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Stable and Consistent *Agrobacterium*-Mediated Genetic Transformation in *Pinus roxburghi* (Chir Pine)

Ravindra B. Malabadi[1,3*] • Jaime A. Teixeira da Silva[2] • K. Nataraja[1]

[1] Division of Plant Biotechnology, Department of Botany, Karnatak University, Pavate nagar, Dharwad-580003, Karnataka state, India
[2] Faculty of Agriculture and Graduate School of Agriculture, Kagawa University, Miki-cho, Ikenobe 2393, Kagawa-ken, 761-0795, Japan
[3] Present Address: Department of Wood Sciences, 4th floor, Forest Sciences centre, # 4030-2424 Main Mall, University of British Columbia, Vancouver, V6T 1Z4, BC, Canada

Corresponding author: * **Ravindra.Malabadi@forestry.ubc.ca**

ABSTRACT

This paper highlights an *Agrobacterium tumefaciens*-mediated transformation protocol, developed for embryogenic cell cultures derived from vegetative shoot apices of mature, 14 years-old trees of *Pinus roxburghii*. The plasmid pBI121, containing the neomycin phosphotransferase II (*npt*II) gene providing kanamycin resistance as a selectable marker and the β-glucuronidase (*uid*A/GUS) reporter gene, was used as a binary vector. Transformation frequencies were dependent on the species, genotype and post-cocultivation procedure. The highest transformation efficiency was obtained in the embryogenic line PR105 (37 transformed lines/g fresh wt) than with the embryogenic lines PR11 and PR521. The transgenic state of the embryogenic tissue was initially confirmed by histochemical GUS assay. Stable integration of the *npt*II gene in the plant genome of *P. roxburghii* was confirmed by polymerase chain reaction (PCR), Southern and Northern blot analyses. These results demonstrated that a stable and enhanced transformation system has been established in chir pine, and that this system would provide an opportunity to transfer economically important genes into other genotypes of *P. roxburghii*.

Keywords: *Agrobacterium tumefaciens*, binary vector, mature trees, somatic embryogenesis, transgenic plants, vegetative shoot buds
Abbreviations: ABA, abscisic acid; **GM**, germination medium; **MM-I**, maintenance medium; **MM-II**, maturation medium

INTRODUCTION

Plant transformation has become an important and promising tool for the genetic improvement of conifers, since their breeding has limitations imposed in general by their heterozygosity, long juvenile periods and auto-imcompatibility (Birch 1997; Cervera *et al.* 1998; Malabadi and Nataraja 2007a, 2007b, 2007c, 2007g, 2007h; Tang *et al.* 2007). Genetic transformation using *Agrobacterium tumefaciens*, a soil plant pathogenic bacterium, is the most efficient procedure for the introduction of foreign genes into plant cells and subsequent regeneration of transgenic plants (Dodueva *et al*. 2007; Malabadi and Nataraja 2007d, 2007e). This system has been effective because only one or few copies of the transfer DNA (T-DNA) are integrated into the host genome, the ability to transfer large segments of DNA, the fewer copies of inserted genes, and higher efficiencies with lower cost (Tinland 1996; Shibata and Liu 2000; Malabadi and Nataraja 2003; Tang *et al.* 2007). Transgenic plants produced *via Agrobacterium*-mediated genetic transformation are generally fertile and the foreign genes are often transmitted to progeny in a Mendelian manner (Rhodora and Thomas 1996). *Agrobacterium* transformation may facilitate the removal of plant-selectable marker genes by segregation (Miller *et al.* 2002; Tang *et al.* 2007). At present, genetic transformation of conifers has been directed towards improving growth rate, wood properties and quality, pest resistance, stress tolerance, herbicide resistance, and increasing cellulose content versus decreasing lignin content, all which will drive forestry to enter a new era of productivity and quality (Tang and Newton 2003, 2005; Tang *et al.* 2006).

Agrobacterium-mediated transformation of conifers has been reported in many species, but transformation remains highly dependent on the genotype. The majority of these reports rely on somatic embryogenesis, usually from cultured mature or immature zygotic embryos of *Larix kaemperi* × *L. deciduas* (Levee *et al.* 1997), *Pinus strobus* (Levee *et al.* 1999; Tang *et al.* 2007), *Picea glauca*, *Picea mariana*, *Picea abies* (Klimaszewska *et al*. 2001), loblolly pine (*Pinus taeda*) (Tang *et al.* 2001), *Pinus radiata* (Cerda *et al.* 2002; Charity *et al.* 2002; Grant *et al.* 2004; Charity *et al.* 2005; Grace *et al.* 2005), *Picea glauca* (Le *et al*. 2001), Norway spruce (*Picea abies*), *Pinus taeda* (Wenck *et al.* 1999), *Pinus nigra* (Lopez *et al.* 2000), *Pinus pinaster* (Tereso *et al.* 2006) and Douglas fir (*Pseudotsuga menziesii*) (Dandekar *et al*. 1987).

