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ORIGINAL ARTICLE

Genetic transformation and regeneration of transgenic plants from protocorm-like bodies of vanilla (*Vanilla planifolia* Andrews) using *Agrobacterium tumefaciens*

S. T. Retheesh · A. Ishwara Bhat

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Abstract An efficient transformation protocol was developed for vanilla (*Vanilla planifolia*) using protocorm-like bodies (PLBs) derived from shoot tips as explants. Of the ten media tested, Murashige and Skoog (MS) medium containing 0.45 μM thidiazuron (TDZ) produced maximum PLBs per shoot tip. Genetic fidelity of PLB-derived plantlets was confirmed by random amplified polymorphic DNA (RAPD) using 23 random primers. PLBs were co-cultured with *Agrobacterium tumefaciens* strain EHA105 harbouring the binary vector pBI121 containing the β-glucuronidase (*gusA*) and neomycin phosphotransferase II (*npt II*) genes for 3 days in MS medium supplemented with acetosyringone and transferred to selective regeneration medium containing 4.43 μM benzyladenine (BA), 2.68 μM naphthalene acetic acid (NAA) supplemented with 50 mg l^{-1}kanamycin and 250 mg l^{-1} cefotaxime. After 15 days of culture, the surviving explants were transferred to the same regeneration medium but with a higher concentration of kanamycin (75 mg l^{-1}). Finally, explants surviving after 30 days were subjected to more stringent selection in the regeneration medium supplemented with 100 mg l^{-1} kanamycin. Strong β glucuronidase activity was detected in the transformed plantlets by histochemical assay. Integration of T-DNA into the nuclear genome of transgenic plants was confirmed by polymerase chain reaction and Southern hybridization, while expression of transgene was confirmed by northern hybridization. This protocol allows effective and high frequency transformation of vanilla.

S. T. Retheesh · A. I. Bhat (✉)
Division of Crop Protection, Indian Institute of Spices Research, Calicut 673 012, Kerala, India
e-mail: aib65@yahoo.co.in

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Abbreviations

BA	6-Benzyladenine
GUS	β-Glucuronidase
NAA	α-Naphthalene acetic acid
MS	Murashige and Skoog
npt II	Neomycin phosphotransferase II
PLB	Protocorm-like body
PCR	Polymerase chain reaction
RAPD	Random amplified polymorphic DNA
TDZ	Thidiazuron
X-glcA	5-bromo-4-chloro-3-indolyl-β-D-glucuronide
YEP	Yeast extract peptone medium

Introduction

Vanilla is one of the most popular flavoring agents for numerous sweetened foods worldwide. It is also used in perfumery and in the cosmetic industry. Vanilla is obtained from fully grown fruit of the orchid, *Vanilla planifolia (*syn. *Vanilla fragrans*) in the Orchidaceae family. It is indigenous to south-eastern Mexico, Guatemala and other parts of Central America. In India, it is grown as mixed crop in arecanut and coconut plantations. There is an ever-growing demand for natural vanilla, the vanilla essence (vanillin) extracted from cured beans. The floral structure of vanilla makes natural self-pollination difficult. Hand pollination results in fruit set but seeds rarely germinate (Dequaire 1976; Soto Arenas 2003). Hence vegetative propagation through stem cuttings is the predominant reproduction mode in vanilla. Consequently, the genetic base of *V. planifolia* is very narrow, with very low levels of variation

within the species (Besse et al. 2004; Divakaran et al. 2006). With low genetic diversity, scope for improvement of vanilla by conventional breeding is very limited. Hence, genetic transformation of vanilla with foreign genes would be useful to impart pest resistance. It could also help to engineer biochemical pathways, e.g., for increased vanillin production. There are many reports of biolistic transformation of orchids and a few reports of *Agrobacterium*-mediated transformation of this species group. However, so far there is only one report of transformation of vanilla by *A. tumefaciens*, but with low efficiency (Malabadi and Nataraja 2007). Here, we describe an efficient protocol for the induction of high frequency protocorm-like bodies (PLBs) and their *Agrobacterium*-mediated genetic transformation with enhanced efficiency.

