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Genetic Transformation of *Vanilla planifolia* by *Agrobacterium-tumefaciens* Using Shoot Tip Sections

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Abstract: A protocol is presented for genetically engineering *Vanilla planifolia* orchid using a routine transformation procedure via *Agrobacterium tumefaciens*. An expression vector containing *nptII* and GUS genes driven by the cauliflower mosaic virus (CaMV) 35S promoter was successfully introduced into the *V. planifolia* genome by *A. tumefaciens*. Protocorm-like bodies (PLB's) derived from protocorms, were the target explants for transformation. The presence of the transformed gene in the transgenic orchid plants was confirmed by the PCR analysis; Southern blot and Northern blot analyses of the PCR product. Therefore, the foreign DNA was successfully integrated into the orchid genome and expressed transcriptionally and translationally in these orchid plants. The present transformation method reported is suitable for improving the *V. planifolia* orchid through genetic engineering.

Key words: Orchid, genetic transformation, protocorm-like bodies, plant regeneration

INTRODUCTION

Vanilla planifolia Andr, a tropical orchid offers excellent scope for cultivation in the high-rainfall regions in Southern India. There is a growing demand for natural vanilla flavor in the global trade. It is commercially cultivated for pods (beans) from which the popular flavoring substance called vanillin is extracted. Vanillin is widely used for flavoring cakes, sweets, chocolates, ice creams, beverages, condiments, oleoresins, in cosmetics and perfumery industries (Goodenough, 1982; George and Ravishankar, 1997; Giridhar and Ravishankar, 2004). The world production of vanilla beans is estimated to be about 3800 tones per annum and nearly 400 tones are used in USA alone. Malagasy Republic grows 70 to 80% of the world's vanilla crop. The world trade is around Rs 360 crores annually, amounting to nearly 7% of the total value of the spice trade (Venkatesha *et al.*, 1998; Geeta and Shetty, 2000). Traditionally Vanilla, a vine orchid is propagated from stem cuttings of mature vines and is raised in polybags. However, this method of propagation is not economical, rather slow, labor-intensive and time-consuming since the collection of stem cuttings leads to arrest the growth and development of mother tissue (Geeta and Shetty, 2000; Giridhar and Ravishankar, 2004). Moreover, the market demand for propagules is hardly met with such cuttings. *In vitro* multiplication of *Vanilla planifolia* has been reported through the callus culture, protocorms, root tips, stem nodes and axillary-bud explants (Stafford, 1991; Knorr *et al.*, 1993; Gu *et al.*, 1987; Davidonis and Knorr, 1991; Philip and Nainar, 1986; Kononowicz and Janick, 1984; George and Ravishankar, 1997; Geeta and Shetty, 2000; Giridhar and Ravishankar, 2004; Giridhar *et al.*, 2001). However, mass propagation for commercial cultivation requires a simple, economical, rapidly multiplying and highly reproducible protocol so as to give rise to true-to-type clones (Stafford, 1991; Knorr *et al.*, 1993).

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There is substantial interest in the genetic improvement of orchids. However, orchids usually have long juvenile periods and reproductive cycles and slow seed maturation (several months) of these plants, which restricts genetic improvement using traditional sexual hybridization. Therefore, the application of genetic engineering techniques to orchid improvement appears to be an attractive alternative. Until recently, transgenic orchid plants have been reported for only a few genera of orchids (Men *et al.*, 2003; Yang *et al.*, 1999; Belarmino and Mii, 2000; Liau *et al.*, 2003; You *et al.*, 2003), there is no report available on the transformation of *V. planifolia* orchid. This paper reports genetic transformation and efficient micropropagation system for *V. planifolia* using thin-shoot tip-sections under the influence of external incorporation of putrescine in the basal medium. Our results for the first time demonstrated that putrescine can be used as a growth regulator for the micropropagation of *V. planifolia* to meet the current trade demand for the large scale synthesis of flavoring substance vanillin. To our knowledge, this is the first report to show *A. tumefaciens*-mediated transformation of the *V. planifolia* orchid, demonstrating GUS expression in transgenic orchid plants.

MATERIALS AND METHODS

Source of Plant Material

Shoot tips of field grown plants of *Vanilla planifolia* Andr, were collected from the Western-Ghat-Forests, South India and were stored in polythene bags at 4°C in the Department of Botany, Karnatak University, Dharwad, India.