Pinus roxburghii Sarg. (Chir pine) is an important Indian pine species of Western Himalayas. Some genotypes have tremendous biomass potential and oleoresin prospects (Malabadi 2006b; Malabadi and Nataraja 2006a, 2006b). Genetic transformation *via* particle bombardment using the embryogenic tissue derived from vegetative shoot apices of mature trees (Malabadi and Nataraja 2007a), and both mature and immature zygotic embryos of *P. roxburghii* has been well established (Parasharami *et al.* 2006). However, genetic transformation of conifers is still remains a slow and tedious process due to factors such as the type and physiological status of explants, the vector system, the *Agrobacterium* strains and the selection scheme for transformants (Le *et al.* 2001; Malabadi and Nataraja 2007g, 2007h). No successful reports of *Agrobacterium*-mediated genetic transformation have been reported in *P. roxburghii*. Methods for the regeneration of plantlets from embryogenic tissue derived from vegetative shoot apices of mature trees of *P. roxburghii* have been well established (Malabadi 2006b; Malabadi and Nataraja 2006ab). This robust and reliable regeneration system has been the basis of a programme for genetic engineering *P. roxburghii* using *A. tumefaciens*-mediated gene transfer. We report here the successful delivery and expression of both the neomycin phosphotransferase gene II (*npt*II) providing resistance to kanamy-

Received: 17 December, 2007. Accepted: 31 January, 2008.

Original Research Paper

cin, and the β-glucuronidase (GUS) gene (*uid*A) into *P. roxburghii* using the embryogenic tissue derived from the vegetative shoot apices of mature trees.

MATERIALS AND METHODS

Plant material and origin of embryogenic tissue

Shoot apical buds from mature trees (14- years old) of three genotypes of *P. roxburghii* (PR11, PR105, and PR521) were collected from the Western Ghat Forests, India (14° 5′ to 15° 25′ N latitude and 74° 45′ to 76° E longitude with an average rainfall of 80 cm) (Malabadi 2006b; Malabadi and Nataraja 2006a, 2006b).

The apical buds were cleansed with commercial NaOCl (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 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 DCR salts on Gupta and Durzan basal medium (Gupta and Durzan 1985), and embryogenic tissue was initiated as described previously (Malabadi *et al.* 2004; Malabadi and van Staden 2005a, 2005b, 2005c; Malabadi 2006b; Malabadi and Nataraja 2006a, 2006b; Malabadi and van Staden 2006; Malabadi and Nataraja 2007f).

Agrobacterium strain and culture conditions

A. tumefaciens strain EHA105 harbouring binary vector pBI121 which contains the *npt*II gene providing resistance to kanamycin and the GUS gene (*uid*A) interrupted with an intron, both driven by the cauliflower mosaic virus (CaMV) 35S promoter was used for transformation studies (Jefferson *et al.* 1987; Hood *et al*. 1993). This disarmed agropine strain EHA 105 (Hood *et al.* 1993) contains an additional 15.8 kb fragment carrying extra copies of the *vir*B, *vir*C and *vir*G regions from the supervirulent plasmid PToK47 (Jin *et al.* 1987). The *A. tumefaciens* was grown in liquid YMB medium (yeast extract, 0.8 gl^{-1}; mannitol, 10.0 gl^{-1}; NaCl, 0.1 gl^{-1}; $MgSO_4 \cdot 7H_2O$, 0.2 gl^{-1}; KH_2PO_4, 0.5 gl^{-1}; pH, 7.0) containing 50 mgl^{-1} kanamycin and 100 mgl^{-1} rifampicin, overnight at 28°C on a shaker at 100 rpm for the selection of the pBI121 vector (Malabadi and Nataraja 2007d, 2007e). The bacterial cells were thereafter pelleted by centrifugation at 4,000 rpm for 10 min and resuspended in liquid DCR basal medium (maintenance medium, MM-I) (Malabadi 2006b; Malabadi and Nataraja 2006ab) to an optical density ($OD_{600\ nm}$) of 0.6.

Agrobacterium-mediated transformation procedure

Prior to transformation experiments, the embryogenic suspension cultures of three embryogenic lines (PR11, PR105, and PR521) were grown in 125 ml Erlenmeyer flasks containing 50 ml of liquid maintenance DCR medium (Malabadi and Nataraja 2006a, 2006b). They were subcultured every week at a 1:5 (v/v) ratio in the dark at 28°C on a rotary shaker at 100 rpm. Subsequently, an equal volume of *A. tumefaciens* culture ($OD_{600\ nm}$) of 0.6 in liquid maintenance medium was added to the cell suspension, resulting in 50 mg fresh mass embryogenic tissue suspended in 1 ml of bacterial culture with an $OD_{600\ nm}$ of 0.3 in liquid maintenance medium in a 125 ml Erlenmeyer flask. In all the embryogenic lines, 100 µM acetosyringone was added to the bacterial suspension just before the inoculation was tested. The control embryogenic lines were treated the same way except that no *A. tumefaciens* was added to the cultures. In the first experiment, the Erlenmeyer flasks with the embryogenic tissue and *A. tumefaciens* were placed on a shaker at 100 rpm for 5 h (infection period). On the other hand, in the second experiment, flasks with embryogenic tissue and *A. tumefaciens* were also placed on a shaker at 100 rpm for 1 h (infection period). Subsequently, 3 ml of the culture (150 mg fresh mass) were poured over a 5.5 cm sterile filter paper (Whatman filter paper, Schleicher and Schuell, qualitative circles) to drain the liquid and the filter paper was placed on a semi-solid DCR basal maintenance medium in a 55 mm diam. Petri dish. Co-cultivation was carried out for 3 days under the conditions described above. A total of 10 Petri dishes of each tested embryogenic lines were used in experiments 1 and 2.