Materials and methods

Plant material and induction of PLBs

Healthy vines of vanilla, originally collected from farmers' fields were vegetatively propagated in a greenhouse by stem cuttings. Shoot tips of 3–4 cm length were collected from these plants and rinsed in distilled water. They were surface sterilized with 0.1% mercuric chloride for 3 min. Shoot tips of approximately 5 mm length were cut using a sterile scalpel and forceps under aseptic conditions and placed in Petri dishes containing different media. A total of ten different media composed of Murashige and Skoog (MS) (1962) basal medium with different growth regulators at varying concentrations were used (Table 1). The media were solidified with 0.3% phytagel. A total of 100 shoot tips were used per medium with three replications. The plates were incubated for 45 days at 25±1°C under a 16 h photoperiod with a light intensity of 40 μM m^{-2} s^{-1} provided by cool white fluorescent tubes. The PLBs were sub-cultured in the same medium every 45 days for three generation for maintenance.

Table 1 Frequency of protocorm-like body (PLB) induction in the different media tested. Data is presented as mean value ± standard error of three experiments. Mean values within the column followed by the same letters are not significantly different according to Duncan's multiple range tests at $P \leq 0.05$. *BA* 6-Benzyladenine, *NAA* α-naphthalene acetic acid, *TDZ* thidiazuron

Medium	Average number of PLBs per shoot tip after 45 days of culture
MS+4.43 μM BA	3±0.4 a
MS+8.86 μM BA	4±0.6 a
MS+13.29 μM BA	4±0.5 a
MS+2.68 μM NAA	0
MS+5.37 μM NAA	0
MS+8.05 μM NAA	0
MS+4.43 μM BA+2.68 μM NAA	0
MS+0.45 μM TDZ	37±2.1 d
MS+2.27 μM TDZ	29±1.8 c
MS+4.54 μM TDZ	24±2.3 b

Genetic fidelity analysis of PLB-derived plantlets

MS medium containing 4.43 μM 6-benzyladenine (BA) and 2.68 μM α-naphthalene acetic acid (NAA) was used for regeneration of PLBs into plantlets. Genetic fidelity of PLB-derived plantlets was tested by random amplified polymorphic DNA (RAPD) using selected 23 random primers (Operon Technologies, Alameda, CA) including the primers known to detect polymorphism in vanilla (Besse et al. 2004; Divakaran et al. 2006). Genomic DNA was isolated from 100 mg leaf tissue using the CTAB method (Doyle and Doyle 1990). The polymerase chain reaction (PCR) contained 50 ng genomic DNA, 15 pM primer, 200 μM dNTPs, 1 unit *Taq* DNA polymerase and 1.5 mM $MgCl_2$ in 1x reaction buffer with a reaction volume of 25 μl and was performed in a thermocycler (Eppendorf Master Cycler, http://www.eppendorf.com) at 92°C for 3 min for initial denaturation followed by 35 cycles of 92°C for 30 s, 40°C for 30 s and 72°C for 1 min. A final extension time of 10 min was allowed at 72°C at the end of 35 cycles. The reaction product was subjected to electrophoresis in 1% agarose gel followed by staining with ethidium bromide and visualization under UV light.

Kanamycin sensitivity study

Untransformed PLBs were cultured in regeneration medium (MS + 4.43 μM BA + 2.68 μM NAA) containing 0, 50, 75, 100 and 150 mg l^{-1} kanamycin to determine the appropriate concentration for selection of transgenic plants. Each of the treatments contained, 120 PLBs (45 days old) with three replications. The PLBs that remained viable without browning were counted after 10, 20 and 30 days of culture.

