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Gene Transfer by Particle Bombardment of Embryogenic Tissue Derived from Vegetative Shoot Apices of Mature Trees of *Pinus roxburghii* (Chir Pine)

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Abstract: This study for the first time was aimed at developing a protocol for the genetic transformation of embryogenic tissue derived from the vegetative shoot apices of mature trees of *P. roxburghii*. This was achieved via the introduction of a bar-GUS cassette under the control of the ubiquitin promoter, through biolistic transfer. Expression of positive histochemical GUS activity (31%) in the bombarded embryogenic tissue was observed. PCR analysis of bar transgenes (54%) transformation efficiency indicated successful genetic modifications of *P. roxburghii* embryogenic tissue by the pAHC25 plasmid. This is the first successful report of genetic transformation in *P. roxburghii* using embryogenic tissue derived from the mature trees indicating that Chir pine is amenable to gene transfer.

Key words: Genetic transformation, chir pine, particle bombardment

INTRODUCTION

Tree breeding and genetic improvement can be greatly enhanced and accelerated by biotechnology. One of the fundamental requisite for the advancement of forest biotechnology and functional genomics is the development of a relatively simple and efficient method to introduce genes into conifer cells, coupled with regeneration of transformed plants (Klimaszewska *et al*., 2001). Historically *Agrobacterium*-mediated transformations were unable to overcome species recalcitrance. 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. However, the transformation frequency by co-culture method is very low for coniferous species. Therefore, direct method of transformation is generally suitable for the pines except with few disadvantages of genes being integrated into the host genome, in fragmentation and gene rearrangements, in high frequency of sterile plants and sometimes in a non-Mendelian inheritance of transgene (s) (Peng *et al*., 1995). Particle bombardment has been successfully used for the genetic transformation of several conifer species, including *Pinus kesiya* (Malabadi and Nataraja, 2007b), *Pinus wallichiana* (Malabadi and Nataraja, 2007c), *Picea glauca* (Ellis *et al*., 1993), Norway spruce (Robertson *et al*., 1992; Haggman *et al*., 1997), white spruce (Bommineni *et al*., 1993), black spruce (Charest *et al*., 1996; Tian *et al*., 2000), larch (Klimaszewska *et al*., 1997), radiata pine (Walter *et al*., 1998), Scots pine (Haggman *et al*., 1997; Aronen *et al*., 2003), *Pinus monticola* and *Pinus griffithii* (Tang and Newton, 2003), *Pinus patula* (Nigro *et al*., 2004) and *Pinus roxburghii* (Parasharami *et al*., 2006).

Transgenic trees are of utmost importance for studying the regulation of genes in specific traits. To our knowledge, there are no published reports of genetic transformation using embryogenic system derived from vegetative shoot apices of mature trees of conifers in the literature. Thus, the aims of this

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study were to develop a biolistic regime for embryogenic tissue derived from vegetative shoot apices of mature trees of *Pinus roxburghii* through the introduction of the pAHC25 plasmid which contains the selectable herbicide resistance *bar* gene and the *uidA* reporter gene. An efficient and reliable transformation method (Malabadi and Nataraja, 2003) is also an important tool for the future applications in commercial forestry. These include: Alteration of tree form and performance, abiotic stress tolerance, insect and herbicide 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, 1999; Tang and Newton, 2003). We report for the first time the successful delivery and expression of both the *uidA* and *bar* genes into *Pinus roxburghii* using the embryogenic tissue derived from the vegetative shoot apices of mature trees.

MATERIALS AND METHODS

Origin of Embryogenic Tissue

Apical shoots (Fig. 1A) from mature (14-years-old) trees of *Pinus roxburghii* from 3 genotypes (PR11, PR105 and PR521) were collected from the Western Ghat forests, India (Latitude-14°5'-15° 25'; Longitude-74°45'-76°E with an average rainfall of 80 cm). The mature material was cleansed with commercial NaOCl (Household bleach containing 3.5% sodium hypochlorite as the active ingredient) for 5 min and then washed 3 times with sterilized distilled water. They were then surface decontaminated with 70% ethanol for 5 min followed by immersion in 0.2% $HgCl_2$ for 2 min and rinsed 4 times with sterile double-distilled water. Transverse sections of approximately 0.5-1.0 mm thick were cut, using a scalpel or a sharp sterilized razor blade from the decontaminated apical shoots for the initiation of embryogenic callus. These apical shoot sections were cultured individually on full strength inorganic salts DCR (Gupta and Durzan, 1985) basal media and embryogenic tissue was initiated according to our existing previous protocols (Malabadi and Nataraja, 2006a, b; Malabadi and Nataraja, 2007a; Malabadi and van Staden, 2006).