After 3-d co-culture on the semi-solid medium, the filter papers with the cells from five Petri dishes were subcultured onto fresh DCR semi-solid maintenance medium (MM-I) with 470 mg l^{-1} cefotaxime (non-washed; Sigma). The filter papers with the cells from the other five Petri dishes were first placed in an Erlenmeyer flask (250 ml) with 100 ml of liquid DCR maintenance medium. The cells were then dislodged by manual shaking and collected on the new filter papers in a Buchner funnel. The filter papers with cells were then placed on fresh medium with 470 mg l^{-1} cefotaxime (Sigma) (washed). The timing of subculture onto selection medium was genotype-dependent and occurred after the first signs of embryogenic tissue growth. Selection medium contained cefotaxime and 35 mg l^{-1} kanamycin depending on the embryogenic lines. After 15 days, the growing embryogenic clumps were transferred to semi-solid DCR maintenance medium supplemented with 35 mg l^{-1} kanamycin (Sigma). The putatively transformed kanamycin-resistant tissues were isolated and maintained for at least 3 subcultures in the presence of cefotaxime.

GUS histochemical assay

Histochemical GUS assays were conducted essentially as described earlier (Jefferson 1987). Briefly, kanamycin-resistant tissues or putative transgenic tissue, somatic embryos, plantlets or needles were incubated overnight at 37°C in a X-Gluc solution composed of 0.1% (w/v) 5-bromo-4-chloro-3-indoyl-β-glucuronic acid, 100 µM sodium phosphate (pH 7.0), 0.5 µM potassium ferrocyanide, 0.5 µM potassium ferricyanide, and 10 µM ethylene diamine tetra acetic acid (EDTA). Plant cells and tissues were scored as GUS-positive for the *uid*A gene if any deep indigo blue colour was present.

PCR analysis of *npt*II

Genomic DNA was extracted from transgenic and non-transgenic embryogenic tissues according to a modified isolation method of Dellaporta *et al.* (1983). PCR amplification of the *npt*II gene was performed with following specific primers (Wenck *et al*. 1999): 5′-ACTGTCCCCTAGT-GGGGAAGGGGACTGGCTGCTATT-3′ and 5′-GATACCG-TACGCCCAAGCGCAGGTCAG-3′ to produce an expected gene fragment of 500 bp. PCR reactions were carried out in a final 25 µl reaction mixture containing 50 ng template DNA, 0.2 µl (1 U) of *Taq* DNA polymerase (Roche, Germany), 0.5 µl of gene-specific primer of *npt*II, 2.0 µl of 10× PCR buffer (Roche, 100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3), 0.5 µl of 10 mM dNTP stock (Operon Technologies). The negative control mixture contained all reagents except the DNA template. Each reaction mixture was overlaid with 25 µl of mineral oil (Sigma) to prevent evaporation. The amplification was performed in a Hybaid Thermal Reactor (Hybaid, UK) programmed with following conditions: 5 min 95°C, followed by 34 cycles (denaturation an extension cycle of 72°C for 5 min. Samples were then stored at 4°C. Amplified DNA was detected by ultraviolet light after electrophoresis on 0.8% agarose/ethidium bromide gels using 1× TAE as running buffer.

Southern blot analysis

Genomic DNA was isolated from 1 g fresh wt of control and putative transgenic plants using modified isolation method of Dellaporta *et al.* (1983). For Southern blot analysis, isolated DNA (50 µg) was digested overnight at 37°C with 150 units of *Hind*III and *Eco*RI (Roche Biochemicals) before separation by electrophoresis on 0.8% (w/v) agarose gel at 70 V for approximately 5 hours. The gels were depurinated, denaturated, neutralized and fragmented DNA was transferred onto a positively charged nylon membrane (Roche Diagnostics GmbH, Mannheim, Germany) by capillary transfer. DNA was bound to the membrane using a UV Stralinker. The prehybridization and hybridizations were performed in Easy Hyb solution (Roche Biochemicals) at 42-45°C. Double-stranded

probe for *npt*II (500 bp or 0.5 kb), which was obtained by digestion of pBI121 with *Hind*III and *Eco*RI, was labeled with digoxigenin-11-dUTP using the PCR conditions according to Roche Diagnostics protocol. After overnight hybridization, the blots were washed twice with 2× SSC (3 M NaCl, 0.3 M Sodium citrate, pH 7.0) containing 0.1% SDS for 5 min at room temperature and at high stringency twice with 0.5× SSC, 0.1% SDS at 68°C for 15 min. The chemiluminescence detection of hybridization products was performed according to manufacturer's (Roche Biochemicals) instructions.

RNA isolation

Needle samples were collected from the control and Southern-positive transgenic *P. roxburghii* plants and immediately frozen in liquid nitrogen and stored at -80°C until further use. Samples of total RNA were isolated from 1.0 g fresh wt of plant material by the modified method of Chang *et al*. (1993).

