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Production of Transgenic Plants via *Agrobacterium tumefaciens*-Mediated Genetic Transformation in *Pinus wallichiana* (Himalayan Blue Pine)

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ABSTRACT

We established for the first time an *Agrobacterium*-mediated genetic transformation system for the Himalayan blue pine. Embryogenic tissue derived from vegetative shoot apices of mature trees of *Pinus wallichiana* were inoculated with *A. tumefaciens* strain EHA105 fused with a binary vector pBI121. The plasmid pBI121, containing the neomycin phosphotransferase II (*npt*II) gene providing kanamycin resistance as a selectable marker and the *β*-glucuronidase (*uid*A) reporter gene, was used in the transformation studies. GUS activity was used to monitor transient expression of the *uid*A gene and to further test lines selected on kanamycin-containing medium. The integration of the transgene (*npt*II) was confirmed by PCR followed by southern and northern blot analyses. These results demonstrated that a stable and enhanced transformation system could be established in *P. wallichiana*. This provides an opportunity to transfer economically important genes into Himalayan blue pines.

Keywords: India, mature trees, somatic embryos, vegetative shoot apices, Western Ghat forest, wood

INTRODUCTION

Wood is the most abundant biological material on earth and also a raw material for a major global industry whose demand is increasing rapidly. Considering the increase of world demand for wood, improving wood quality to better fit industrial requirements becomes a major objective for tree breeders (Pena and Seguin 2001; Grant *et al*. 2004; Grace *et al*. 2005). Hitherto, breeding for wood traits has been hampered by the cost of traditional assays, and the need to wait until the trees are nearly mature to be valuated (Poupin and Arce-Johnson 2004). Tree improvement is a long term and costly process which implies the addressing of many problems (Tzfira *et al*. 1998). Biotechnology offers a strategy for addressing this challenge. The recent development of molecular tools for genomic analyses of woody species makes it possible to introduce foreign genes into plants controlling wood traits. The sufficient production of superior quality wood without disturbing primary forest will be one of the most important issues for the near future. A range of targets are of interest for genetic engineering in trees, e.g. lignin and/or cellulose modification, pest resistance, and tolerance to abiotic stress (Tang and Newton 2003; Tereso *et al*. 2006; Malabadi and Nataraja 2007a, 2007b, 2007c).

Genetic transformation has become one of the important research tools in plant biology (Birch 1997; Hansen and Wright 1999; Malabadi and Nataraja 2003; Malabadi 2006; Malabadi and Nataraja 2007e, 2007f). Most conifers have been considered to be recalcitrant to *Agrobacterium*-mediated transformation. The number of species in this category is gradually being reduced as developments in methodology occur (Grant *et al*. 2004). Introduction of foreign genes through the co-cultivation of cells or organ explants with *A. tumefaciens* has been considered as the most effective method due to its relative simplicity and efficiency and simple transgene integration pattern, compared to microprojectile transformation (Birch 1997; Grant *et al*. 2004; Tereso *et al*. 2006). Efficient and reproducible transformation systems have been reported in few conifers of *Pinus strobus* (Levee *et al*. 1999; Tang *et al*. 2007), *Picea glauca*, *Picea mariana*, *Picea abies* (Klimaszewska *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), *Pinus taeda* (Wenck *et al*. 1999), *Pinus pinaster* (Tereso *et al*. 2006), *Larix kaemperi* × *L. deciduas* (Levee *et al*. 1997), *Picea glauca*, *Picea mariana*, loblolly pine, (*Pinus taeda*) (Tang *et al*. 2001), Douglas fir (*Pseudotsuga menziesii*) (Dandekar *et al*. 1987), and Norway spruce (*Picea abies*), *Pinus nigra* (Lopez *et al*. 2000).

Pinus wallichiana AB. Jacks (Himalayan Blue pine or Bhutan pine) is a native of the outer Himalayas and prevalent in the Northern Himalayan range. Himalayan blue pine or Bhutan pine is an important indigenous pine species in India, Bhutan and Nepal (Malabadi and Nataraja 2007d). Genetic transformation via particle bombardment using mature zygotic embryos has been reported in *P. wallichiana* (Malabadi and Nataraja 2007c). No successful reports of *Agrobacterium*-mediated genetic transformation have been reported in *P. wallichiana*. Methods for the regeneration of plantlets from embryogenic tissue derived from vegetative shoot apices of mature trees of many conifers (Malabadi *et al*. 2004; Malabadi and van Staden 2005a, 2005b, 2005c; Malabadi and Nataraja 2006; Malabadi and van Staden 2006; Malabadi and Nataraja 2007g) including *P. wallichiana* (Malabadi and Nataraja 2007d), and somatic embryogenesis in *P. sylvestris* (Scots pine) (Aronen *et al*. 2007) have been well established.

