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Agrobacterium tumefaciens-Mediated Genetic Transformation of *Pinus kesiya* Royle ex Gord (Khasi Pine)

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

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

Corresponding author: * **ravindra. malabadi@forestry.ubc.ca**

ABSTRACT

This study highlights, for first time, an *Agrobacterium*-mediated gene transfer method for the genetic improvement of *Pinus kesiya*. The genetic transformation of *P. kesiya* has been limited by difficulty in selection efficiencies and a low transformation frequency. Embryogenic cultures were established from zygotic embryos according to our previous protocol (Malabadi *et al.* 2005). During transformation events, rapidly growing embryogenic tissue of three genotypes were co-cultivated with disarmed *A. tumefaciens* strain EHA105 fused with a binary vector pBI121, which contains 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. All the transgenic lines exhibited very low maturation potential compared to the control. 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 one of the transgenes, *npt*II, was confirmed by PCR followed by Southern and Northern blot analyses. *Agrobacterium*-mediated gene transfer was found to be a very useful technique for large-scale generation of transgenic *P. kesiya*, and may prove useful for other recalcitrant conifer species.

Keywords: DNA transformation, somatic embryogenesis, zygotic embryos
Abbreviations: ABA, abscisic acid; **AS**, acetosyringone; **GM**, germination medium; IM, initiation medium; **MM-I**, maintenance medium; **MM-II**, maturation medium

INTRODUCTION

Genetic transformation of woody plants is a very important technique for their genetic improvement (Dunwell 2000; Grace *et al.* 2005; Malabadi and Nataraja 2007a, 2007b, 2007c, 2007f, 2007g). Traditional breeding has been widely used in forestry. However, this technique is inefficient because trees have a long and complex life cycle that is not amenable to strict control by man (Poupin and Johnson 2004). Some desirable traits of commercial value, such as insect and herbicide resistance, are available neither in the breeding population nor in the genetic resource (Grace *et al.* 2005). Improved genetic engineering and tissue cultue technologies now offer alternative methodologies for the fast and efficient introduction of desirable traits directly into genotypes from elite families (Dunwell 2000; Grace *et al.* 2005; Malabadi and Nataraja 2007a, 2007b, 2007c). The introduction of selected traits can be used to increase the productivity and commercial value of trees and other plants (Malabadi and Nataraja 2003; Malabadi 2006). It has also been possible to introduce genes that modify plant development, wood quality, and induce sex sterility (Birch 1997; Grant *et al.* 2004; Poupin and Johnson 2004; Malabadi and Nataraja 2007a, 2007b, 2007c). The development of transgenic trees has required the implementation of *in vitro* regeneration techniques such as organogenesis or somatic embryogenesis (Poupin *et al.* 2004). Techniques to genetically engineer most of the conifers have been successfully developed during the last decade, and the transfer of foreign genes to trees has become increasingly routine via either *Agrobacterium* or biolistic gene transfer systems (see reviews by: Tzfira *et al.* 1998; Peña and Seguin 2001; Walter 2002; Tang and Newton 2003). Genetic transformation through the co-cultivation of cells or organ explants with *Agrobacterium tumefaciens* has been the method of choice due to its relative simplicity and efficiency, and apparantly simple transgene integration pattern, compared to microprojectile transformation (see reviews by: Birch 1997; Hansen and Wright 1999 and references therein; Gelvin 2000; Klimaszewska *et al.* 2003).

Agrobacterium-mediated transformation for the introduction of genes has been reported in many conifers. However, many difficulties have been encountered in attempts to regenerate transgenic woody plants, and in many cases appropriate regeneration systems have not yet been established. Efficient and reproducible transformation systems have been reported in few conifers of Douglas fir (*Pseudotsuga menziesii*) (Dandekar *et al.* 1987), *Picea glauca* (Le *et al.* 2001), *Pinus taeda* (Wenck *et al.* 1999), *Pinus pinaster* (Tereso *et al.* 2006), Norway spruce (*Picea abies*), *Pinus nigra* (Lopez *et al.* 2000), *Larix kaemperi* × *L. deciduas* (Levee *et al.* 1997), *Picea abies* (Klimaszewska *et al.* 2001), *Pinus strobus* (Levee *et al.* 1999; Tang *et al.* 2007), *Picea glauca*, *Picea mariana*, loblolly pine (*Pinus taeda*) (Tang *et al.* 2001; Tang 2003), and *Pinus radiata* (Cerda *et al.* 2002; Charity *et al.* 2002; Grant *et al.* 2004; Charity *et al.* 2005; Grace *et al.* 2005). *Pinus kesiya* Royle ex Gord (Khasi pine) is one of the most important pines of North-Eastern region of India. The wood is mainly used in the paper industry, for building construction and plays an important role in commercial forestry. *In vitro* regeneration system using mature, immature, and apical meristematic tissue of mature trees have been well established in *P. kesiya* (Malabadi *et al.* 2002, 2003, 2004, 2005). We have previously reported the genetic improvement of *P. kesiya via* biolistic gene transfer method (Malabadi and Nataraja 2007b). However, this