Northern blot analysis

Northern-blot analyses were performed to confirm the presence of the transgene (*npt*II) in the *P. roxburghii* genome. Fifteen µg of total RNA was separated on a 1.2% agarose gel containing 2.9% formaldehyde following denaturation of samples at 100°C for 2 min in formaldehyde and formamide. The electrophoretically separated RNAs were transferred to a nylon membrane (Roche Diagnostics, GmbH) by capillary transfer in 20× SSC overnight. After blotting, the membrane was washed twice in 2× SSC at room temperature for 10 min and crosslinked by UV-illumination. The efficiency of the RNA transfer was determined by staining the membrane in methylene blue (0.02% w/v methylene blue, 0.3 M sodium acetate, pH 5.5) for 3 min. Before hybridization, the membrane was de-stained in 0.1× SSC, 0.5% SDS at 68°C for 15 min. The pre-hybridization was performed in Easy Hyb Solution (Roche Biochemicals) at 50°C for 1-2 hrs. For hybridization, a fresh Easy Hyb Solution containing denatured *npt*II probe for the detection of the corresponding mRNAs (500 bp or 0.5 kb) was used. The probe used for detection of the *npt*II mRNA was the same one as that applied in Southern blot analyses, and was labeled with digoxigenin-11-dUTP by PCR using the same set of primers. After overnight hybridization at 50°C, the blots were washed twice with 2× SSC containing 0.1% SDS for 5 min at room temperature and at high stringency twice with 0.2× SSC, 0.1% SDS at 68°C for 15 min. The chemiluminescence detection of hybridization products was performed according to manufacturer's (Roche Biochemicals) instructions.

Maturation of somatic embryos

For maturation, embryogenic tissue clumps of each of the transformed lines obtained were isolated from filter paper discs, and subcultured on fresh DCR maintenance medium without filter paper disc. Transgenic tissue was proliferated for another 15 days and cultures were incubated in the dark at room temperature. One gram fresh weight of transgenic tissue of each embryogenic line was transferred to sterile empty Petri dishes (60 mm diam.) containing two sterile Whatman filter paper disks (50 mm) (Schleicher and Schuell, qualitative circles). The Petri dishes were sealed with Parafilm and kept at 25 ± 2°C in the dark for 24 hr to obtain the desired extent of desiccation. After desiccation, the partially desiccated transgenic tissue of each embryogenic line was transferred to maturation medium to induce cotyledonary embryo development. The full strength DCR medium with 60 g l^{-1} maltose, 37.84 µM abscisic acid (ABA) and 5 g l^{-1} Gellan gum (maturation medium, MM-II) was tested for this purpose. All the cultures were placed in the dark at 25 ± 2°C and these maintained for 8 to 12 weeks (Malabadi 2006b; Malabadi and Nataraja 2006a, 2006b).

Germination and plantlet recovery

After 6 to 8 weeks of maturation in the presence of ABA and higher concentrations of maltose (60 g l^{-1} maltose) the transgenic cotyledonary somatic embryos were recovered from the cultures for germination. Histochemical GUS assay was carried out on mature somatic embryos of each transgenic line at the same time as another subset of somatic embryos from the same Petri dish was placed on the germination medium. Before germination, the transgenic cotyledonary somatic embryos of the all embryogenic lines were cold pre-treated at 2°C and kept in dark for 5 days. The germination medium (GM) used was half DCR medium with 2 g l^{-1} Gellan gum (Malabadi and Nataraja 2006a, 2006b). In the first week of germination, cultures were kept in darkness, then transferred to diffuse light in the second week, and thereafter to a 16-hr photoperiod under a light intensity of 50 µmol m^{-2} s^{-1} for hardening. Somatic embryos were considered germinated as soon as radical elongation occurred and conversion to plantlets was based on the presence of epicotyls. After 4 to 6 weeks on germination medium, plantlets were transferred to vermiculite in a controlled growth room. Needles of transgenic plants were then collected for the GUS assay and PCR analysis.

Statistical analysis

In the above experiments, each replicate contained 50 cultures and one set of experiments is made up of two replicates (total 100 cultures for one experiment) for each genotype (PR11, PR105, PR521) of *P. roxburghii.* All the experiments were repeated 3 times and Table 1 represents the average of 3 independent experiments. 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 the Chi-square test. Further, the differences in means were contrasted using Duncan's multiple range test (a=0.05) following ANOVA. All statistical analyses were performed using the SPSS statistical software package.

RESULTS AND DISCUSSION

In the present study, genetic transformation has been achieved through the introduction of two transgenes into the *Pinus roxburghii* genome. During transformation, an infection period of 5 h was found optimum in all the embryogenic lines tested (**Table 1**). There was no significant effect on the growth of cultures during a 3-day co-cultivation of cultures. After 3 days co-cultivation of embryogenic tissue with the *Agrobacterium*, there was only a slight growth of the bacterium around the edges of the filter paper. Bacterial growth was inhibited by culture on a medium with a higher concentration (470 mg l^{-1}) of cefotaxime (**Table 2**). The rest of the bacteria might be eliminated due the washing of embryogenic tissue with liquid medium, although no experimental data exists at present o prove our hypothesis. This was also observed in other conifers such as *P. glauca*, *P. mariana* and *P. abies* (Klimaszewska *et al.* 2001). A lower concentration of cefotaxime (100-350 mg l^{-1}) did not significantly affect the bacterial growth compared to the control without antibiotic (**Table 2**). Therefore, the optimum concentration of cefotaxime (470 mg l^{-1}) was crucial for the recovery of embryogenic tissue growth after co-cultivation. It was also reported that a higher concentration (600 to 800 mg l^{-1}) of cefotaxime inhibited the growth of bacteria during selection of embryogenic lines of *P. glauca*, *P. mariana* and *P. abies* (Klimaszewska *et al.* 2001). On the other hand the post-cocultivation of embryogenic tissue in all the embryogenic lines tested that had not been washed, but that were transferred directly with filter papers onto medium with cefotoxime did not show any abundant bacterial growth compared to the control. Cefotaxime at 470 mg l^{-1} was required for at least 3 subcultures to ensure complete elimination of the bacteria. Alternately, a transgenic, chimeric colony could arise that would be a mixture of cells representing different transformation events. To eliminate this possibility cultures were not washed after cocultivation in other conifer species (Klimaszewska *et al.* 2001). The co-cultivation procedure with *A. tumefaciens*, particularly the post-coculture treatment of embryogenic tissue played a role in the recovery of transgenic tissue (Klimaszewska *et al.* 2001; Le *et al.* 2001; Tang *et al.* 2007). Subsequent subculture onto selective medium with kanamycin was done