This paper highlights for first time the successful delivery and expression of the neomycin phosphotransferase gene II (*npt*II) providing resistance to kanamycin, and transient expression of the *β*-glucuronidase (GUS) gene

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(*uid*A) into *P. wallichiana* genome using embryogenic tissue derived from the vegetative shoot apices of mature trees. Results described in this paper provide useful information for the establishment of an enhanced and efficient *A. tumefaciens*-mediated transformation system for future stable integration of economically important genes into Himalayan blue pine species.

MATERIALS AND METHODS

Plant material and initiation of embryogenic tissue

Apical shoots from mature trees (13 year-old) of *Pinus wallichiana* AB. Jacks of 3 genotypes (PW10, PW39 and PW120) 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 85 cm) (Malabadi and Nataraja 2007d). They were washed with 1% citramide (sodium hypochlorite 3.5%) for 5 min and rinsed thoroughly with sterilized distilled water. They were then surface decontaminated with 70% ethanol for 5 min followed by immersion in 0.5% $HgCl_2$ for 2 min and rinsed 4-times with sterile double distilled water. Transverse-thin sections, approximately 0.5-1.0 mm thick, were cut using a sharp sterilized blade or a scalpel from apical shoots (upper part with 2 to 3 thin-sections only) for the initiation of embryogenic tissue. These apical- shoot-sections were cultured individually on full strength inorganic salts DCR (Gupta and Durzan 1985) basal medium containing 0.2 g l^{-1} polyvinyl pyrollidine (PVP) (Sigma, USA), 2 g l^{-1} Gellan gum (Sigma), 30 g l^{-1} maltose (Sigma) and 0.3% activated charcoal (Sigma) without growth regulators (Pre-culture medium I). The initiation medium (II) was supplemented with 10% of smoke-saturated water, and embryogenic tissue was initiated as described previously (Malabadi and Nataraja 2007d).

Agrobacterium strain and culture conditions

A. tumefaciens strain EHA105 harboring binary vector pBI121 which contains the *nptII* gene providing resistance to kanamycin and the GUS gene (*uidA*) 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 EHA105 (Hood *et al*. 1993) containing 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 2007e, 2007f). The bacterial cells were thereafter pelleted by centrifugation at 4,000 rpm for 10 min and resuspended in liquid DCR basal medium (Maintenance medium) (II) (Malabadi and Nataraja 2007d) to an optical density ($OD_{600\ nm}$) of 0.6.

Agrobacterium-mediated transformation procedure

This method was adapted from the protocol described by Klimaszewska *et al*. (2001). Prior to transformation experiments, the embryogenic suspension cultures of 3 embryogenic lines (PW10, PW39 and PW120) were grown in 125 ml Erlenmeyer flasks containing 50 ml of liquid maintenance DCR medium (Malabadi and Nataraja 2007d). They were subcultured every week at a ratio (1:5 (v/v) in 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 ($OD_{600\ nm}$) of 0.3 in liquid maintenance medium in a 125 ml Erlenmeyer flask. In all the embryogenic lines, the addition of 100 µM acetosyringone to the bacterial suspension just before 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). Subsequently, 3 ml of the culture (150 mg fresh mass), poured over a 5.5 cm sterile filter paper, was applied to drain the liquid and the filter paper was placed on a semi-solid DCR basal maintenance medium in a 55 mm Petri dish. Co-cultivation was carried out for 3 days under the conditions described above. There were a total of 10 Petri dishes of each tested embryogenic line.

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 (Malabadi and Nataraja 2007d) with 470 mg l^{-1} cefotaxime (Sigma) (non-washed). 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 (Malabadi and Nataraja 2007d). 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 (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 semisolid DCR maintenance medium supplemented with 35 mg l^{-1} kanamycin. The putatively transformed kanamycin-resistant tissues were isolated and maintained for at least 5 subcultures in the presence of cefotaxime.