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direct procedure of transformation generally resulted in multiple copies of genes and gene rearrangements and in high frequency of sterile plants (Finnegan and McElroy 1994; Flavell 1994) and sometimes in a non-Mendelian inheritance of transgens(s) (Scheid *et al.* 1991; Peng *et al.* 1995; Le *et al.* 2001). At present, there are no reports of gene transfer using *A. tumefaciens* in *P. kesiya* available in the literature. This paper highlights the *Agrobacterium*-mediated transformation for the expression of both the neomycin phosphotransferase gene II (*npt*II) providing resistance to kanamycin, and the β-glucuronidase (GUS) gene (*uid*A) into the *P. kesiya* genome.

MATERIALS AND METHODS

Plant material

Seeds of three genotypes (PK405, PK04 and PK214) of open-pollinated *Pinus kesiya* (Royle ex. Gord) trees were collected from the Forest Department, East Khasi Hills, Shillong, Meghalaya, India. Seeds were surface cleansed with 1% citramide (Sigma) for 2 min, and washed thoroughly with sterilized distilled water three times. Seeds were further treated with sodium hypochlorite solution (Sigma) (4-5% available chlorine) for 2 min, rinsed 5 times with sterile distilled water and treated with 6% hydrogen peroxide (Sigma) for 24 h. Prior to dissection of embryos, seeds were surface decontaminated sequentially with 0.1% $HgCl_2$ (Sigma) for 2 min, immersed in 70% ethanol for 3 min and finally rinsed thoroughly with sterile distilled water (Malabadi *et al.* 2002, 2003).

Initiation of embryogenic tissue

Embryogenic tissue was established according to our previous protocols (Malabadi *et al.* 2005). Mature zygotic embryos of three genotypes (PK405, PK04 and PK214) were cultured individually on half strength inorganic salts MSG basal medium (Becwar *et al.* 1990) containing 2.0 $g.l^{-1}$ Gellan gum (Sigma), 90 mM maltose (Hi-media, Mumbai), 1 $g.l^{-1}$ L-glutamine (Sigma), 1 $g.l^{-1}$ casein hydrosylate (Sigma), 0.5 $g.l^{-1}$ *myo*-inositol (Sigma), 0.2 $g.l^{-1}$ *p*-aminobenzoic acid (Sigma) and 0.1 $g.l^{-1}$ folic acid (Sigma). The medium was supplemented with 10 $\mu g.l^{-1}$ TRIA (Sigma) (Malabadi *et al.* 2005). The cultures were raised in 25 mm × 145 mm glass culture tubes (Borosil) containing 15 ml of medium and maintained in the dark for 4-6 weeks at 25 ± 3°C. Nutrient medium without TRIA (Sigma) served as the control. The pH of the medium was adjusted to 5.8 with NaOH or HCl before Gellan gum was added. The medium was then sterilized by autoclaving at 121°C and 1.08 $Kg.cm^{-2}$ for 15 min. L-glutamine, *p*-aminobenzoic acid and TRIA (Sigma) were filter sterilized (Whatman, pore size = 0.3 µm; diameter of paper = 25 mm) and added to the media after it had cooled to below 50°C.

All the cultures were examined for the presence of embryonic suspensor masses by morphological and cytological observations of callus. Cultures showing white mucilaginous embryogenic tissue were identified and subcultured on initiation medium (IM) for a further two weeks for the improved development of embryonal suspensor masses. Initiation medium was defined by Malabadi *et al.* (2005) as half-strength (inorganic salts) MSG basal medium (Becwar *et al.* 1990) supplemented with 9.0 µM 2,4-dichlorophenoxy acetic acid (2,4-D) and 10 $\mu g.l^{-1}$ triacontanol (TRIA).