Initiation of Cultures

Shoot tips (0.5-0.8 cm) harvested from mother plants were carefully washed in distilled water. They were surface decontaminated sequentially with 0.1% streptomycin (20 sec), 70% (v/v) ethanol (50 sec) and 0.1% (w/v) HgCl₂ (2 min) and thoroughly rinsed with sterilized double distilled water. Transverse-thin sections of 1-2 mm thick were cut from shoot tips and these sections were cultured on Murashige and Skoog (MS) (1962) basal medium with 3.0% sucrose (Hi media, Mumbai), 0.7% agar (Sigma), 0.5 g L⁻¹ meso inositol, 1.0 g L⁻¹ casein hydrosylate, 0.5 g L⁻¹ L-glutamine, 250 mg L⁻¹ peptone, 0.2 g L⁻¹ p-aminobenzoic acid and 0.1 g L⁻¹ biotin. The medium was supplemented with 4.0 mg L⁻¹ putrescine without any other growth hormones in 25×145 mm glass culture tubes (Borosil) containing 15 mL of the medium under cool white fluorescent light (100 μmol m⁻² s⁻¹) at 25±3°C with a relative humidity of 55-60%. The pH of the media was adjusted to 5.8 with NaOH or HCl before agar was added. Medium without putrescine (Sigma, St Louis, USA) was served as control. The media were then sterilized by autoclaving at 121°C at 1.04 kg cm⁻² for 15 min. L-glutamine, biotin, p-aminobenzoic acid and putrescine were filter sterilized and added to the media after autoclaving when the medium had cooled to below 50°C. The cultures were maintained for 6-10 weeks for the initiation of PLB's or proliferating shoot buds. These cultures are used for the following genetic transformation experiments.

Agrobacterium Strain and Culture Medium

Agrobacterium-tumefaciens strain LBA4404 harbouring binary vector pCAMBIA2301, which contains a neomycin phosphotransferase gene (*nptII*) and β-glucuronidase (GUS) gene (*gusA*) interrupted with an intron, both driven by the Cauliflower Mosaic Virus (CAMV) 35S promoter was used for transformation studies. The *A. tumefaciens* was grown in liquid YMB medium (yeast extract-0.8 g L⁻¹; mannitol-10.0 g L⁻¹; NaCl-0.1 g L⁻¹; MgSO₄·7H₂O-0.2 g L⁻¹; KH₂PO₄-0.5 g L⁻¹; pH-7.0) containing 10 mg L⁻¹ kanamycin and 10 mg L⁻¹ rifampicin, overnight at 28°C on a shaker at 300 rpm.

Genetic Transformation

Bacteria were pelleted at 4,000 rpm for 10 min and suspended in a liquid MS basal medium supplemented with 4.0 mg L⁻¹ putrescine. Callus tissue showing protocorm-like-bodies (PLB's) precultured in a liquid MS basal medium supplemented with 4.0 mg L⁻¹ putrescine was infected by the bacteria for 3 h (infection period) on rotating shaker at 300 rpm. After 3 h of infection, callus tissue containing PLB's was blotted out on sterile filter paper and co-cultured on semi-solid MS basal medium containing 4.0 mg L⁻¹ putrescine under a 16 h photoperiod at 25±2°C for 3 days. After 3 days (co-cultivation period), co-cultured callus tissue containing PLB's on filter papers were transferred on semi-solid MS basal medium containing 4.0 mg L⁻¹ putrescine supplemented with 75 mg L⁻¹ kanamycin and 500 mg L⁻¹ cefotaxime. This step was repeated at least for 3 times to inhibit the growth of bacteria.

Histochemical Test

GUS activity was determined immediately after co-cultivation of callus tissue with PLB's by histochemical GUS assay according to Jefferson (1987). Callus tissue with PLB's was incubated overnight at 37°C in 2 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) solution buffered with 50 mM sodium phosphate buffer at pH 7.0.