β-glucuronidase 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; Sigma). Plant cells and tissues were scored as GUS-positive for the *uid*A gene if any deep indigo blue color was present.

PCR analysis of *npt*II

Genomic DNA was extracted from putatively 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: 5′-ACTGTC CCCTAGT-GGGGAAGGGGACTGGCTGCTATT-3′ and 5′-GAT ACCGTACGCCCAAGCGCAGGTCAG-3′. 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 10X 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 at 95°C, followed by 34 cycles (denaturation for 1 min at 94°C, annealing for 1 min at 57°C, and extension for 2 min at 72°C). Cycling was followed by final extension cycle of 72°C for 5 min. All these conditions were applied for amplification of *npt*II only. Samples were then stored at 4°C. Amplified DNA was detected by ultraviolet light after electrophoresis on 0.8% agarose/ethidium bromide gels using 1X 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 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), which was obtained by digestion of pBI121 with *Hind*III and *Eco*RI, was labeled with digoxigenin-11-dUTP in the PCR conditions according to the Roche Diagnostics protocol. After overnight hybridization, the blots were washed twice with 2X 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 0.5X SSC, 0.1% SDS at 68°C for 15 min. The chemiluminescence detection of hybridization products was performed according to the manufacturer's (Roche Biochemicals) instructions.

RNA isolation

Needle samples were collected from the control and Southern-positive transgenic *P. wallichiana* plants (10-12 week-old 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 a modified method of Chang *et al*. (1993).

Northern blot analysis

Northern-blot analyses were performed to confirm the presence of transgene in the *P. wallichiana* genome. Fifteen µg of total RNA was separated on 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 20X SSC overnight. After blotting, the membrane was washed twice in 2X SSC at room temperature for 10 min and cross linked 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 0.1X 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 hours. For hybridization, a fresh Easy Hyb Solution containing denatured *npt*II probe for the detection of the corresponding mRNAs (500 bp) was used. The probe used for detection of the *npt*II mRNA was the same one as applied in Southern blot analyses, and was labeled with digoxigenin-11-dUTP by PCR using the set of primers as used for PCR. After overnight hybridization at 50°C, the blots were washed twice with 2X 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 0.2X SSC, 0.1% SDS at 68°C for 15 min. The chemiluminescence detection of hybridization products was performed according to the 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 dark at room temperature. One gram fresh weight of transgenic tissue of each embryogenic line was transferred to sterile empty Petri dishes (60 mm) containing two sterile Whatman filter (Schleicher and Schuell, qualitative circles) paper disks (55 mm). 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 ABA and 5 g l^{-1} Gellan gum (III) was used for this purpose. All the cultures were placed in the dark at 25 ± 2°C and these were maintained for 8 to 12 weeks (Malabadi and Nataraja 2007d).

Germination and plantlet recovery

After 12 weeks of maturation in presence of ABA and higher concentrations of 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 the dark for 4 weeks. The germination medium (IV) used was half DCR medium with 2 g l^{-1} Gellan gum (Malabadi and Nataraja 2007d). In the first week of germination, cultures were kept in darkness, and then transferred to diffuse light in the second week. Thereafter followed 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 plantlet 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 experiment was made up of two replicates (total 100 cultures for one experiment) for each genotype (PW10, PW39 and PW120) of *P. wallichiana*. All the experiments were repeated 3 times and **Tables 1-3** represent the average of 3 independent experiments. Data presented in **Tables 1-3** 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 a Duncan's multiple range test ($\alpha=0.05$) following ANOVA. All statistical analyses was performed using the SPSS statistical software package.