Maintenance of embryogenic tissue

White mucilaginous embryogenic tissue that developed on initiation medium (IM) was subcultured on maintenance medium (MM-I), defined as half-strength (inorganic salts) MSG basal medium containing 130 mM maltose, 4 $g.l^{-1}$ Gellan gum and supplemented with 2 µM 2,4-D and 2 $\mu g.l^{-1}$ TRIA (Malabadi *et al.* 2005). On MM, embryogenic tissue containing embryonic suspensor masses was maintained for three weeks with two subcultures in the dark.

Agrobacterium strain and culture conditions

Agrobacterium tumefaciens strain EHA105 harbouring binary vector pBI121 which contains the *npt*II gene providing resistance to kanamycin and the *uid*A gene interrupted by an intron, both driven by the cauliflower mosaic virus (CAMV) 35S promoter (Jefferson *et al.* 1987; Hood *et al.* 1983) was used for transformation studies. This disarmed agropine strain EHA105 (Hood *et al.* 1983) 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). *A. tumefaciens* was grown overnight in liquid YMB medium (yeast extract, 0.8 $g.l^{-1}$; mannitol, 10.0 $g.l^{-1}$; NaCl, 0.1 $g.l^{-1}$; $MgSO_4.7H_2O$, 0.2 $g.l^{-1}$; KH_2PO_4, 0.5 $g.l^{-1}$; pH 7.0) containing 50 $mg.l^{-1}$ kanamycin and 100 $mg.l^{-1}$ rifampicin, at 28°C on a shaker at 100 rpm for the selection of pBI121 (Malabadi and Nataraja 2007d, 2007e). The bacterial cells were thereafter pelleted by centrifugation at 4,000 rpm for 10 min and resuspended in MM to an optical density ($OD_{600\ nm}$) of 0.6.

Agrobacterium-mediated transformation

The embryogenic suspension cultures of three embryogenic lines (PK405, PK04 and PK214) were grown in 125 ml Erlenmeyer flasks containing 50 ml of liquid MM-I. 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 MM was added to the cell suspension, resulting in 50 mg fresh mass of embryogenic tissue suspended in 1 ml of bacterial culture ($OD_{600\ nm}$ = 0.3) in liquid MM in a 125 ml Erlenmeyer flask. In all the embryogenic lines, the addition of 100 µM acetosyringone (AS) to the bacterial suspension just before inoculation was tested. The control embryogenic lines were treated in the same way except that no *A. tumefaciens* was added to the cultures. In this 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) were poured over a 5.5 cm sterile Whatman filter paper (Schleicher and Schuell, qualitative circles) to drain the liquid and the filter paper was placed on semi-solid MM-I containing 4.0 $g.l^{-1}$ Gellan gum (Sigma), 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 in the above mentioned experiments.

After 3-d co-culture on semi-solid medium, filter papers with the cells from five Petri dishes were subcultured onto fresh semi-solid MM-I with 470 $mg.l^{-1}$ cefotaxime (non-washed; Sigma). Filter papers with cells from the remaining five Petri dishes were first placed in an Erlenmeyer flask (250 ml) with 100 ml of liquid MM. The cells were then dislodged by manual shaking and collected on 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 semi-solid MM 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 470 $mg.l^{-1}$ cefotaxime and 35 mg l^{-1} kanamycin for at least 2 weeks.

GUS histochemical assay

Histochemical GUS assays were conducted essentially as described by 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 if any deep indigo blue colour was observed.

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 was performed with

primers: 5′-ACTGTCCCCTAGT-GGGGAAGGGGACTGGCTGC TATT-3′ (forward) and 5′-GATACCG-TCACGCCCAAGCGCAG GTCAG-3′ (reverse) for the *npt*II gene. 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 for the DNA template. Each reaction mixture was overlaid with 25 µl of mineral oil (Sigma) to prevent evaporation. The amplification was performed in a 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 a final extension at 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 1X TAE as running buffer.

Southern blot analysis

Genomic DNA was isolated from 1 g fw of control and putative transgenic plants using a 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 (0.5 kb), which was obtained by digestion of pBI121 with *Hind*III and *Eco*RI, were labeled with digoxigenin-11-dUTP in the above PCR conditions according to 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 manufacturer's (Roche Biochemicals) instructions.