Agrobacterium tumefaciens strain and vector

Agrobacterium tumefaciens strain EHA 105 (Hood et al. 1993) harboring plasmid binary vector pBI121 was used for transformation. The vector, in its transfer DNA (T-DNA) of 6,193 bp, contains *npt II* (neomycin phosphotransferase II) (kanamycin resistance) for selection of transformed plant cells and the *gusA* gene, which encodes β-glucuronidase (GUS), to permit histochemical assay of transformed tissue. The *gusA* and *npt II* were driven by

CaMV 35S and NOS promoters, respectively, and the ends of T-DNA are flanked by 25 bp direct repeats, *viz*., left and right border.

Inoculation and co-cultivation

A single colony of *Agrobacterium* was transferred to 5 ml yeast extract peptone (YEP) broth containing 25 mg l^{-1} rifampicin and 50 mg l^{-1} kanamycin in a vial. The culture was incubated at 28°C in a rotary shaker at 200 rpm for 24 h; removed at mid-log phase (OD_{600}=0.5) when the cell density was approximately 10^8 ml^{-1} and diluted to 1/10 with half-strength MS basal medium. The PLBs were separated and used as explants for transformation experiments. A total of five different infection durations, *viz*. 5, 15, 30, 45 and 60 min were used. After infection, explants were blotted dry on a sterile filter paper and co-cultivated on MS regeneration medium supplemented with 0.3% sucrose, 4.43 μM BA, 2.68 μM NAA and 100 μM acetosyringone at 25±1°C in dark for 3 days.

The effect of acetosyringone in transformation was investigated separately by co-cultivating 100 explants in the regeneration medium with and without 100 μM acetosyringone. The experiment was repeated three times independently.

Regeneration and selection of transformants by kanamycin

After co-cultivation, the explants were washed in MS liquid medium, blotted dry on sterile filter paper, and transferred to MS regeneration medium containing 0.3% sucrose, 4.43 μM BA, 2.68 μM NAA supplemented with 50 mg l^{-1}kanamycin and 250 mg l^{-1} cefotaxime. After 15 days of culture, the surviving explants were transferred to the same regeneration medium but with a higher concentration of kanamycin (75 mg l^{-1}). Finally, explants surviving after 30 days were subjected to more stringent selection in regeneration medium supplemented with 100 mg l^{-1} kanamycin.

GUS histochemical assay

GUS histochemical assay (Jefferson 1987) was carried out on PLBs of five distinguishable developmental stages growing in the selection medium using a GUS Reporter Gene Staining Kit (Sigma, St. Louis, MO) by following the manufacturer's instructions. Each developmental stage was represented by three randomly selected independent lines. 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-GlcA) was used as the substrate. After staining for 16 h at 37°C, the tissues were incubated in 70% ethanol for 3 h to remove chlorophyll prior to observation.

Screening of transgenic lines using PCR

All the plantlets regenerated in the stringent selection medium were subjected to PCR. Total genomic DNA was isolated from leaf tissue by the CTAB method and subjected to PCR using *gusA* gene-specific oligonucleotide primer pairs: a forward primer 5′-GTTGGGGTTTCTA CAGGACG-3′ and a reverse primer 5′-GCAACTGGA CAAGGCACTAG-3′ with an expected amplicon of 686 bp. Each 25 μl PCR reaction mixture contained 50 ng genomic DNA as template, 200 μM dNTPs, 1.5 mM $MgCl_2$, 15 pM each of forward and reverse primers, and 1 unit *Taq* DNA polymerase in 1x reaction buffer. The reaction was carried out in a thermocycler (Eppendorf Master Cycler) at 92°C for 3 min for initial denaturation followed by 35 cycles of 92°C for 30 s, 56°C for 30 s and 72°C for 1 min. A final extension time of 10 min was allowed at 72°C at the end of 35 cycles to allow the completion of synthesis of any incomplete fragments. A reaction containing pBI121 as template served as a positive control and genomic DNA isolated from non-transgenic vanilla regenerated from PLB served as a template in the negative control. The reaction product was subjected to electrophoresis in 0.8% agarose gel and DNA bands were visualized under ultraviolet light after staining with ethidium bromide.