RESULTS AND DISCUSSION

Research with conifers has benefited from the development of *Agrobacterium tumefaciens*-mediated transformation methods (Wenck *et al*. 1999; Tang *et al*. 2007; Trontin *et al*. 2007). However, Himalayan blue pine is one of the commercially important coniferous species that has not received attention relative to its genetic engineering. That is precisely why this transformation study was conducted using our most reliable tissue culture regeneration method (Malabadi and Nataraja 2007d). In the present study transgenic plants were obtained via *Agrobacterium*-mediated genetic transformation of embryogenic tissue derived from vegetative shoot apices of mature trees of *P. wallichiana*. During the transformation events, an infection period of 5 h was found to be optimum for the embryogenic lines tested (**Table 1**). There was no significant effect on the growth of cultures during 3-d co-cultivation of cultures. Bacterial growth was inhibited by culture onto a medium with higher concentration (470 mg l^{-1}) of cefotaxime. The rest of the bacteria might be eliminated due the washing of embryogenic tissue with liquid medium. This was also observed in other conifers such as *P. glauca*, *P. mariana* and *P. abies* (Klimaszewska *et al*. 2001, 2007; Vooková and Kormuťák 2007). A lower concentration of cefotaxime has not significantly affected the bacterial growth compared to control without antibiotic (data not shown (**Table 2**). The optimum concentration of cefotaxime (470 mg l^{-1}) was crucial for the recovery of embryogenic tissue growth after co-cultivation. Therefore, the technique developed here is robust and genotype-independent. It was also reported that higher concentration of cefotaxime inhibited the growth of bacteria during selection of embryogenic lines of *P. glauca*, *P. mariana* and *P. abies* (Klimaszewska *et al*. 2001, 2007). On the other hand the post-cocultivation of embryogenic tissue in all the embryogenic lines tested that was not washed, but transferred directly with filter papers onto the medium with cefotaxime did not show any abundant bacterial growth. The antibiotic cefotaxime at 470 mg l^{-1} was required for at least

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

Embryogenic lines tested	*Agrobacterium*-infection period (h)	Acetosyringone (µM)	GUS spots/g fw of transgenic tissue	Recovery of transgenic colonies/g fw of tissue
PW10	0	0	0.0 ± 0.0 c	0.0 ± 0.0 c
	1	25	2.0 ± 0.1 b	0.0 ± 0.0 c
	2	50	2.0 ± 0.1 b	0.0 ± 0.0 c
	3	70	4.0 ± 0.1 b	2.0 ± 0.1 b
	4	80	6.0 ± 0.4 b	3.0 ± 0.2 b
	5	100	14.0 ± 1.3 b*	16.0 ± 2.4 b*
	6	150	5.0 ± 0.2 b	1.0 ± 0.0 b
PW39	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	2.0 ± 0.1 b	1.0 ± 0.1 b
	4	80	2.0 ± 0.1 b	3.0 ± 0.2 b
	5	100	57.0 ± 4.0 a*	43.0 ± 2.8 a*
	6	150	13.0 ± 1.6 b	8.0 ± 0.5 b
PW120	0	0	0.0 ± 0.0 c	0.0 ± 0.0 c
	1	25	1.0 ± 0.1 b	0.0 ± 0.0 c
	2	50	5.0 ± 0.1 b	1.0 ± 0.0 c
	3	70	8.0 ± 0.1 b	1.0 ± 0.1 b
	4	80	16.0 ± 1.4 b	2.0 ± 0.1 b
	5	100	36.0 ± 3.0 a*	30.0 ± 2.0 a*
	6	150	9.0 ± 1.2 b	3.0 ± 0.2 b

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

Definition of a transgenic colony: 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.

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

Embryogenic lines tested	Cefotaxime (mgl^{-1})	Kanamycin (mgl^{-1})	Recovery effect during selection	№ of transgenic colonies recovered/g fw of tissue
PW10	0	0	–	0.0 ± 0.0 c
	100	5	–	0.0 ± 0.0 c
	250	15	–	0.0 ± 0.0 c
	350	25	–	2.0 ± 0.1 b
	400	30	+	3.0 ± 0.2 b
	470	35	++++	16.0 ± 2.4 b*
	600	50	–	0.0 ± 0.0c
PW39	0	0	–	0.0 ± 0.0 c
	100	5	–	0.0 ± 0.0 c
	250	15	–	0.0 ± 0.0 c
	350	25	–	1.0 ± 0.1 b
	400	30	+	3.0 ± 0.2 b
	470	35	++++	43.0 ± 2.8 a*
	600	50	–	8.0 ± 0.5 b
PW120	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	+	2.0 ± 0.1 b
	470	35	++++	30.0 ± 2.0 a*
	600	50	–	3.0 ± 0.2 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.

Definition of a transgenic colony: See **Table 1**.