RNA isolation

Needle samples were collected from the control and Southern-positive transgenic *P. kesiya* 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. kesiya* 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 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 destained in 0.1X SSC, 0.5% SDS at 68°C for 15 min. Pre-hybridization was performed in Easy Hyb Solution 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 mRNA's (0.5 kb) was used. This probe was the same as that used in Southern blot analyses, and was labeled with digoxigenin-11-dUTP by PCR using the above set of primers. After overnight hybridization at 50°C, the blots were washed twice with 2X SSC 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 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 MM-II (maturation medium) without filter paper disc. Before maturation, 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) containing two sterile Whatman filter paper disks (50 mm). The Petri dishes were sealed with Parafilm and kept at 25 ± 2°C in the dark for 24 h 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. Half-strength (inorganic salts) MSG basal medium supplemented with 180 mM maltose, 60 µM abscisic acid (ABA) and 8 $g.l^{-1}$ Gellan gum (MM-II) was used as the maturation medium (Malabadi *et al.* 2005). All the cultures were again maintained in the dark for 8 to 12 weeks. Somatic embryos further matured to exhibit a hypcotyl and cotyledonary development.

Germination and plantlet recovery

After maturation, 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 germination medium (GM): half-strength MSG medium with 2 $g.l^{-1}$ Gellan gum (Malabadi *et al*. 2005). During germination, cultures were kept in darkness for one week, and thereafter to a 16-hr photoperiod under a light intensity of 50 $\mu mol.m^{-2}.s^{-1}$ for hardening. After 4 to 6 weeks on GM, plantlets were transferred to vermiculite in a controlled growth room.

Statistical analysis

In the above experiments, each replicate contained 50 cultures and one set of experiments consisted of two replicates (total 100 cultures for one experiment) for each genotype (PK405, PK04 and PK214) of *P. kesiya.* All the experiments were repeated in triplicate and **Table 1** represents the average of three 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 analysis was performed using the SPSS statistical software package.

RESULTS AND DISCUSSION

In the present study, we were able to establish an *Agrobacterium*-mediated transformation method for *P. kesiya* as part of a genetic improvement programme. This is the first successful report of transgenic *P. kesiya* plantlets using *A. tumefaciens*-mediated transformation. During the transformation events, the infection period of 5 h was found to be optimum for the embryogenic lines tested (**Table 1**). Bacterial growth was inhibited by culture onto medium with a higher concentration (470 $mg.l^{-1}$) of cefotaxime (**Table 2**). The remaining bacteria could be eliminated by washing 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). A lower concentration of cefotaxime did not significantly affect bacterial growth compared to controls without antibiotic (**Table 2**). The optimum concentration of cefotaxime (470 $mg.l^{-1}$) was crucial for the recovery of embryogenic tissue growth after co-cultivation (**Table 2**). This is in conformity with other stu-

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

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
PK04	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 ± 0.0 c	0.0 ± 0.0 c
	3	70	4.0 ± 0.1 b	0.0 ± 0.0 c
	4	80	10.0 ± 1.2 b	2.0 ± 0.1 b
	5	100	38.0 ± 2.3 a*	11.0 ± 1.2 a*
	6	150	6.0 ± 0.1 b	1.0 ± 0.1 b
PK214	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	1.0 ± 0.1 b	0.0 ± 0.0 c
	3	70	0.0 ± 0.0 c	0.0 ± 0.0 c
	4	80	3.0 ± 0.1 b	0.0 ± 0.0 c
	5	100	17.0 ± 2.0 a*	8.0 ± 0.3 a*
	6	150	2.0 ± 0.1 b	0.0 ± 0.0 c
PK405	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 ± 0.0 c	0.0 ± 0.0 c
	3	70	0.0 ± 0.0 c	0.0 ± 0.0 c
	4	80	6.0 ± 0.2 b	0.0 ± 0.0 c
	5	100	56.0 ± 4.0 a*	13.0 ± 1.4 a*
	6	150	5.0 ± 0.3 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 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. kesiya*.