Table 1 Effect of *Agrobacterium*-infection period and various concentrations of acetosyringone on genetic transformation of *P. roxburghii*.

Embryogenic lines tested	*Agrobacterium* infection period (h)	Acetosyringone (µM)	GUS spots/g fresh wt of transgenic tissue	Recovery of transgenic colonies/g fw of tissue
PR11	0	0	0.0 ± 0.0 c	0.0 ± 0.0 c
	1	25	4.0 ± 0.2 b	0.0 ± 0.0 c
	2	50	1.0 ± 0.1 b	0.0 ± 0.0 c
	3	70	2.0 ± 0.1 b	1.0 ± 0.1 b
	4	80	5.0 ± 0.2 b	1.0 ± 0.1 b
	5	100	24.0 ± 1.3 a*	10.0 ± 0.2 a*
	6	150	3.0 ± 0.1 b	0.0 ± 0.0 c
PR105	0	0	0.0 ± 0.0 c	0.0 ± 0.0 c
	1	25	0.0 ± 0.0 c	0.0 ± 0.0 c
	2	50	4.0 ± 0.0 c	0.0 ± 0.0 c
	3	70	0.0 ± 0.0 c	0.0 ± 0.0 c
	4	80	1.0 ± 0.1 b	0.0 ± 0.0 c
	5	100	27.0 ± 4.0 a*	13.0 ± 0.5 a*
	6	150	4.0 ± 0.1 b	0.0 ± 0.0 c
PR521	0	0	0.0 ± 0.0 c	0.0 ± 0.0 c
	1	25	0.0 ± 0.0 c	0.0 ± 0.0 c
	2	50	0.0 ± 0.1 c	0.0 ± 0.0 c
	3	70	2.0 ± 0.1 b	0.0 ± 0.0 c
	4	80	6.0 ± 0.1 b	0.0 ± 0.0 c
	5	100	26.0 ± 3.0 a*	9.0 ± 0.4 a*
	6	150	2.0 ± 0.1 b	0.0 ± 0.0 c

*Mean (±SE) followed by the same letter in each column were not significantly different at P<0.05 using DMRT.

A mass of cells containing a transgene, which is easily seperated from the rest of callus due to the resistance to the antibiotics such as cefotaxime and kanamycin. This resistance is mainly due to the presence of transgene, and it is called as transgenic colony. A transgenic colony is also positive for GUS assay. A non-transgenic colony will not survive the selection pressure and ultimately resulted in the browning of callus leading to the death of cells.

Table 2 Effect of various concentrations of cefotaxime and kanamycin on genetic transformation of *P. roxburghii*.

Embryogenic lines tested	Cefotaxime (mgl^{-1})	Kanamycin (mgl^{-1})	Recovery effect during selection	Number of transgenic colonies recovered/g fw of tissue
PR11	0	0	–	0.0 ± 0.0 c
	100	5	–	0.0 ± 0.0 c
	250	15	–	0.0 ± 0.0 c
	350	25	–	4.0 ± 0.2 b
	400	30	+	3.0 ± 0.1 b
	470	35	++++	36.0 ± 5.0 a*
	600	50	–	0.0 ± 0.0 c
PR105	0	0	–	0.0 ± 0.0 c
	100	5	–	0.0 ± 0.0 c
	250	15	–	0.0 ± 0.0 c
	350	25	–	0.0 ± 0.0 c
	400	30	+	2.0 ± 0.1 b
	470	35	++++	30.0 ± 3.1 a*
	600	50	–	10.0 ± 1.8 b
PR521	0	0	–	0.0 ± 0.0 c
	100	5	–	0.0 ± 0.0 c
	250	15	–	1.0 ± 0.0 c
	350	25	–	1.0 ± 0.1 b
	400	30	+	3.0 ± 0.1 b
	470	35	++++	26.0 ± 4.0 a*
	600	50	–	2.0 ± 0.1 b

(*Mean (±SE) followed by the same letter in each column were not significantly different at P<0.05 using DMRT).

Note: In the table – indicates negative selection pressure where unable to recover the transgenic callus, whereas +++ indicates positive selection pressure leading to the recovery of transgenic tissue.

A mass of cells containing a transgene, which is easily separated from the rest of callus due to the resistance to the antibiotics such as cefotaxime and kanamycin. This resistance is mainly due to the presence of transgene, and it is called as transgenic colony. A transgenic colony is also positive for GUS assay. A non-transgenic colony will not survive the selection pressure and ultimately resulted in the browning of callus leading to the death of cells.

only after the first sign of growth of the embryogenic tissue, which was determined visually. It was necessary to vary the concentration of kanamycin in the medium for the selection of transformed embryogenic lines, depending upon the species. Second, the timing of selection was also important and varied among the species (Klimaszewska *et al.* 2001). After the first subculture onto selection medium, putatively transformed lines could be individualized either from the original clumps or from the filter paper.