Recovery of Transgenic Seedlings

Finally, the callus tissue with PLB's on filter paper was subcultured on MS basal medium supplemented with 4.0 mg L⁻¹ putrescine. Healthy shoots were developed within 10-12 weeks. They were subcultured on the same medium for another 2 weeks for further shoot development. The well-developed shoot buds were transferred on fresh MS medium supplemented with 4.0 µM TRIA for rooting. After rooting, they were removed from the media, freed of agar by washing in running tap water and planted in a sand-compost mixture (1:2) at about 80% relative humidity under polyethylene hoods in the green house. The plants were watered daily and fertilized at weekly intervals with a foliar spray of a mixture of commercial DAP (Di Ammonium phosphate) and NPK (Nitrogen 20: phosphorous 10: Potassium 10) (Malabadi *et al.*, 2004, 2005). The plantlets were hardened for 65 days and then well maintained in the green house.

PCR Amplification

Genomic DNA was extracted from putatively transformed and non-transformed (control) plants with the method of Dellaporta *et al.* (1983). The DNA pellet was dissolved in TE buffer and DNA concentration was measured spectrophotometrically at 260 and 280 nm. The purity of DNA was checked by using agarose gel electrophoresis and by determining the ratio of 260/280 nm. PCR amplification was carried out with gene-specific primers and template DNA prepared from transgenic plants, control plants and respective construct as positive control. Transformants were screened for the presence of the *nptII* gene. The 540 bp coding region of *nptII* was amplified using 20 base oligonucleotide primers (I: 5'-CCACCATGATATTCGGCAAC-3' and II: 5'-GTGGAGAGGCTATTCGGCTA-3'). PCR reactions were carried out in a final 25 µL reaction mixture containing 25 ng template DNA, 0.2 µL (1 U) of *Taq* DNA polymerase (Roche, Germany), 0.5 µL of gene-specific-primer, 2.0 µL of 10x PCR buffer (Roche, 100 mM Tris-HCl, 15 mM MgCl₂, 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 for 1 cycle of 94°C, 1 min; 36°C, 20 sec; 72°C, 2 min, followed by 45 cycles of 94°C, 10 sec; 36°C, 20 sec; 72°C 2 min and finally an extension cycle of 72°C, 5 min; 35°C, 1 min as described in Malabadi *et al.* (2006) and Malabadi and Nataraja (2006).

Southern Blot Hybridization

Southern blot analyses were performed to verify the integration of the transgenes into the *V. aconitifolia* genome. DNA was extracted from putatively transformed and non-transformed (control) plants with the method of Dellaporta (1983). Ten microgram of genomic DNA was digested with *KpnI* (www.fermentas.com) (having a unique restriction site within the constructs) and fractioned on a 0.8% (w/v) agarose gel at 70 V for approximately 5 h. The gels were depurinated, denaturated, neutralized and fragmented DNA was transferred to a nylon membrane (Roche Diagnostics GmbH, Mannheim, Germany) by capillary transfer. The prehybridizations and hybridizations were performed in Easy Hyb solution (Roche biochemicals) at 42-45°C. Double stranded probe for *nptII* (540 bp or 5.4 kb) was labeled with digoxigenin-11-dUTP in the PCR conditions according to our previous protocols. For *nptII* the sense primer was (I: 5'-CCACCATGATATTCGGCAAC-3') and the antisense primer was II: 5'-GTGGAGAGGCTATTCGGCTA-3'. After overnight hybridization, the blots were washed twice with 2X SSC (3M 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 X SSC, 0.1% SDS at 68°C for 15 min. The chemiluminescence detection of hybridization products was performed according to manufacture's (Roche biochemicals) instructions.

RNA Isolation

Total RNA was isolated from the transformed plantlets by the modified method of Chang *et al.* (1993). One gram plant material was homogenized in the presence of liquid nitrogen. The homogenate was mixed in 15 mL of 65°C pre-warmed extraction buffer (25 µM EDTA pH 8.0, 100 µM Tris-HCl pH 8.0, 2.0 M NaCl, 2% CTAB, 2% PVP-K30 and 2% freshly added β-mercaptoethanol). After vigorous shaking for 15 min, the sample was incubated at 65°C for 5 min and thereafter at room temperature for 10 min. The sample was extracted once with chloroform and the RNA was precipitated with 10 M LiCl overnight. Next day, the RNA was harvested by centrifugation, dissolved in SSTE (1 µM EDTA pH 8.0, 10 µM Tris-HCl pH 8.0, 1 M NaCl, 0.5% SDS pH 7.2) and re-extracted with chloroform. Finally, total RNA was precipitated with ethanol, harvested by centrifugation and dissolved in diethylpyrocarbonate-treated water (DEPC-treated water).