Embryogenic lines tested	Cefotaxime ($mg.l^{-1}$)	Kanamycin ($mg.l^{-1}$)	Recovery effect during selection	Number of transgenic colonies recovered/g fw of tissue
PK04	0	0	–	0.0 ± 0.0 c
	100	5	–	0.0 ± 0.0 c
	250	15	–	0.0 ± 0.0 c
	350	25	–	9.0 ± 0.4 b
	400	30	+	18.0 ± 1.1 b
	470	35	++++	51.0 ± 3.0 a*
	600	50	–	0.0 ± 0.0 c
PK214	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 ± 0.1 b
	400	30	+	12.0 ± 0.8 b
	470	35	++++	43.0 ± 2.1 a*
	600	50	–	0.0 ± 0.0 c
PK405	0	0	–	0.0 ± 0.0 c
	100	5	–	0.0 ± 0.0 c
	250	15	–	0.0 ± 0.0 c
	350	25	–	11.0 ± 0.2 b
	400	30	+	18.0 ± 0.6 b
	470	35	++++	46.0 ± 3.0 a*
	600	50	–	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).

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 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.

dies in which a higher concentration of cefotaxime inhibited the growth of bacteria during the selection of embryogenic lines of *P. glauca*, *P. mariana*, *P. abies* and *P. wallichiana* (Klimaszewska *et al.* 2001; Malabadi and Nataraja 2007f). The antibiotic cefotaxime at 470 $mg.1^{-1}$ was required for at least three subcultures (2 weeks each) to ensure complete elimination of bacteria. Alternately, a chimeric 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). Subsequent subculture onto selective medium with kanamycin was done 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 species. Kanamycin sensitivity appears to depend on the explant and species (Klimaszewska *et al.* 2001). On the basis of studies carried out (**Table 2**), the best kanamycin concentration for selection of transgenic tissue was 35 $mg.1^{-1}$ kanamycin. During the first two weeks after inoculation, there was no visible effect of selection; on the contrary, all cultures proliferated. Selective

pressure was only significant after three weeks of culture onto medium containing cefotaxime and kanamycin. This trend was noticeable in all conifer transformation studies reported earlier (Le *et al.* 2001; Klimaszewska *et al.* 2001; Tereso *et al.* 2006). Kanamycin is an aminoglycosidase derivative antibiotic and is widely used to select *npt*II transformed cells (Terakami *et al.* 2007).

The viability of cultures, bacterial strains and cocultivation conditions were all important in making the cells accessible to *Agrobacterium* binding, T-DNA transfer, and integration into the host cell genome (Wenck *et al.* 1999; de Buck *et al.* 2000; Lelu and Pilate 2000; Klimaszewska *et al.* 2001). These results of selection of transgenic tissue on 35 mg l^{-1} kanamycin 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 analyzed in the putatively transformed lines by the GUS assay. Activity of GUS enzyme was not detected in control tissues. Most transformed lines showed variable GUS intensity in blue cell aggregates. 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 and Matzke 1998; Tereso *et al.* 2006). Gene expression could also vary 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 calculated 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 fw 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 did not apply this method since our starting material for the transformation study was embryogenic tissue. Therefore, we 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 at all developmental stages of embryogenic tissue; 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 was also previously observed in *P. radiata* (Walter *et al.* 1999; Grace *et al.* 2005), *P. abies* (Walter *et al.* 1999) and *P. wallichiana* (Malabadi and Nataraja 2007f). The highest transformation efficiency (highest number of GUS-blue-color spots) was obtained in the embryogenic line PK405 (56 transformed lines/g fw) than with the embryogenic lines PK04 and PK214. In PK214, the lowest transformation efficiency was recorded (17 transformed lines/g fw). On the other hand PK04 showed 38 transformed lines/g fw (**Table 1**). These results suggest that the success of the transformation process in *P. kesiya* 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 report of biolistic gene transfer (Malabadi and Nataraja 2007b), in all the embryogenic lines of *P. kesiya* tested (PK11, PR105, and PR521) the transformation frequency

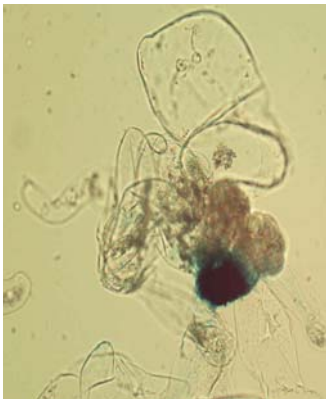

Fig. 1 Evidence for the presence of the *uidA* gene. GUS activity was visible as blue staining in the embryogenic tissue as seen under a microscope at 40X magnification following GUS histochemical assay.