3 subcultures to ensure complete elimination of bacteria. Alternately, a transgenic colony could arise that would be a mixture of cells representing different transformation events. To eliminate these possibilities cultures were not washed after cocultivation in other conifer species (Klimaszewska *et al*. 2001). The cocultivation procedure with *A. tumefaciens*, particularly the post-coculture treatment of the embryogenic tissue played a significant role in the recovery of transgenic tissue (Klimaszewska *et al*. 2001; Le *et al*. 2001; Tang *et al*. 2007; Trontin *et al*. 2007). Subsequent subculture onto selective medium with kanamycin was done only after the first sign of growth of the embryogenic tissue, which was determined visually. The timing of selection was also important and varied among the species. Kanamycin sensitivity appears to depend on the explant and species (Klimaszewska *et al*. 2001, 2003, 2007).

On the basis of preliminary studies (**Table 2**), the best kanamycin concentration for selection of transgenic tissue was 35 mg l^{-1} kanamycin. Kanamycin is an aminoglycosidase derivative antibiotic and is widely used to select *npt*II transformed cells (Terakami *et al*. 2007). During the first 2 weeks of period, 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 (Le *et al*. 2001; Klimaszewska *et al*. 2001; Tereso *et al*. 2006).

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. In other reports, the frequency of transformation was calculated by dividing the number of genetically independent transformants by the number of infected explants. This method holds good for the explants like cotyledon/leaf explants or embryo, when used directly instead of callus for the transformation events (Nishiguchi *et al*. 2006). In our present study, we have not applied this method since our starting material for the transformation study was embryogenic tissue. Therefore, we have counted the number of GUS-blue color spots in one gram fresh wt of transgenic tissue (**Table 1; Fig. 1**). As evidenced by blue staining, all the transgenic lines expressed the *uid*A gene in all the developmental stages of embryogenic tissue (**Fig. 1**); however staining intensity varied among the lines, ranging from pale blue to a more intense blue in developing embryos (**Fig. 1**). This variation in staining intensity has also been observed previously in *P. radiata* (Walter *et al*. 1999; Grace *et al*. 2005) and *P. abies* (Walter *et al*. 1999). The viability of cultures, bacterial strains and cocultivation conditions were all important in making the cells accessible to the *Agrobacterium* binding, T-DNA transfer, and integration into a cell genome (Wenck *et al*. 1999; de Buck *et al*. 1999, 2000; Lelu and Pilate 2000; Klimaszewska *et al*. 2001). These results are in agreement with Klimaszewska *et al*. (2001), Tereso *et al*. (2006), and Le *et al*. (2001). GUS reporter system using an exogenous substrate for histochemical visualization plays an important role in functional genomics (Tang *et al*. 2007). GUS expression can be monitored in any type of tissue. Most transformed lines showed a variable GUS intensity in blue cell aggregates (**Fig. 1**). Activity of GUS enzyme was not detected in control tissues. In other conifers a mixture of GUS positive and GUS-negative embryogenic aggregates was also observed at least in some transformed lines (Ellis *et al*. 1993; Walter *et al*. 1998; Tian *et al*. 2000; 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 *et al*. 1994; Matzke and Matzke 1998; Tereso *et al*. 2006). Gene expression could also very due to copy number effect (Cervera *et al*. 2000). A high copy number may lead to gene silencing (Matzke *et al*. 1994). In our present study, we found that a single copy number of the *npt*II gene was no-

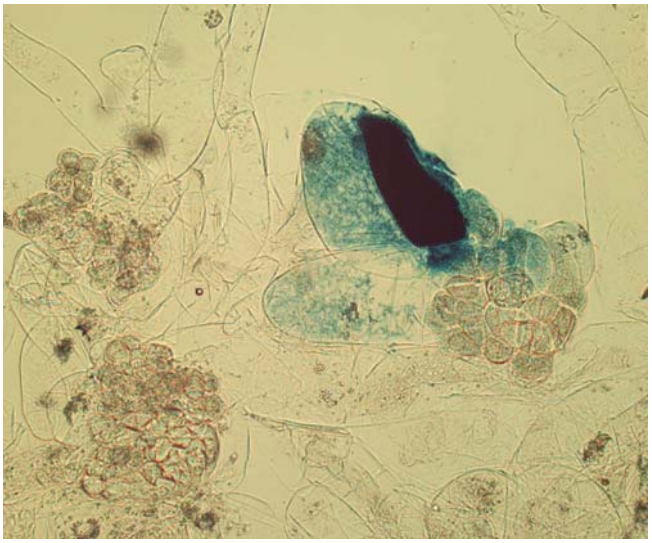

Fig. 1 Evidence for the presence of the *uid*A gene. The GUS activity was visible as dark blue to pale blue spot in the embryogenic tissue as seen under the microscope at 40X higher magnification following GUS histochemical assay.