Southern and northern blot hybridization

Total genomic DNA isolated from leaf tissue by the CTAB method was subjected to Southern blot hybridization analysis using standard protocols (Sambrook and Russell 2001). DNA (10 μg) was restricted using *Sac*I and size-fractionated in 0.7% agarose gel by electrophoresis, subsequently transferred to a Hybond N + nylon membrane (Amersham Biosciences, Buckinghamshire, UK) by capillary method (Sambrook and Russell 2001). Plants that were positive in Southern analysis were screened for transcription of the transgene by northern hybridization. Total RNA was isolated from 300 mg leaf tissue by the method described by Chomczynski and Sacchi (1987). RNA (20 μg) was subjected to formaldehyde-agarose gel electrophoresis followed by transfer to Hybond N + nylon membrane by capillary method (Sambrook and Russell 2001). In both Southern and northern analyses, genomic DNA or total RNA isolated from a PLB-derived non-transgenic plant served as a negative control. Plasmid vector pBI121, after digestion with *Sac*I, served as a positive control in the Southern blot.

Probe preparation, hybridization and detection were performed using the DIG High Prime DNA Labelling and Detection Starter Kit II (Roche Applied Science, Indianapolis, IN) following the manufacturer's instructions. The

DNA probe was prepared by PCR amplification of *gusA* using pBI121 as template and *gusA* gene-specific oligonucleotide primer pairs. The amplified DNA fragment was isolated after gel fractionation, purified and labelled with digoxigenin (DIG) using the random primed labelling method as specified in the kit. Hybridization procedures and incubation with the chemiluminescence substrate CSPD were carried out in accordance with the manufacturer's instructions. Tracks of chemiluminescence were captured, developed and fixed in X-ray film (Kodak) as per standard procedures.

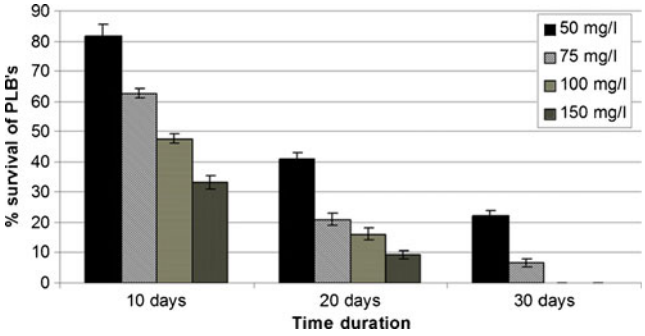

Fig. 2 Effect of increasing concentrations of kanamycin on proliferating PLBs

Results

Production of PLBs and their regeneration into plantlets

Out of ten media tested, MS medium supplemented with 0.45 μM thidiazuron (TDZ) induced maximum PLBs (37.2 ±2.1 PLBs per shoot tip) after 45 days of culture (Fig. 1; Table 1). TDZ at concentrations above 0.45 μM retarded growth, and resulted in lower numbers of PLB produced. It was observed that BA induced less PLBs at all the tested concentrations (4.43, 8.86 and 13.29 μM), whereas addition of NAA did not induce PLB production at any tested concentration, even after 60 days of culture. The MS medium containing TDZ did not support regeneration of PLB into plantlets. However, when the PLBs were transferred to MS medium containing 4.43 μM BA and 2.68 μM NAA, plantlets were formed after 60–75 days.

Genetic fidelity of PLB-regenerated plantlets

All plantlets derived through PLBs were genetically identical as revealed by RAPD profiles. Of the total of 238 loci analyzed by 23 random primers, none of the loci exhibited any polymorphism. This demonstrated that there was no genetic variation between regenerants and the parent plant from which they originated.