was very low compared to *Agrobacterium*-mediated genetic transformation in this protocol. A similar discrepancy 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 (Hood *et al.* 1993; Humara *et al.* 1999; Le *et al.* 2001; Terakami *et al.* 2007). Another beneficial factor for the higher transformation frequency in all the tested embryogenic lines of *P. kesiya* might be due to the addition of AS (100 µM) during transformation. In another separate experiment, lower transformation efficiency was observed when AS was not added (**Table 2**). AS is one of the low MW phenolic compounds naturally released by wounded plant cells and acts as an inducer of the *vir* genes (Winans *et al*. 1994). For *P. kesiya*, the use of AS probably increases T-DNA transfer and resulted in a higher transformation frequency. In some other conifers, an increased transformation efficiency of embryogenic tissues by adding AS 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* (50 µM, Le *et al.* 2001), but in *L. kaempferi* × *L. deciduas* (100 µM, Levee *et al.* 1997), and French with 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*-mediated 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 by Southern blot analyses (**Fig. 3**). Genomic DNA was digested with *Eco*RI and *Hind*III, which recognize separate sites within the T-DNA. Transformed lines contained at least one gene copy of the T-DNA inserted in different loci. When T-DNA is introduced into plant cells a wound res-

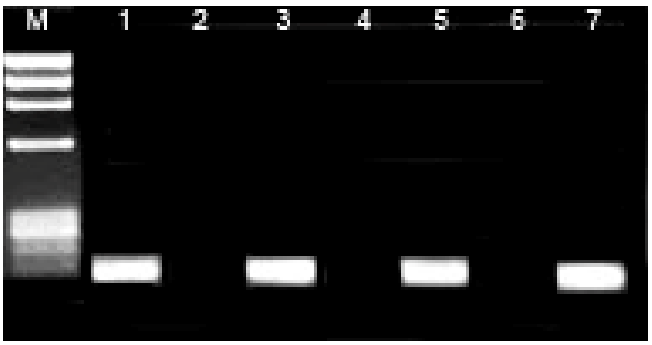

Fig. 2 PCR analysis of transformed embryogenic tissues of *P. kesiya*. DNAs were amplified with specific primers for the *npt*II gene. M, molecular marker. Lanes 1, 3, 5, 7: Amplified DNA fragment of *npt*II gene (500 bp) from four independent transformed lines of PK04. Lanes 2, 4 and 6: Untransformed control tissue of 3 independent lines.

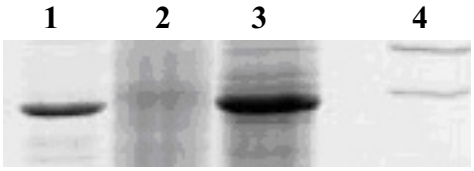

Fig. 3 Southern blot analysis of 3 putatively transformed tissue sample. Genomic DNA was digested with *Hind*III and *Eco*RI. Lanes 1 3, 4: Genomic DNA from independently transformed tissues showing single copies of *npt*II genes (500 bp) integrated into the genome of *P. kesiya* PK04. Lane 2: No signal detected in the genomic DNA from untransformed tissue (control).

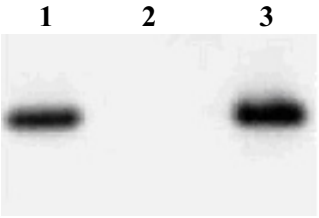

Fig. 4 Northern blot analysis of two putatively transformed somatic seedlings showing PCR/Southern positive signal. Lanes 1 and 3: RNA from 2 transgenic seedlings showing the integration of *nptII* gene in the *P. kesiya* genome of PK04. Lane 2: No signal in untransformed seedling (control)