Establishment of Suspensor Cultures for Transformation

Embryogenic tissue of 3 genotypes of *Pinus roxburghii* was induced and subcultured as described previously (Malabadi and van Staden, 2006). Cell suspension cultures were initiated by selecting and transferring 1 g fresh mass of white mucilaginous embryogenic tissue to 150 mL Erlenmeyer flasks with 50 mL full strength DCR (Gupta and Durzan, 1985) liquid basal medium supplemented with 20 μM 2,4-D, 25 μM NAA and 9 μM BA initiation medium (I) (Malabadi and Nataraja, 2006a, b, 2007a; Malabadi and van Staden, 2006). The cultures were maintained on a horizontal rotatory shaker at 100 rpm in the dark at 25±2°C. The suspension cultures were maintained for 2 weeks and the resulting suspension culture was settled by centrifugation for 5 min (70 x g). The settled cell aggregates were resuspended in a fresh liquid DCR basal medium for further proliferation. After filtering 3 times, an embryogenic suspension culture was established and then subcultured every week by transferring 1 mL of cell suspensions to the solid DCR maintenance basal medium (Malabadi and Nataraja, 2006a, b, 2007a).

Gene Construct for Transformation

The plasmid construct 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).

DNA Coating of Microparticles

One hundered 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 x g. The ethanol was removed and the micropaticles were washed twice with 2 mL sterile dH_2O. 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 delivary 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 Gene booster chamber. The microcarrier travel distance was 70 mm from the stopping plate to the target tissue. After the bombardment, the tissue was maintained in dark for the incubation for 24 h. It was then tested for the following histochemical test for the GUS expression.

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, 5 mM K-ferricyanide, 0.005% Triton X-100 (v/v), 100 mM Na-phosphate buffer (0.5 M NaH_2PO_4. $2H_2O$, 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.

Selection of Potentially Transformed Material

The Whatman No-1 filter paper supports carrying transgenic tissue was placed onto solid DCR maintenance basal medium (Malabadi and van Staden, 2006; Malabadi *et al*., 2005). The selection medium was consisting of BASTA incorporated in the DCR maintenance basal medium. 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}. The tissue was maintained on selection medium for at least 3 to 4 weeks with regular subculture. After 4 weeks, the transgenic tissue was separated and again maintained on the selection medium for the further analysis.

Maturation of Somatic Embryos

The partially desiccated (24 h) transgenic tissue was transferred to maturation medium to induce cotyledonary development (Malabadi and Nataraja 2006a; Malabadi and van Staden, 2006). The DCR basal medium with 175 mM maltose, 80 μM ABA and 9 g L^{-1} Gellan gum (maturation medium IV) was used for this purpose. All the cultures were placed in the dark at 25±2°C and maintained for 10 to 14 weeks.

Germination and Plantlet Recovery

After 10 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 (IV) used was half DCR basal medium with 2 g L^{-1} Gellan gum and 2.0 μM

triacontanol (TRIA) (Malabadi and Nataraja, 2006a). 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.

DNA Extraction

Genomic *P. roxburghii* DNA for PCR amplification was extracted after bombarded material had undergone selection at 3 mg L^{-1} BASTA® boactive 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 μL 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 (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 x g), 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 x g 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 μL ultra water at -20°C until further use. DNA from untreated tissue is served as control.

PCR-Mediated Gene Detection

The bar amplification cocktail, consisted of a 50 μL reaction with 50 ng genomic template DNA, 1.25 units of Taq DNA polymerase (Roche biochemicals), 0.5 μM of each primer, 10 mM of each dNTP: dATP, dTTP, dCTP and dGTP and 5 μL 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 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 analyzed 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. 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), a 60°C annealing step (30 sec) and a 72°C extension step (45 sec). 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 above experiments each culture tube received a single explant. Each replicate contained 50 cultures and one set of experiment is made up of two replicates (total 100 cultures for one experiment) for each genotype of *Pinus roxburghii*. Data presented in the tables were arcsine transformed before being analyzed for significance using ANOVA (analysis of variance, $p<0.05$) or evaluated for independence using Chi-square test. Further, the differences contrasted using a Duncan's multiple range test ($a = 0.05$) following ANOVA. All statistical analysis was performed using the SPSS statistical software package.