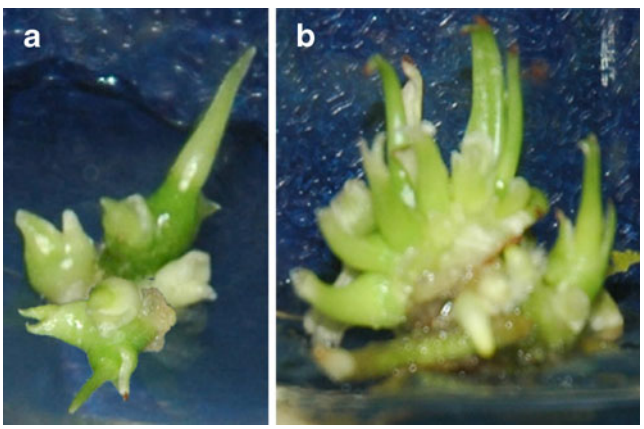

Fig. 1 Induction and proliferation of protocorm-like bodies (PLBs) in Murashige-Skoog (MS) medium + 0.45 μM thidiazuron (TDZ) after **a** 30 and **b** 45 days of culture

Kanamycin sensitivity study

Kanamycin sensitivity was studied by culturing PLBs on MS regeneration medium supplemented with 0, 50, 75, 100 and 150 mg l^{-1} kanamycin. Concentrations >100 mg l^{-1} kanamycin inhibited regeneration leading to complete death of PLBs after 30 days of culture (Fig. 2). At 75 mg l^{-1} kanamycin, 6.6±1.15% of PLBs survived until 30th day of culture, but PLBs were malformed and died later without regenerating into plantlets.

Inoculation and co-cultivation

To determine the optimum time duration for *Agrobacterium* infection, explants were subjected to five different infection durations, i.e., 5, 15, 30, 45 and 60 min. The results showed that 30 min was optimum as it yielded the highest number of transgenic events (43±2%) (Table 2). A gradual decline in transformation frequency was observed when the duration of infection was either increased or decreased.

Table 2 Effect of duration of *Agrobacterium* infection time on transformation frequency. Data is presented as mean value ± standard error of three experiments. Mean values within the column followed by the same letters are not significantly different according to Duncan's multiple range tests at $P \leq 0.05$

Infection duration (min)	No. of PLBs inoculated	No. of kanamycin-resistant PLBs recovered	Transformation efficiency %
5	100	28±2	28±2 a
15	100	33±3	33±3 bc
30	100	43±2	43±2 d
45	100	36±1	36±1 c
60	100	31±2	31±2 ab

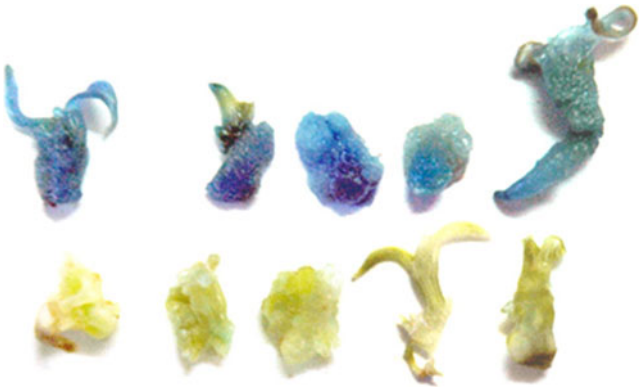

Fig. 3 Histochemical staining to detect β-Glucuronidase (GUS) activity in transformed tissue. *Upper line* Transgenic lines, *lower line* non-transgenic control plants

Results from experiments to test the effect of acetosyringone in transformation showed that addition of acetosyringone enhanced transformation frequency. Addition of 100 μM acetosyringone to the regeneration medium resulted in a high number of transgenic events (47.33± 0.57%) compared to medium without acetosyringone (12.66±1.15%). It was observed that, in the presence of acetosyringone, explants showed a delayed (1–2 days) response to culture conditions, indicating stress due to *Agrobacterium* infection.

Elimination of *Agrobacterium* after co-cultivation was effective when 250 mg l^{-1} cefotaxime was used in the selection medium. At this concentration, no *Agrobacterium* growth was seen in the medium even after 15 days of culture, and there was no apparent effect on PLBs developing in the regeneration medium.