Based on preliminary studies (**Table 2**), it becomes obvious that for selection of transformed *P. roxburghii* embryogenic lines tested, the best kanamycin concentration for selection of transgenic tissue was 35 mg l^{-1}. During the first 2 weeks of period from the start of the experiment, there was no visible sign of selective effect; on the contrary, all cultures proliferated. The selective pressure was significant after 3 weeks of culture onto medium containing cefotaxime and kanamycin. This trend was noticeable in all the other conifers reported earlier (Klimaszewska *et al.* 2001; Le *et al*. 2001; Tereso *et al.* 2006). In other conifers embryogenic cells of some genotypes were highly competent for T-DNA uptake and integration under suitable conditions (Klimaszewska *et al.* 2001). Our results also showed that embryogenic lines of three different genotypes tested showed different transformation frequencies, unless we assume that the cultures differed in their cellular composition.

The cells for the transformation experiments in this study came from vigorously growing embryogenic tissue derived from apical shoot buds of mature trees, collected from filter paper, and characterized by their ability to produce mature somatic embryos and plants. The viability of cultures, bacterial strains and co-cultivation conditions were all important in making the cells susceptible to *Agrobacterium* binding, T-DNA transfer, and integration into a cell genome (Wenck *et al.* 1999; de Buck *et al.* 2000; Lelu and Pilate 2000; Klimaszewska *et al.* 2001). Pieces of embryogenic tissue that showed vigorous and continuous growth were subjected to histochemical GUS assay. Stable kanamycin-resistant lines produced enough tissues that could be used in the GUS assay, whereas the non-transformed tissues turned brown without further growth within three weeks of selection, depending on the embryogenic lines. These results are in agreement with those of Klimaszewska *et al.* (2001), Le *et al.* (2001) and Tereso *et al.* (2006). The expression of the GUS gene, *uid*A, was analysed in the putatively transformed lines by the GUS assay. In the present experiments with *P. roxburghii*, an overall 95% of the embryogenic tissues visually selected on the basis of growth on the medium with kanamycin were found to be GUS-positive (**Fig. 1**). Activity of the GUS enzyme was not detected in control tissues. The GUS-expressing

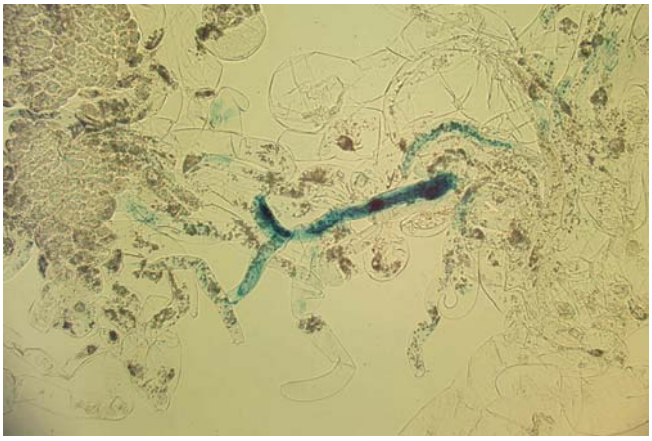

Fig. 1 Evidence for the presence of the *uid*A gene. GUS activity was visible as dark blue to pale blue spots in embryogenic tissue as seen under a microscope at 40X magnification following GUS histochemical assay.

embryogenic lines showed no reduction in growth when cultured with or without kanamycin over a 12-week period, and this was also confirmed with a previous study in eastern white pine with or without hygromycin (Tang *et al.* 2007). Most of transformed lines showed variable GUS intensity in blue cell aggregates. In other conifers a mixture of GUS-positive and -negative embryogenic aggregates was also observed at least in some transformed lines (Ellis *et al.* 1993; Walter *et al.* 1998; Tian *et al.* 2000; Tereso *et al.* 2006). This could be explained by the existence of different proportions of transformed to non-transformed cells (Tereso *et al.* 2006). On the other hand, variations in the expression level of the *uid*A gene are possible in different transformed lines, which can be explained by phenomena such as the position effect of the insertion (Matzke and Matzke 1998; Tereso *et al.* 2006). Gene expression could also be very due to copy number effect (Cervera *et al.* 2000). A high copy number may lead to gene silencing (Matzke *et al.* 1994).