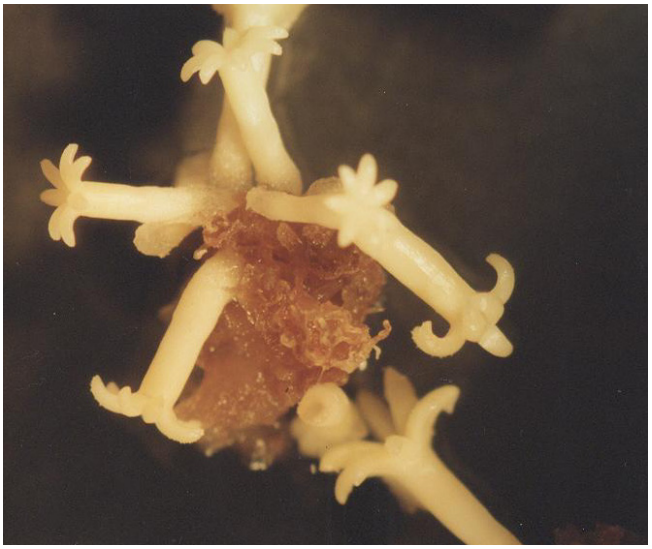

Fig. 5 Transgenic *P. kesiya* PK04 somatic embryos at various developmental stages on maturation medium as seen under the microscope.

ponse is elicited, which involves the activation of nucleases and DNA repair enzymes that maintain the integrity of the host genome (Tinland 1996; de Buck *et al.* 1999; Vergunst and Hooykaas 1999; Gelvin 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). 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). 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 (*P. taeda*) and Norway spruce (*P. 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 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).

All the three tested transgenic genotypes of *P. kesiya* showed a varied percentage of somatic embryogenesis (**Table 3**). A total of six somatic seedlings recovered per gram fw of transgenic tissue compared against control in a genotype PK214 (**Table 3**). Genotype PK405 displayed 19% somatic embryogenesis, with a total of seven somatic seedlings recovered per gram fw of transgenic tissue (**Table 3**). The highest percentage somatic embryogenesis (22%) was recorded in genotype PK04 with a total of 14 transgenic somatic seedlings recovered per gram fw of transgenic tissue (**Table 3**). The maturation potential 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 same embryogenic lines (PK214, PK405, PK04) by using biolistic gene transfer, the percentage of somatic embryogenesis was very low and transgenic seedlings were recovered only in one genotype PK04. In the remaining two genotypes, transgenic tissues had lost their maturation potential and failed to produce somatic embryos on MM (Malabadi and Nataraja 2007b). The reason why the transgenic lines obtained by biolistic gene transfer method were unable to regenerate somatic embryos reamains unclear. This phenomenon 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-positive. After 14 weeks of maturation, advanced (i.e. with well developed or fully developed cotyledons) cotyledonary somatic embryos were picked up for germination (**Fig. 5**). GUS/PCR assays were negative in non-transformed control plants. After 4-6 weeks on germination medium (GM), the transgenic plantlets were recovered and hardened. All the transgenic plants of tested embryogenic lines survived, showed poor growth for 3 weeks, then regained normal growth after 5 weeks. The growth of plants was very poor between weeks 3 and 5 and only after 5 weeks did the plants regain

Table 3 Recovery of transgenic seedlings following *Agrobacteriuum*-mediated genetic transformation of embryogenic tissue in three genotypes of *P. kesiya*. 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
PK04	*22.0 ± 1.0 c	19.0 ± 1.5 c	17.0 ± 0.6 c	14.0 ± 0.2 c
Control 1	88.0 ± 3.4 a	48.0 ± 2.1 b	40.0 ± 2.9 b	39.0 ± 3.0 b
PK214	16.0 ± 1.0 c	13.0 ± 1.8 c	9.0 ± 0.4 c	6.0 ± 0.1 c
Control 2	43.0 ± 2.4 b	21.0 ± 2.0 c	17.0 ± 1.4 c	14.0 ± 0.3 c
PK405	19.0 ± 3.0 c	16.0 ± 3.1 c	12.0 ± 2.5 c	7.0 ± 1.9 c
Control 3	68.5 ± 2.9 a	21.0 ± 1.3 c	18.0 ± 1.0 c	14.0 ± 1.5 c

PK214, PK405, PK 04- Transgenic seedlings (% of somatic embryogenesis in transgenic lines= 5 grams 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

normal growth.

In this study, we developed a simple protocol or the genetic transformation of *P. kesiya.* This improved transformation system is sufficient to generate many transgenic plants. Transgene integration was confirmed by PCR, Southern and Northern analyses. This protocol will allow the genetic improvement of this pine species by the introduction of relevant genes that regulate growth, flowering, and resistance to insects and disease, among other characteristics. This method of pine transformation is clearly applicable to a range of genotypes, and it will prove to be useful for introducing valuable traits for the genetic improvement of *P. kesiya*.

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