Northern Blot Analysis

Northern-blot analyses were performed to confirm the transgene in the *V. planifolia* genome. Fifteen microgram 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 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 methyleneblue, 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 at 50°C for 1-2 h. For hybridization, a fresh Easy Hyb Solution containing denatured *nptII* probe for the detection of the corresponding mRNA's (5.4 kb) was used. The probe used for detection of the *nptII* mRNA was the same one as applied in Southern blot analyses and was labeled with digoxigenin-11-dUTP by the PCR using the set of primers. After overnight hybridization at 50°C, the blots were washed twice with 2X SSC (3M 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 0.2 X SSC, 0.1% SDS at 68°C for 15 min. The chemiluminescence detection of hybridization products was performed according to manufacture's (Roche biochemicals) instructions.

Statistical Analysis

In all the above experiments each culture tube received a single explant. Each replicate contained 50 cultures and one set of experiment is made up of 2 replicates (100 leaf segments were cultured for one set of experiment). All the experiments were repeated 3 times. Data presented in the tables were arcsine transformed before being analyzed for significance using ANOVA (analysis of variance, $p < 0.05$) or evaluated for independence using Chi-square test. Further, the differences contrasted using a Duncan's multiple range test ($\alpha = 0.05$) following ANOVA. All statistical analysis was performed using the SPSS statistical software package.

RESULTS AND DISCUSSION

Orchids are substantially different from other plants in their requirements for a transformation system. Orchid cells have a low rate of proliferation. Secondly, the orchid cells are recalcitrant to tissue culture manipulations (Yang *et al.*, 1999; Malabadi *et al.*, 2004, 2005). Plant regeneration from de-differentiated cells has not been achieved for orchids. Finally, the orchid cells in tissue culture exude a large quantity of phenolics that become toxic to the cells when oxidized. However, a very few reports of *Agrobacterium*-mediated transformation has been reported for orchids. The co-culture of organized and easily regenerable tissue such as PLB's allowed us to regenerate transgenic *V. planifolia* orchids. The advantage in this approach is that shoots develop directly from the primary and secondary meristems without an intervening explant-organogenesis phase. This minimizes the necessity for treatment with phytohormones and thus the opportunity for somaclonal variation. In the present investigation, putrescine has a high potential to induce proliferating shoot buds or PLB's with callusing from thin shoot tip sections of *V. planifolia*. The highest percentage of explants (67.0%) (After co-culture with *A. tumefaciens*) producing PLB's (29.6 ± 2.0) was recorded on 4.0 mg L^{-1} putrescine in a period of 10-12 weeks. These PLB's or proliferating shoot buds formed the maximum number of healthy shoots (2.4 ± 0.1) (Table 1). Lower or higher concentrations of putrescine resulted in the browning of explants and failed to produce PLB's (data not shown). PLB's were further subcultured on the same medium and maintained for 10-12 weeks. After nearly 12 weeks, small bud-like structures formed healthy shoots. Shoots formed rooting successfully on MS basal medium supplemented with $4.0 \text{ }\mu\text{M}$ TRIA (data not shown). Protocorm-like Bodies (PLB's) derived from protocorms are the most easily obtained materials for most orchids that are capable of regenerating plants. Therefore, developing a routine and effective transformation system for gene transfer into PLB's is important for producing new horticulturally and economically important varieties of *V. planifolia*.

Polyamines, which include putrescine, cadaverine, spermidine and spermine, are small polycations found in most organisms and are essential for cellular proliferation and normal cellular function (Kevers *et al.*, 2002; Kakkar *et al.*, 2000). Bagni and Serafini-Fraccassin (1985) have reported that exogenous polyamines serve as mere nitrogen source for plants. Several studies have indicated that polyamines may be important for cell growth and somatic embryogenesis of *Daucus carota* (Montague *et al.*, 1979) and formation of floral buds from thin layer explants of *Nicotiana tabacum*

Table 1: Recovery of transgenic seedlings following *Agrobacterium*-mediated genetic transformation of *V. planifolia*