RESULTS AND DISCUSSION

The growth of bombarded embryogenic tissue of all the three genotypes of *P. roxburghii* was inhibited at 4 mg L^{-1} of glufosinate ammonium (BASTA). Lower concentrations (1, 2 and 3 mg L^{-1}) of BASTA showed the slow growth of bombarded embryogenic tissue (data not shown). However, in order to reduce the toxicity to regenerating or recovering bombarded embrogenic tissue, the entire selection medium was incorporated with 2 mg L^{-1} of BASTA active ingredient. The white mucilaginous transgenic tissue was subcultured on the maintenance medium for the further development of embryonal suspensor masses. 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 transgenic tissue was transferred to maturation medium to induce cotyledonary development (Malabadi and Nataraja, 2006a; Malabadi and van Staden, 2006) and it has developed somatic embryos on maturation medium after a period of 10 to 14 weeks (Fig. 1A-D).

The percentage of somatic embryogenesis was not similar in all the three transgenic genotypes of *Pinus roxburghii* (Table 1). Highest percentage of somatic embryogenesis (22%) was recorded in a genotype PR105, with a total number of 13 transgenic seedlings recovered per gram fresh weight of transgenic tissue (Fig. 1C-E) (Table 1). On the other hand in the rest of the two genotypes (PR11 and

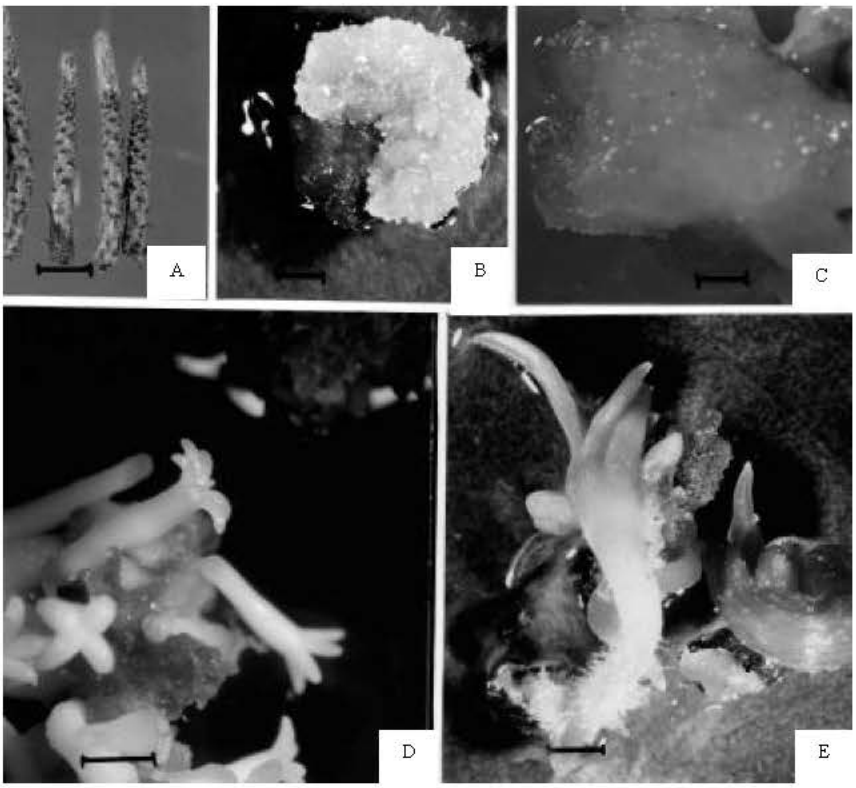

Fig. 1: Recovery of transgenic plants of genotype PR105. A- Vegetative shoot apices harvested from mature trees (scale bar 10 mm = 0.95 mm). B- Initiation of embryogenic tissue on initiation medium. C- Re-growth of transgenic callus on maintenance medium (scale bar 10 mm = 8 mm). D- Development of advanced cotyledonary somatic embryos on maturation medium after bombardment (10 mm = 9.3 mm). E- Trasgenic somatic seedlings on germination medium

Table 1: Recovery of transgenic somatic seedlings following the bombardment of embryogenic tissue cultured on DCR basal medium supplemented with 2 mg L^{-1} of BASTA in three genotypes of *Pinus roxburghii*

