous embryogenic tissue developed on the above initiation medium (I) was subcultured on maintenance medium (II). The half-strength (inorganic salts) MSG basal medium containing 130 mM maltose, 4 g L⁻¹ Gellan gum and supplemented with 2 μM 2, 4-D and 0.5 μM 24-epiBrassinolide (maintenance medium) was used for this purpose. On the maintenance medium, the embryogenic tissue containing embryonal suspensor masses was maintained for 3 weeks with two subcultures. All the cultures were maintained in dark and microscopic observation of cultures was conducted to ensure the development of pro-embryo.

Maturation of somatic embryos: After partial desiccation of 24 h (Malabadi and van Staden, 2005a-d; Malabadi *et al.*, 2004; Malabadi and Nataraja, 2006a,b), the embryogenic tissue was transferred to maturation medium to induce cotyledonary embryo development (Fig. 1-E). The half strength (inorganic salts) MSG basal medium supplemented with 180 mM Maltose, 60 μM ABA and 8 g L⁻¹ Gellan gum (maturation medium) was tested for this purpose (Malabadi *et al.*, 2005). All the cultures were again maintained in the dark for 12 to 14 weeks.

Germination and plantlet recovery: After 12 to 14 weeks of maturation in presence of ABA and higher concentrations of maltose, advanced cotyledonary somatic embryos were picked from the cultures for germination. The germination medium used was half strength (inorganic salts) MSG basal medium with 2 g L⁻¹ Gellan gum. Somatic embryos were considered germinated as soon as radical elongation occurred and conversion to plantlet was based on the presence of epicotyls. After 4 to 6 weeks on germination medium, plantlets were transferred to vermiculite. Plantlets were placed in growth room under a 16 h photoperiod (50 μmol m⁻² sec⁻¹) for hardening.

Gene construct for transformation: The plasmid constructs pAHC25 (Christensen and Quail, 1996) 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 embryogenic tissue: Using the embryogenic tissue derived from suspension culture, embryonal suspensor masses, were filtered in 1.5 mL aliquots onto Whaman No. 1 filter paper supports and placed onto MSG solid medium supplemented with 90 mM maltose as an osmoticum (Malabadi *et al.*, 2003) or no maltose (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® herbicide, a bioactive ingredient (glufosinate ammonium) in the medium at each subculture. BASTA is a water-soluble and contains an active ingredient of glufosinate ammonium at 200 g L⁻¹.

DNA coating of microparticles: One hundred milligram 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 dH₂O. The sterile particles were stored in sterile 50% glycerol (v/v) solution at -20°C. Macro-particles 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 the experiments were performed using a gene gun (Gene booster, Germany) 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-glucuronide (X-glcA) (w/v) buffer (X-glcA, Sigma), 5 mM K-ferrocyanide, 5mM K-ferricyanide, 0.005% Triton X-100 (v/v), 100mM Na-phosphate buffer (0.5 M NaH₂PO₄ · 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.

DNA extraction: Genomic *P. wallichiana* 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 embryogenic tissue 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 μ L urea extraction buffer (7 M urea crystals, 5M 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⁻¹ (Nigro *et al.*, 2004). 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 xg), the supernatant was then transferred to fresh microfuge tubes. The nucleic acids were precipitated 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 xg and subsequently purified using 70% ethanol, washed with 100% ethanol prior to air-drying for 3-5 min on a laminar flow bench (Nigro *et al.*, 2004). Isolated genomic DNA was stored in 20 μ L ultra water at -20°C until further use