Regeneration and selection of transformants by kanamycin

After co-cultivation with *Agrobacterium* for 3 days, explants were transferred to MS regeneration medium with a stepwise increase in the kanamycin concentration (50 mg l^{-1} kanamycin up to 15 days, 75 mg l^{-1} kanamycin from 16–30 days, and 100 mg l^{-1} kanamycin from 31–120 days) for selection of transformants. The results showed that, out of 280 co-cultivated PLBs, 134 (47.86± 1.85%) plantlets were regenerated in selection medium containing increasing amounts of kanamycin. After about 30–45 days in regeneration medium, PLB explants produced shoots and roots without an intervening callus phase. During direct adventitious shoot formation, the explants turned green and produced green nodular structures at the apical ends. Simultaneously, root initials were originated from the opposite ends of the PLBs. PLBs took 90–110 days for complete regeneration into plantlets.

GUS histochemical assay

Histochemical staining of 15 putative transformants showed uniform blue color, indicating expression of the *gusA* gene in contrast to the colorless negative control (Fig. 3). The entire plantlets were uniformly stained blue without any mosaic appearance, indicating that the plantlets had been regenerated from a single transformed cell and were not chimeric for the trait. There was no detectable endogenous GUS activity in non-transformed control plants.

Screening of plants by PCR, Southern and northern hybridization

Total genomic DNA isolated from all 134 regenerated plantlets was subjected to PCR using *gusA* gene-specific primer pairs. All produced the expected amplicon of 686 bp, whereas no amplification was observed in the non-transformed control plants, indicating that all putative transformed plants were positive for the transgene (Fig. 4). A set of 20 plants was randomly selected for Southern hybridization to confirm the integration of T-DNA into genomic DNA. Of these, 14 plants (70%) showed a positive reaction confirming stable integration of the *gusA* (Fig. 5). Out of the 14 Southern-positive plants, 11 produced a single band indicating the presence of single copy of the transgene; the remaining three had two bands implying two insertion events within the same genome. All the detected bands were above the expected minimum size of 5.9 kb as indicated by its position in the blot. No hybridization signal was observed in the non-transgenic plants.

Expression of the transgene was confirmed by northern hybridization where all the six plants tested showed the presence of transcript (Fig. 6). The non-transformed control

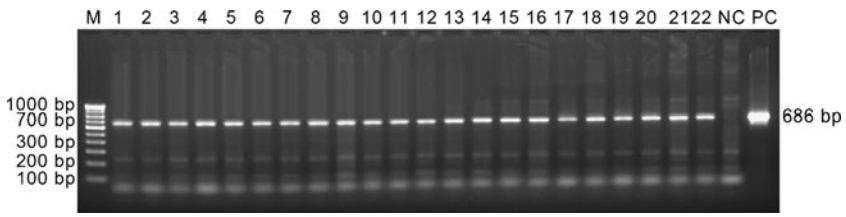

Fig. 4 PCR screening of the putative transgenic plants using a *gusA* gene-specific primer pair. Lanes: *M* Molecular weight marker, *1–22* putative transgenic plants, *NC* negative control, *PC* positive control

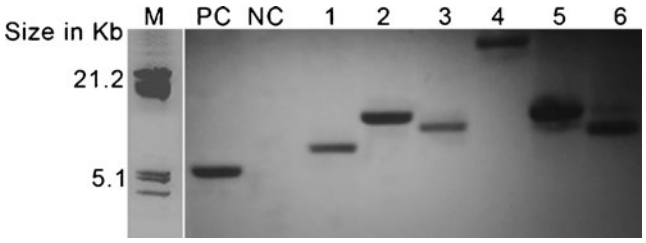

Fig. 5 Southern hybridization of putative transgenic plants using a DIG-labelled *gusA* gene as probe. Lanes: *PC* Positive control, *NC* negative control, *1–6* putative transgenic plants

plant did not show the presence of the *gusA* mRNA as no band was detected in the northern blot. This result clearly revealed that the transgene was integrated stably in the genome and was actively transcribed.