The transformation frequency was considered as the number of transformed lines per gram fresh weight of embryogenic tissue. This is directly related to the total number of GUS blue color spots in one gram fresh wt of transgenic tissue. The highest transformation efficiency (highest number of blue-color GUS spots) was obtained in the embryogenic line PR105 (37 transformed lines/g fresh wt) than with the embryogenic lines PR11 and PR521. In PR11, the lowest transformation efficiency was recorded (16 transformed lines/g fresh wt). On the other hand PR521 showed 26 transformed lines/g fresh wt. These results suggest that the success of the transformation process in *P. roxburghii* embryogenic lines is genotype-dependent. This was also reported for French and Portuguese *P. pinaster* embryogenic lines (Trontin *et al.* 2002; Tereso *et al.* 2006) with four out of six lines transformed, and for L. *kaempferi* × *L. deciduas* (Levee *et al.* 1997) with four out of seven lines transformed with variable efficiencies based on GUS assays. The transformation frequency obtained in the present study is also higher than those obtained in similar experiments on *P. glauca* (Le *et al.* 2001), hybrid larch (Levee *et al.* 1997) and white pine (Levee *et al.* 1999). In our earlier reports of biolistic gene transfer, for all the embryogenic lines of *P. roxburghii* tested (PR11, PR105, and PR521), the transformation frequency was very low (Malabadi and Natarja 2007a) when compared to *Agrobacterium*-mediated genetic transformation in this study. A similar difference in the transformation frequency was also noticed with biolistic gene method in other conifers (Bommineni *et al.* 1993; Ellis *et al.* 1993; Charest *et al.* 1996). Among the parameters evaluated in this study, plant genotype and physiological state of embryogenic tissue were the most important factors for the success of transformation. Another beneficial factor for the higher transformation frequency in all the tested embryogenic lines of *P. roxburghii* might be due to the addition of acetosyringone (AC; 100 µM) during the transformation. In another separate experiment, lower transformation efficiency was observed without the addition of AC (**Table 1**). AC is a low MW phenolic compound naturally released by wounded plant cells and acts as an inducer of the virulence genes. For *P. roxburghii*, the use of AC probably increased T-DNA transfer and resulted in the higher transformation frequency. In some other conifers, an increased transformation efficiency of embryogenic tissues by adding AC has been reported, such as in *P. strobes* (100 µM, Levee *et al.* 1999), *P. abies* and *P. taeda* (25-50 µM, Wenck *et al.* 1999), *P. glauca* (50 µM, Le *et al.* 2001), but in *L. kaempferi* × *L. deciduas* (100 µM, Levee *et al*. 1997) and French and Portuguese genotypes of *P. pinaster* (100 µM, Trontin *et al.* 2002; Tereso *et al.* 2006), no improvement could be achieved.

The transformed state of the kanamycin-resistant embryogenic lines obtained by the *Agrobacterium* transformation method were analyzed by PCR amplification of the expected fragment band of 500 bp for the *npt*II gene, whereas no amplification was detected in the sample from untransformed tissue (**Fig. 2**). Integration of T-DNA into the genome of all the GUS/PCR-positive lines was confirmed

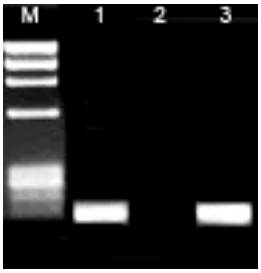

Fig. 2 PCR analysis of transformed embryogenic tissues of *P. roxburghii*. DNAs were amplified with specific primers for the *npt*II gene. M: Molecular marker. Lanes 1 and 3: Amplified DNA fragment of the *npt*II gene (500 bp) from two independent transformed lines of PR11. Lane 2: Untransformed control tissue.

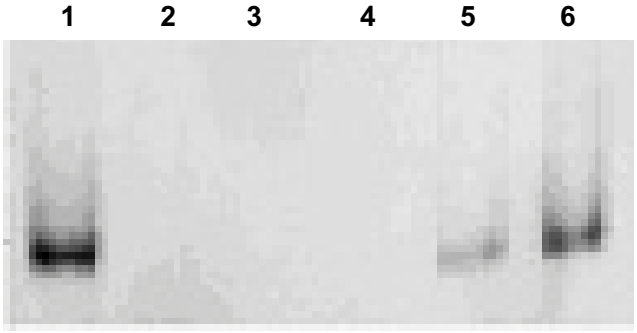

Fig. 3 Southern blot analysis of 3 putatively transformed tissue samples. Genomic DNA was digested with *Hind*III and *Eco*RI. Lanes 1, 5, 6: Genomic DNA from independently transformed tissues showing single copies of the *npt*II gene (500 bp) integrated into the genome of *P. roxburghii* PR11. Lanes 2, 3, 4: Genomic DNA from untransformed tissue (control).

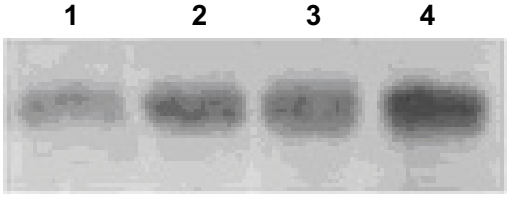

Fig. 4 Northern blot analysis of four putatively transformed somatic seedlings showing PCR/Southern positive signal. Lanes 1-4: RNA from 4 transgenic seedlings showing integration of the *npt*II gene into the genome of *P. roxburghii* PR11.

by Southern blot analyses (**Fig. 3**). Genomic DNA was digested with *Eco*RI and *Hind*III recognizing separate sites within the T-DNA. Transformed lines contained at least one gene copy of the T-DNA inserted in different loci. No hybridization signal was detected in non-transformed embryogenic tissue (**Fig. 3**). These stably transformed cultures and plants exhibited expression of GUS/PCR/Southern blot-positive signal were also confirmed by Northern blot analysis (**Fig. 4**). Transient expression of the *uid*A gene has also been observed in both loblolly pine (*Pinus taeda*) and Norway spruce (*Picea abies*) and transformed embryogenic tissues were obtained from Norway spruce (Wenck *et al.* 1999). The method of transformation had no effect on the integration pattern of T-DNAs. In the present study, however, the majority of the transgenic lines had a relatively simple T-DNA integration pattern; a case also noted for *Pinus strobus* (Levee *et al.* 1999), *Picea abies* (Wenck *et al.* 1999), and *Picea glauca*, *P. mariana* and *P. abies* (Klimaszewska *et al*. 2001).