PCR-mediated gene detection: The *bar* gene was successfully amplified as described by Vickers *et al.* (1996) and Nigro *et al.*, (2004) using Expand™ High Fidelity *Taq* DNA polymerase (Roche biochemical's) with the *bar* primer 5'-ATATCCGAGCGCCTCGTGCATGCG-3' (Roche products) designed for use with pAHC25 construct by Wan and Lemaux (1994). This has yielded a 0.34 kb fragment (Fig. 2) 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 MEDTA, pH 8.5) in TAE buffer after PCR. A *bar* amplification cocktail, consisted of a 50 μ L reaction with 50 ng genomic template DNA, 1.25 units of *Taq* DNA polymerase (Roche biochemicals), 0.5 μ M of each primer, 10 mM of each dNTP: dATP, dTTP, dCTP and dGTP and 5 μ L PCR buffer (Roche biochemicals). To enhance the efficiency of the PCR, 10% dimethyl sulphoxide (DMSO) (v/v) was also included in the reaction mixture (Winship, 1989; Nigro *et al.*, 2004). 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 sec⁻¹), a 60°C annealing step (30 sec) and a 72°C extension step (45 sec⁻¹). The final stage employed the same denaturation and annealing conditions as described above but the last primer extension step was increased to a 5 min (Nigro *et al.*, 2004).

Statistical analysis: In all the above experiments each culture tube received a single explant. Each replicate

contained 50 cultures and one set of experiment is made up of 2 replicates (100 zygotic embryos were cultured for each genotype for one set of experiment). All the experiments were repeated 3 times (total 900 cultures for 3 independent experiments of three genotypes). Data presented in the tables were arcsine transformed before being analyzed for significance using ANOVA and the differences contrasted using a Duncan's multiple range test. All statistical analysis was performed at the 5% level using the SPSS statistical software package.

RESULTS AND DISCUSSION

Mature zygotic embryos (Fig. 1A) produced embryogenic tissue on half strength MSG basal medium supplemented with 9.0 μ M 2, 4-D and 2.0 μ M 24-epiBR (Initiation medium) in all three genotypes of *P. wallichiana*. Pullman *et al.* (2003) reported that use of brassinolide at a concentration of 0.1 μ M has improved the percentage of embryogenic cultures in loblolly pine, Douglas-fir (*Pseudotsuga menziesii*) and Norway spruce (*Picea abies*). They have also showed that brassinolide increased the weight of loblolly pine embryogenic tissue by 66% and stimulated initiation in the more recalcitrant families of loblolly pine and Douglas-fir, thus compensating somewhat for genotypic differences in initiation (Pullman *et al.*, 2003). Sasaki (2002) used brassinolide to increase adventitious shoot production on cauliflower hypocotyls segments. Wang *et al.*, (1992) obtained embryogenic cotton cultures from seedling hypocotyls with the aid of 0.02 μ M brassinolide. These results indicated ample evidence that brassinosteroids possess a broad spectrum of biological activities compared to the known plant hormones, including gibberellin, auxin and cytokinin- like activities (Brosa 1999; Yopp *et al.*, 1981).

In this study, lower concentrations (1, 2 and 3 mg L⁻¹) of BASTA showed the growth of bombarded embryogenic tissue (data not shown). The growth of bombarded embryogenic tissue of all the three genotypes of *P. wallichiana* was inhibited at 4 mg L⁻¹ of glufosinate ammonium (BASTA). However, in order to reduce the toxicity to regenerating or recovering bombarded embryogenic tissue, the entire selection medium was incorporated with 3 mg L⁻¹ BASTA active ingredient. The white mucilaginous bombarded embryogenic tissue was subcultured on the initiation medium for the further development of embryonal suspensor masses (Fig. 1-B). The pro-embryos developed on the maintenance medium could not grow further, until they were transferred on a medium with enhanced maltose, ABA and Gellan gum, respectively. The half strength (inorganic salts) MSG basal medium supplemented with 180 mM Maltose, 60 μ M

Table 1: Recovery of transgenic somatic seedlings following the bombardment of embryogenic tissue cultured on MSG basal medium supplemented with 3 mg L⁻¹ of BASTA in three genotypes of *Pinus wallichiana*

Genotypes	Somatic embryogenesis (%)	Total No. of somatic embryos recovered per gram fresh weight of embryogenic tissue	Total No. of somatic seedlings recovered per gram fresh weight of embryogenic tissue
PW145	10.0±1.1a*	23.0±2.1a	18.0±1.6a
PW106	0±0	0±0	0±0
PW21	0±0	0±0	0±0