Genotypes	Somatic embryogenesis (%)	Total number of somatic embryos recovered per gram fresh weight of embryogenic tissue	Total number of somatic seedlings recovered per gram fresh weight of embryogenic tissue
PR11	0.0±0.0	0.0±0.0	0.0±0.0
PR105	22.0±2.8a*	29.0±2.9a	13.0±0.2b
PR521	5.0±0.1b	22.0±2.3a	8.0±0.2b

*Mean (±SE) followed by the same letter(s) were not significantly different at $p \leq 0.05$

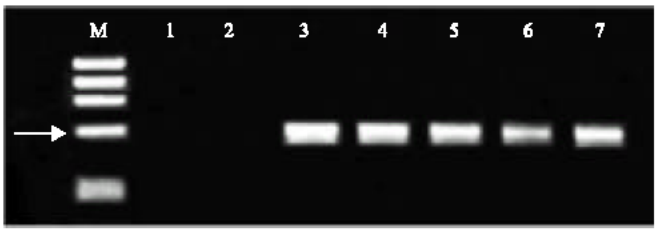

Fig. 2: PCR mediated amplification for *bar* gene products described by Vickers *et al*. (1996) and Nigro *et al*. (2004). The DNA contents of lane are: Molecular weight marker M (Roche Biochemicals). Lanes 1, 2 = Genomic samples of unbombarded *Pinus roxburghii*. Lanes 3, 4, 5, 6, 7 = Genomic samples of bombarded *Pinus roxburghii* of genotype PR105 showing the integration of *bar* gene at 340 bp (3.4 kb)

PK521), the transgenic tissue showed decreased maturation potential and tissue becoming highly mucilaginous was observed after several months in culture, irrespective of particle transfer. However, genotype PR521 showed decreased percentage (5% in PR521) of somatic embryogenesis, with a total number of 8 somatic seedlings recovered per gram fresh weight of transgenic tissue (Table 1). Furthermore, the transgenic tissue of genotype PR11 failed to produce somatic embryos and resulted in the browning and ultimate death of the tissue on the maturation medium (Table 1). After 10 to 14 weeks of maturation, the advanced cotyledonary somatic embryos were picked up for the germination. After 4-6 weeks on germination medium (Fig. 1E), the transgenic plantlets were recovered and hardened. Furthermore, the transgenic seedlings of a genotype PR521 showed very poor growth and were not survived and discarded. On the other hand, transgenic plants of a genotype PR105 were survived and recovered and showed normal growth.

These results indicated that embryogenic tissue of this genotype PR105 was amenable to genetic transformation and the GUS reporter gene could be incorporated and expressed in the *P. roxburghii* genome (Fig. 1A-E). Transgenic 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). 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, 54 contained positive bar amplicons resulting in higher transformation efficiency (54%) (Fig. 2) than GUS (31%). 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. Stably transformed shoots of Chir pine have been obtained after transfer by particle bombardment of plasmid DNA containing the bar and gusA genes and selection on medium contain the herbicide BASTA (Parasharami *et al*., 2006). They have also mentioned that the rooting efficiency of the regenerants was very low as very few transformed shoots were rooted by *in vitro* rooting method (Parasharami *et al*., 2006). On the other hand in our present investigation, rooting

efficiency of transformed seedlings was improved by the incorporation of TRIA (2.0 μM) in the germination medium and this has helped in the survival rate of transformed seedlings (Malabadi *et al*., 2005). Another method of recovery at high frequency of regenerants is the micrografting, which is applied for many forest trees (Jonard, 1986; Parasharami *et al*., 2006). Regenerants without roots can serve as *scions* which can be multiplied *in vitro* easily and could be rooted on rootstocks from seedlings grown *in vitro* (*in vitro* mirografting method). These stock plants could be used for plantation forestry programmes in the future (Parasharami *et al*., 2006).

In conclusion, this study has successfully established a biolistic gene transfer regime. Transformation was confirmed by PCR and histochemical tests for GUS and bio-tests for continued BASTA resistance. This would extend the scope of genetic transformation of *P. roxburghii* 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. roxburghii*. Therefore, facilitate Chir pine improvement via genetic engineering and physiological studies through the use of genetic manipulation. This is the first report of production of transgenic plants using embryogenic tissue derived from vegetative shoot apices of mature trees of *P. roxburghii* (Fig. 1A-E).

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