The percentage of somatic embryogenesis was not similar in all the three transgenic genotypes of *P. roxburghii* (**Table 1**). The highest percentage of somatic embryogenesis (42%) was recorded in PR11, with a total number of 23 transgenic somatic seedlings (**Fig. 5**) recovered per gram fresh weight of transgenic tissue compared against control 1 (**Table 1**). On the other hand, in the remaining two genotypes (PR105 and PR521), the transgenic tissue showed decreased maturation potential. However, PR521 showed a lower percentage (31%) of somatic embryogenesis, with a total of 15 somatic seedlings recovered per gram fresh

Table 3 Recovery of transgenic seedlings following *Agrobacteriuum*-mediated genetic transformation of embryogenic tissue in three genotypes of *Pinus roxburghii*. This table represents the comparison between control (non-transgenic) and transgenic tissue.

Embroygenic lines tested	Somatic embryogenesis (%)	Somatic embryos recovered / g fresh wt of embryogenic tissue	Somatic embryos germinated / g fresh wt of embryogenic tissue	Somatic seedlings recovered / g fresh wt of embryogenic tissue
PR11	*42.0 ± 3.1 b	33.0 ± 3.4 b	27.0 ± 1.9 c	23.0 ± 1.2 c
Control 1	71.0 ± 2.4 a	42.0 ± 3.1 b	36.0 ± 2.9 b	26.0 ± 1.8 c
PR105	26.0 ± 3.4 c	14.0 ± 2.8 c	12.0 ± 1.4 c	9.0 ± 0.1 c
Control 2	56.5 ± 6.4 a	20.0 ± 3.0 c	15.0 ± 2.0 c	13.0 ± 0.6 c
PR521	31.0 ± 3.0 b	23.0 ± 3.1 c	18.0 ± 2.5 c	15.0 ± 1.9 c
Control 3	73.3 ± 3.4 a	31.0 ± 2.3 b	26.0 ± 2.0 c	23.0 ± 1.4 c

PR11, PR105, PR521- Transgenic seedlings (% of somatic embryogenesis in transgenic lines = 5 g of transgenic tissue of each embryogenic lines was taken aseptically and chopped into 100 pieces and subcultured on the selection medium for the growth of callus. Out of 100 pieces, growth of the number of pieces was recorded and calculated in terms of percentage of SE).
Control 1, 2, 3: Non-transgenic seedlings
*Mean (±SE) followed by the same letter in each column were not significantly different at P≤0.05

Fig. 5 Transgenic *P. roxburghii* PR11 somatic embryos at various developmental stages on maturation medium as seen under a dissecting microscope. Bar = 9.3 mm.

weight of transgenic tissue against control 3 (**Table 3**). Least (26%) somatic embryogenesis was recorded in PR105, with a total of nine somatic seedlings recovered per gram fresh weight of transgenic tissue compared against control 2 (**Table 3**). These results suggest that maturation ability was partially lost with extended time in culture, a phenomenon that was also observed in control tissues from the same embryogenic lines used in this work. This was also frequently observed in *P. radiata* embryogenic lines maintained for 12-18 months in culture (Walter *et al.* 1998), and in Portuguese *P. pinaster* embryogenic lines (Tereso *et al.* 2006). In our previous reports with the same embryogenic lines (PR11, PR105, PR521) by using biolistic gene transfer, the percentage of somatic embrygenesis was very low and also the recovery of transgenic seedlings was also poor compared to the present *Agrobacterium*-mediated transformation (Malabadi and Nataraja 2007a). This also confirmed the superiority of *Agrobacterium*-mediated gene transfer over biolistic gene transfer method (Malabadi and Nataraja 2007a, 2007b, 2007c). The mature somatic embryos obtained from transformed lines were all GUS/PCR/Southern-positive. After 10 to 14 weeks of maturation, the advanced cotyledonary somatic embryos were picked up for germination. After 4-6 weeks on germination medium, the transgenic plantlets were recovered and hardened. Furthermore, the transgenic seedlings of PR105 showed very poor growth and could not survive and were discarded. On the other hand, transgenic plants of PR11 and PR521 survived, and showed normal growth compared to control plants. The histochemical GUS assay performed on mature somatic embryos, whole somatic seedlings and needles of transgenics confirmed the expression of the GUS gene during the process of plant regeneration. GUS/PCR was found to be negative with non-transformed control plants.

In conclusion, a simple and efficient transformation procedure has been developed for embryogenic tissue of *P. roxburghii* using disarmed *Agrobacterium* strain EHA105 containing copies of *vir*B, *vir*C and *vir*C genes from the supervirulent plasmid pToK47. Foreign gene transfer, integration and expression were successfully achieved, and confirmed by Southern and Northern blot analysis. This is the first report of *Agrobacterium*-mediated T-DNA integration in *P. roxburghii* using embryogenic lines derived from apical meristematic tissue of mature trees. This procedure will therefore, permit Chir pine (*P. roxburghii*) improvement via genetic engineering and facilitate physiological studies through the use of genetic manipulation.

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