Discussion

The present study reports a good medium for the production of PLBs (from shoot tips) of vanilla, their genetic transformation using *Agrobacterium* harbouring *gusA* and *npt II* reporter genes and regeneration of transgenic plants. The integration and expression of the *gusA* gene in transgenic plants were confirmed through histochemical assay, PCR, and Southern and northern hybridization tests. Previously, Malabadi and Nataraja (2007) reported production of PLBs (29.6 per explant) in the presence of putrescine from thin section culture of shoot tips of vanilla. In the present study, we showed that the use of TDZ can further enhance the number of PLBs (37.2) produced per explant. RAPD results also confirmed the genetic fidelity of vanilla plantlets generated from PLBs. The direct induction of PLBs from shoot tip without going through a callus phase probably explained the genetic fidelity of the regenerated plants. Similar observations have been made in other orchids such as hybrid *Cymbidium* (Silva et al. 2006) and *Syngonium podophyllum* (Cui et al. 2008), in which PLB-derived plantlets were found to be genetically true to the type and uniform. Plantlet regeneration of vanilla was also reported from callus (Davidonis and Knorr 1991), root tips (Philip and Nainar 1986) and node explants (Kononowicz and Janick 1984), although these tissues have not been used as explants for genetic transformation so far. At all concentrations tested, TDZ did not cause any genetic change in vanilla in vitro, and the PLBs induced were amenable to *Agrobacterium*-mediated genetic transformation and regeneration.

The optimum time for *Agrobacterium* infection of explants was found to be 30 min. Increasing or decreasing the time led to decreased frequency of transformation. The decreased frequency of transformation observed with decrease in time duration (<30 min) of *Agrobacterium* infection may be due to the limited number of bacterial cells that could interact with the explant during this period. Similarly, the gradual decline observed when the duration of infection was >30 min may be due to overcrowding of bacterial cells that outgrew the explant. Similar findings have been reported in *Galega orientalis* (Collen and Jarl 1999), *Cajanus cajan* (Singh et al. 2002), *Cicer arietinum* (Husnain et al. 1997) and *Vigna unguiculata* (Muthukumar et al. 1996).

Explants co-cultivated for 3 days in regeneration medium supplemented with acetosyringone showed a higher frequency of transformation compared to medium without acetosryringone. Acetosyringone is a known inducer of the *vir* operon and enhances expression of genes involved in transformation, leading to high frequency transformation. The importance of acetosyringone in *Agrobacterium*-mediated transformation was reported in *Dendrobium* orchid (Men et al. 2003) and also in the transformation of monocot crops like golden pothos (Kotsuka and Tada 2008), maize (Ishida et al. 1996) and rice (Hei et al. 1994). The delayed response of explants observed in presence of acetosyringone may be due to stress because of the *Agrobacterium* infection on explants. Cefotaxime at 250 mg l^{-1} was able to control *Agrobacterium* growth after co-cultivation. Malabadi and Nataraja (2007) used 500 mg l^{-1} cefotaxime for vanilla transformation while other workers have used concentrations varying from 200 to 500 mg l^{-1} . Perez-Clemente et al. (2004) used 500 mg l^{-1} in transformation of *Prunus persica*; 250 mg l^{-1} for *Dendrobium* orchid transformation (Men et al. 2003) and 250 mg l^{-1} for transforming *Brassica juncea* (Dutta et al. 2008).