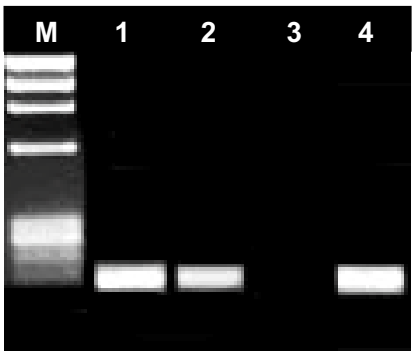

Fig. 2 PCR analysis of transformed embryogenic tissues of *P. wallichiana*. DNAs were amplified with specific primers for the *npt*II gene. M: Molecular marker. Lanes 1, 2, 4: Amplified DNA fragment of *npt*II gene (500 bp) from three independent transformed lines of PW39. Lane 3: Non-transformed control tissue of one independent line (control 2 in **Table 1**).

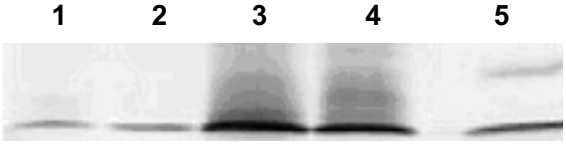

Fig. 3 Southern blot analysis of 5 putatively transformed tissue samples. Genomic DNA was digested with *Hind*III and *Eco*RI. Lanes 1-5: Genomic DNA from independently transformed tissues showing single copies of the *npt*II gene (500 bp) integrated into the genome of *P. wallichiana* PW39.

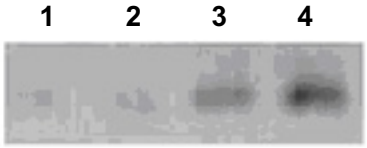

Fig. 4 Northern blot analysis of two putatively transformed somatic seedlings showing PCR/Southern positive signal. Lanes 3 and 4: RNA from 2 transgenic seedlings of PW39 lines showing the integration of *npt*II gene in the *P. wallichiana* genome. Lanes 1 and 2: No signal in untransformed seedling (control 2 in **Table 1**).

ticed in the transgenic plants as evidenced by Southern blot analysis. However, the integration of one copy of a transgene does not ensure the same level of expression in different transgenics due to the different sites of integration into the plant genome, commonly called the position effect (Matzke *et al*. 1994; Matzke and Matzke 1998; Klimaszewska *et al*. 2003; Tereso *et al*. 2006). Variability in the transgene expression levels between individual transgenic plants or cell lines is a general phenomenon described in many plant transformation studies (Walter *et al*. 1999; Charity *et al*. 2002; Klimaszewska *et al*. 2003). Due to this variability, it is necessary to screen the number of transformants for the most useful level of expression that would preferably be ascribed to the integration of one copy number of a transgene. Additionally, integration of more than one transgene copy may lead to a higher or lower expression leading to silencing mechanisms (Matzke *et al*. 1994). It is clear that for large-genome conifers, the detection of single transgene copy integrations by Southern hybridization is difficult. The alignment of PCR and Southern results is not stringent, and reliance on PCR alone may lead to the false identification of putative transgenics. On the basis of the present study and other published investigations, it appears difficult to establish a true relation between transgene copy number and expression level in our transformed plant material.