*Mean (±SE) followed by the same letter were not significantly different at p≤0.05

ABA and 8 g L⁻¹ Gellan gum (maturation medium III) was tested for this purpose (Malabadi *et al.*, 2005). The bombarded embryogenic tissue developed somatic embryos on maturation medium after a period of 12 to 14 weeks (Fig. 1C). The percentage of somatic embryogenesis was not similar in all the three genotypes of *Pinus wallichiana* (Table 1). Highest percentage of somatic embryogenesis (10%) was recorded in a genotype PW145, with a total number of 18 somatic seedlings recovered per gram fresh weight of bombarded embryogenic tissue (Fig. 1B and C) (Table 1). On the other hand in the rest of the two genotypes (PW21 and PW106), the bombarded embryogenic tissue showed decreased maturation potential and tissue becoming highly mucilaginous was observed after several months in culture, irrespective of particle transfer. Furthermore, the bombarded embryogenic tissue of genotypes PW106, PW21 failed to produce somatic embryos and resulted in the browning and ultimate death of the tissue on the maturation medium (Table 1). After 12 to 14 weeks of maturation, the advanced cotyledonary somatic embryos were picked up for the germination. After 6 weeks on germination medium (Fig. 1D), the plantlets were recovered and hardened.

Bombarded embryogenic tissue samples exhibited a range of expression strength of the β-glucuronidase enzyme, although higher magnification revealed that the embryonal heads had expressed the transient GUS activity and had turned a turquoise-blue color (data not shown). These results indicated that embryogenic tissue of this genotype was amenable to genetic transformation and the GUS reporter gene could be incorporated and expressed in the *P. wallichiana* genome. The smaller *bar* amplicon was resolved at 0.34 kb (Fig. 2) using the PCR regime described by Vickers *et al.* (1996) and Nigro *et al.* (2004). Of the 100 samples tested, 52 contained positive *bar* amplicons resulting in higher transformation efficiency (52%) (Fig. 2) than GUS (39%). Perhaps the smaller gene was easier to incorporate into the genome and was expressed at high rate during selection. This indicated that co-integration of both the reporter GUS gene and the herbicide resistant *bar* gene did not always occur.

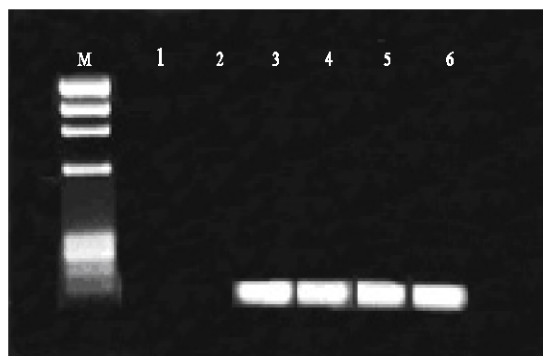


Fig. 2: PCR mediated amplification for *bar* gene products described by Vickers *et al.* (1996) and Nigro *et al.* (2004). The DNA contents of lane are: Molecular weight marker M (Roche Biochemicals). Lanes 1 and 2 = Genomic samples of unbombarded *Pinus wallichiana*. Lanes 3, 4, 5, 6 = Genomic samples of bombarded *Pinus wallichiana* of genotype PW145 showing the integration of *bar* gene at 340 bp (3.4 kb)

This is the first study that describes the stable integration and expression of marker genes through biolistic gene transfer regime. This would extend the scope of genetic transformation of *P. wallichiana* as with other investigations in a variety of tree species (Holland *et al.*, 1997; Tang *et al.*, 2001; Nigro *et al.*, 2004; Parasharmi *et al.*, 2006). This transformation protocol for the first time provides a platform towards the commercial exploitation of transgenic *P. wallichiana* for the genetic improvement of Himalayan blue pine. This provides an additional routine tool to augment breeding programmes and develops lines with improved disease resistance, altered lignin biosynthesis and selection of best clones, resulting in a new potential for its use in commercial forestry.

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