Experiment details	PLB formation with shoot buds (%)	GUS (%)	Transformation (%)	Total No. of shoot buds recovered per gram fresh wt. of callus	Total No. of transgenic seedlings recovered per gram fresh wt. of callus
I	41.0 \pm 2.0a	26.0 \pm 2.1a	60.0 \pm 3.8a	4.0 \pm 0.1b	2.0 \pm 0.2b
II	32.0 \pm 1.3a	33.0 \pm 2.0a	43.0 \pm 2.0a	7.0 \pm 0.4b	5.0 \pm 0.1b
III	16.0 \pm 1.0a	11.0 \pm 1.1a	15.0 \pm 1.7a	1.0 \pm 0.6b	0.0 \pm 0.0b
Mean	29.6 \pm 2.0a	23.4 \pm 2.1a	39.4 \pm 2.1a	4.0 \pm 0.1b	2.4 \pm 0.1b

I, II, III represents 3 independent experiments conducted separately; Mean \pm SE followed by the same letter(s) in each column were not significantly different at $p \leq 0.05$

(Torrigiani *et al.*, 1987). Furthermore, treatments of putrescine in several systems have promoted shoot multiplication (Chi and Pua, 1989). In plants polyamines are always present in amounts varying from micro molar to more than mill molar (Galston and Kaur-Sawhney, 1990). Recent reports have indicated that polyamines could enhance somatic embryogenesis in several plant species (Kakkar and Sawhney, 2002; Kevers *et al.*, 2002; Sakhanokho *et al.*, 2005).

The success of *Agrobacterium*-mediated transformation depends in part on the efficient interaction of *Agrobacterium* with the host plant cells (You *et al.*, 2003). In addition to efficient interaction between target plant cells and *Agrobacterium*, efficient regeneration from these target plant cells is also required for the recovery of transgenic plants. One approach to obtain homogeneously transformed individuals from co-cultured meristematic tissues is by selfing the treated generation and selecting for the added trait. The other is to insert DNA into meristematic tissues in the early stages of organization and then stimulate continued meristem development during antibiotic selection. The former approach is limited in orchid transformation mainly due to the long generation time and slow growth. However, later can be successfully applied to orchid transformation if proper culture regimes and transformation conditions can be devised. Previous reports on the transformation of *Dendrobium* and *Vanda* orchids showed that the general approach of the particle-mediated transformation of plant tissues can be successfully applied to orchids. However, despite its commercial value, *Cymbidium* orchids have been up to now recalcitrant to transformation (Yang *et al.*, 1999). The choice of PLB's may also be an important factor in the successful transformation and regeneration of transformed PLB's. The pre-wounding treatment is critical for transformation, possibly because it circumvents the attachment step or releases phenolic inducers of *Agrobacterium vir* functions, stimulates host DNA replication for T-DNA integration. This disrupts tissue organization such that somatic embryos can occur near the wounded surface or provides access to target cells.

The standard transformation procedure involves the co-culturing of the explant for 2-3 days followed by selecting the putative transgenics on selection medium that is supplemented with antibiotics to eliminate *A. tumefaciens* and untransformed explants. In our present study, after 3 days (co-cultivation period), co-cultured callus tissue containing PLB's on filter papers were transferred on semi-solid MS basal medium containing 4.0 mg L⁻¹ putrescine supplemented with 75 mg L⁻¹ kanamycin and 500 mg L⁻¹ cefotaxime. This step was repeated at least for 3 times to inhibit the growth of bacteria. We assume that the foreign DNA from *A. tumefaciens* may still be transferred into the actively growing PLB's during this long term transformation process. However, during the selection and regeneration stage, *A. tumefaciens* was completely inhibited at higher concentrations of kanamycin and cefotaxime, were used to select the transformed PLB's. These results indicated that the long coculture period was essential for successful transformation and this is in accordance with the report of Liao *et al.* (2003). The histochemical assay of leaf explants of transgenic seedlings for GUS activity demonstrated the presence of blue-spots in the colorless background. The β -glucuronidase enzyme catalyses the conversion of 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (colorless) to 5-bromo-4-chloro-3-indol (blue). Therefore, the unique appearance of blue spot in transgenic lines indicated that the GUS enzyme is expressed under the control of the 35S promoter. Of the 100 samples tested, 39.4 (Average of 3 experiments) (Table 1) contained positive neomycin phosphotransferase gene (*nptII*) resulting in higher transformation efficiency (39.4%) than GUS (23.4%). These values are the mean of 3 independent experiments tested with *V. planifolia* (Table 1). Further average number of shoot buds recovered per gram fresh wt of tissue was found to be 4.0 \pm 0.1. 2.0 transgenic seedlings were recovered per gram fresh wt of tissue (Table 1).