The highest transformation efficiency (highest number of GUS-blue-color spots) was obtained in the embryogenic line PW39 (57 transformed lines/g fresh wt) than with the embryogenic lines PW10 and PW120. In PW10, the lowest transformation efficiency was recorded (14 transformed lines/g fresh wt). On the other hand PW 120 showed 36 transformed lines/g fresh wt. These results suggest that the success of the transformation process in *P. wallichiana* embryogenic lines is genotype-dependent. These results are consistent with that in the transformation of 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. 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, all the embryogenic lines obtained from zygotic embryos of *P. wallichiana* tested (PW145, PW21 and PW106) (Malabadi and Natarja 2007c), the transformation frequency was very low as compared against the present study of *Agrobacterium*-mediated genetic transformation in *P. wallichiana*. A similar difference in the transformation frequency was also noticed with biolistic gene method in other conifers (Ellis *et al*. 1993; Bommineni *et al*. 1993; Charest *et al*. 1996). These results suggest that higher transformation frequency might be due the *Agrobacterium* strain EHA105. Several studies have reported that strain EHA105 was found to be the most effective one for the transformation studies in Italian stone pine (*P. pinea*) (Humara *et al*. 1999), and *P. glauca* (Le *et al*. 2001). This strain is more infectious than other strains because EHA105 is a disarmed derivative of the supervirulent strain A281 (Hood *et al*. 1993), and also it is very difficult to eliminate EHA105 from plant tissues (Terakami *et al*. 2007). Another beneficial factor for the higher transformation frequency in all the tested embryogenic lines of *P. wallichiana* might be due to the addition of acetosyringone (100 µM) during transformation. For *P. wallichiana*, the use of acetosyringone probably increases T-DNA transfer and resulted in the higher transformation frequency. In another separate experiment, lower transformation efficiency was observed without addition of acetosyringone (**Table 2**). In some other conifers, an increased transformation efficiency of embryogenic tissues by adding acetosyringone has been reported, such as in *P. strobus* (100 µM, Levee *et al*. 1999), *P. abies* and *P. taeda* (25-50 µM, Wenck *et al*. 1999), *P. glauca* (Le *et al*. 2001, 50 µM), but in *L. kaempferi* × *L. deciduas* (Levee *et al*. 1997, 100 µM), and French with Portuguese genotypes of *P. pinaster* (Trontin *et al*. 2002; Tereso *et al*. 2006, 100 µM), no improvement was noted.

No PCR amplification was detected in the sample from

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

Embroygenic lines tested	Somatic embryogenesis (%)	Somatic embryos recovered/g fw of embryogenic tissue	Somatic embryos germinated/g fw of embryogenic tissue	Somatic seedlings recovered/g fw of embryogenic tissue
PW10	7.0 ± 0.2 b	23.0 ± 1.2 a	14.0 ± 0.6 b	9.0 ± 0.2 b
Control 1	13.0 ± 1.2 b	37.0 ± 1.5 a	35.0 ± 2.4 a	30.0 ± 2.7 a
PW39	11.0 ± 0.4 b	20.0 ± 1.7 a	10.0 ± 0.4 b	7.0 ± 0.1 b
Control 2	21.0 ± 1.4 a	34.0 ± 3.7 a	31.0 ± 2.5 a	28.0 ± 2.0 a
PW120	16.0 ± 1.0 b	26.0 ± 2.1 a	22.0 ± 1.5 a	12.0 ± 1.0 b
Control 3	27.0 ± 2.1 a	39.0 ± 2.4 a	33.0 ± 2.1 a	27.0 ± 1.3 a

PW10, PW39 and PW120: Transgenic seedlings (% of somatic embryogenesis in transgenic lines = 5 g of transgenic tissue of each embryogenic line was taken aseptically and chopped into 100 pieces and sub-cultured 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).
Controls 1, 2, 3: Non-transgenic seedlings
Means (±SE) followed by the same letter in each column were not significantly different at P≤0.05 using DMRT.

untransformed tissue (**Fig. 2**) although integration of T-DNA into the genome of all the GUS/PCR-positive lines was confirmed by Southern blot analyses (**Fig. 3**). The kanamycin-resistant embrygenic lines were analyzed by PCR amplification of the expected fragment band of 500 bp for the *npt* II gene (**Fig. 2**). Genomic DNA was digested with *Eco*RI recognizing sites within the T-DNA and *Hind*III with other recognizing sites within the T-DNA. Transformed lines contained at least one gene copy of the T-DNA inserted in different loci. It was well reported that when T-DNA is introduced into plant cells a wound response is elicited, which involves the activation of nucleases and DNA repair enzymes that maintain the integrity of the host genome (Grant *et al*. 2004). The extra T-DNA tends to be integrated into the same position as the first copy-a hotspot-and separated by genomic filler DNA (Kohli *et al*. 1998; de Buck *et al*. 2000; Kumar and Fladung 2000; Grant *et al*. 2004). Usually one intact copy is integrated by illegitimate recombination. A single intact copy may be accompanied by a variable number of extra copies, which may be rearranged into head-to-head or head to tail concentration, incomplete copies and/or truncated fragments (Grant *et al*. 2004). Models of this process have been developed, although a detailed understanding of the molecular events that occur during the interaction of *Agrobacterium* within plant cells is still not well understood (Tinland 1996; de Buck *et al*. 1999; Vergunst and Hooykaas 1999; Gelvin 2000). These stably transformed cultures and plants exhibited expression of GUS/PCR/Southern blot-positive signal were also confirmed by northern blot analysis (**Fig. 4**). The method of transformation had no effect on the integration pattern of T-DNAs. Transient expression of the *uid*A gene has also been observed in our present study (**Fig. 1**), and in both loblolly pine (*P. taeda*) and Norway spruce (*P. abies*), and transformed embryogenic tissues were obtained from Norway spruce (Wenck *et al*. 1999). In the present study, however, the majority of the transgenic lines had a relatively simple T-DNA integration pattern; a case also noted for *P. strobus* (Levee *et al*. 1999), *P. abies* (Wenck *et al*. 1999), and *P. glauca*, *P. mariana* and *P. abies* (Klimaszewska *et al*. 2001, 2003, 2007).

The percentage of somatic embryogenesis in all the tested transgenic genotypes was not similar (**Table 3**). The total number of transgenic seedlings recovered per gram fresh wt of embryogenic tissue was also found very low when compared against control (**Table 3**). A total number of 9 somatic seedlings recovered per gram fresh weight of transgenic tissue in a genotype PW10 (**Table 3**). The highest percentage of somatic embryogenesis (16%) was recorded in a genotype PW120 with a total number of 12 transgenic somatic seedlings recovered per gram fresh weight of transgenic tissue (**Table 3**). Genotype PW39 showed 11% of somatic embryogenesis, with a total number of 7 somatic seedlings recovered per gram fresh weight of transgenic tissue (**Table 3**). Maturation potentiality of transgenic tissues was very poor in all the tested embryogenic lines. 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 other embryogenic lines from zygotic embryos of *P. wallichiana* (PW145, PW21 and PW106) by using biolistic gene transfer, the percentage of somatic embryogenesis was very low and transgenic seedlings were recovered only in one genotype PW145 (Malabadi and Nataraja 2007c). In the remaining two genotypes, transgenic tissues lost their maturation potential and failed to produce somatic embryos on maturation medium (Malabadi and Nataraja 2007c). The reason why the transgenic lines by biolistic gene transfer method were unable to regenerate somatic embryos remains unclear. This phenolmenon of non-regeneration of plants from some transgenic lines was also observed in *P. radiata* (Walter *et al*. 1998). The mature somatic embryos obtained from transformed lines were all GUS/PCR/Southern/Northern-positive (**Figs. 1-4**). The advanced cotyledonary somatic embryos were selected for germination (**Fig. 5**). GUS/PCR was found negative with non-transformed control plants. After 4 weeks the transgenic plantlets were recovered and hardened. During the first 4 weeks, the transgenic plants of tested embryogenic lines showed poor growth. Growth was regained after 6 weeks.

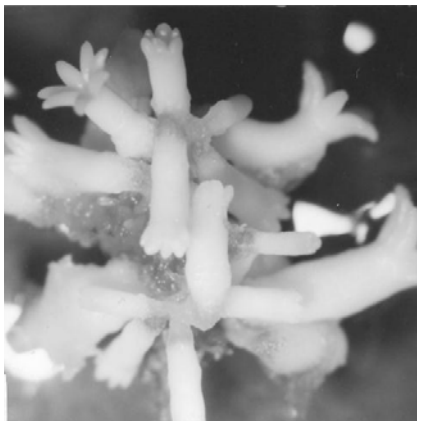

Fig. 5 Transgenic *P. wallichiana* PW39 somatic embryos at various developmental stages on maturation medium.

In this paper, we have demonstrated for the first time that *Agrobacterium*-mediated transformation is a viable means of transforming *P. wallichiana*. Transformation using *Agrobacterium* has several advantages over previous methods such as biolistics. By this method, usually no rearrangements occur during transgene integration. Additionally, the insert is often a single insertion event. The genetic transformation protocol developed in the present study can be successfully applied to many genotypes of *P. wallichiana*. This technique is very useful to add to the suite of plant gene technologies designed to improve the characteristics of Himalayan blue pine for plantation forestry.

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