The integration of transgenes in plant genomes was shown by the PCR amplification of the transgene with gene-specific primers from the chromosomal DNA (Fig. 1) and by the Southern hybridization (Fig. 2) of total genomic DNA with gene-specific DNA probes. Southern analysis of transgenic plants revealed different patterns of junction fragments between the T-DNA and the plant

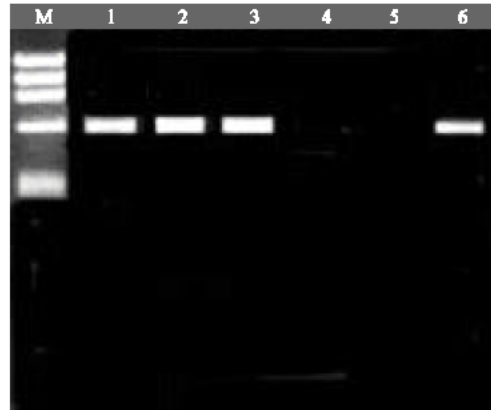


Fig. 1: PCR mediated amplification for neomycin phosphotransferease gene (*nptII*) products described by Saini and Jaiwal (2005). The DNA contents of lane are: Molecular weight marker M Lanes 1, 2, 3 and 6 = Genomic samples of transgenic *V. planifolia* seedlings showing the integration of *nptII* gene at 530bp (5.3 kb). Lane 4, 5 = Genomic samples of control (non-transgenic) plants of *V. planifolia*



Fig. 2: Southern blot analysis of DNA of 2 independent transformed *V. planifolia* plantlets. Lanes 1 and 2 showing multiple copies of genes integrated into the plant genome following successful transformation

genome, depending upon the integration site (Fig. 2). This clearly indicates that these plants were derived from independent transformation events. The T-DNA or pCAMBIA2301 (5.3 kb) contains a single *KpnI* site at the multiple cloning site located in the *lacZ* alpha region. The number of hybridization signals indicated that plants have two copies of T-DNA integrated into their genome (Fig. 2). DNA isolated from non-transformed plants did not hybridise with the *nptII* probe. This is also in conformity with the results of other transformed orchids (Liau *et al.*, 2003; Yang *et al.*, 1999; Belarmino and Mii, 2000; Men *et al.*, 2003). Northern blot analysis with the *nptII* gene probe was also done to determine if the *nptII* gene was transcribed (Fig. 3). Present results showed that the levels of mRNA transcripts in the transgenic lines were high in all the transgenic *V. planifolia* plants, whereas

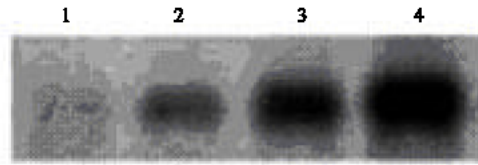


Fig. 3: Northern blot analysis of putative transgenic plants of *V. planifolia*. A RNA gel blot containing 15 µg of total RNA and hybridized with the probe recognizing *nptII* mRNA's. Lanes 2, 3, 4 showing integration of genes into the plant genome. Lane-1: Non transformed control plant

no signals were detected in untransformed plants (Fig. 3). These results also confirm the integration of gene in the transgenic plants. Therefore, foreign gene can be expressed transcriptionally and translationally in the transgenic orchid plants (Liau *et al.*, 2003; Yang *et al.*, 1999; Men *et al.*, 2003; Belarmino and Mii, 2000).

In conclusion, we successfully transformed *V. planifolia* at a relatively high efficiency. The protocols reported here are simple, repeatable and effective and they can be applied to other varieties of *V. planifolia*, providing a very useful basis for further genetic improvements of orchid. Furthermore, this transformation system is also expected to be applicable for transfer of other useful transgenes into *V. planifolia*, benefiting both basic and applied floral plant research.

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