When Malabadi and Nataraja (2007) used 75 mg l^{-1} kanamycin for the selection of vanilla transformants, only 50% of the plantlets were transgenic. In the present study, it was observed that kanamycin at 100 mg l^{-1} could eliminate all untransformed PLBs after 30 days of culture. Assuming that such a high concentration may simultaneously cause sudden death or slow proliferation or malformation, a stepwise increase in kanamycin concentration followed during the selection of transformants. After co-cultivation with *Agrobacterium* for 3 days in regeneration medium

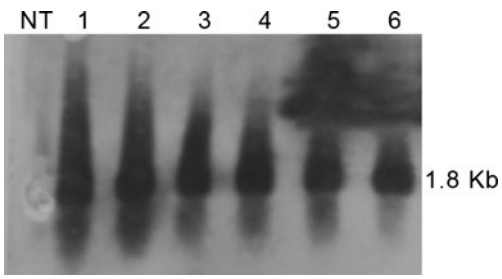

Fig. 6 Northern hybridization of transgenic vanilla using DIG-labelled *gusA* gene as a probe. Lanes: *NT* Non transgenic, *1–6* putative transgenic plants

devoid of antibiotic, PLBs were subjected to selection in three stages with increasing concentration of kanamycin; initially at 50 mg l^{-1} for 15 days followed by 75 mg l^{-1} for the next 15 days, and finally at 100 mg l^{-1} from the 30th day onwards. This procedure apparently reduced the sudden shock to the explants and increased the frequency of transgenic lines as shown by GUS histochemical assay (100%), PCR (100%) and Southern hybridization (70%). A similar selection strategy with increasing concentrations of antibiotic has been reported in the transformation of *Dendrobium phalaenopsis*, *D. nobile* (Men et al. 2003), peach (Perez-Clemente et al. 2004), mustard (Dutta et al. 2008), *Juncus accuminatus* (Nandakumar et al. 2007) and *Lathyrus sativus* (Barik et al. 2005). The regeneration efficiency of PLBs is 100% in a medium devoid of selection agent (kanamycin). In the present study, transformation efficiency was determined through the ability of PLBs to regenerate in selection medium; PCR test showed 47% efficiency, while it was 36.6% based on Southern hybridization. Malabadi and Nataraja (2007) reported a transformation frequency of 39% in vanilla, although the methodology (whether regeneration in selection medium or PCR or Southern) used to determine the transformation frequency was not indicated.

All 134 putative transformed plantlets showed a positive reaction in PCR, indicating the presence of the transgene. However, screening transgenics by PCR is not foolproof, as reported by many workers (Barik et al. 2005; Hei et al. 1994; Shekhawat et al. 2008) due to the possible presence of *Agrobacterium* in the transformed plants. Hence, in order to confirm the stable integration of the transgene, Southern hybridization is necessary. In the present study, Southern analysis confirmed the stable integration of *gusA* gene in 14 of the 20 putative transgenic plants, which apparently indicates that 70% of the PCR positive plants were also positive in Southern analysis. Bands detected by this probe would be expected to contain 5.9 kb DNA from the T-DNA and an unknown length of flanking plant DNA. This also allowed us to determine the number of transgenes integrated in a given plant. One to two copies of the transgene were detected in the transformed plants, with single copy transformation being predominant. All the detected bands were above the expected minimum size of 5.9 kb, as indicated by their position in the blot, thus ruling out integration of the truncated T-DNA. Overall, the insertion patterns observed on the Southern blot are consistent with the relatively simple T-DNA insertions typical of *Agrobacterium*-mediated transformation.

All six plants tested by northern hybridization showed a positive reaction, indicating that the integration event occurred in actively transcribed regions of the chromosome. Similar observations were reported by earlier workers in vanilla (Malabadi and Nataraja 2007) and *Dendrobium* (Men et al. 2003). Neither the antibiotic resistance gene (*npt II*) nor the β-glucuronidase gene (*gusA*) had any apparent effect on the normal development and morphology of the transgenic vanilla plants as all plants maintained in the greenhouse exhibited normal growth and morphology and had a uniform appearance. The method described in this paper and the efficiency of the transformation would allow production of transgenic vanilla—an otherwise a 'limited gene pool crop'—with genes of desirable characters.

The results presented here demonstrate an efficient methodology for induction of high frequency PLBs from shoot tips, and genetic transformation of vanilla plants expressing kanamycin antibiotic resistance and GUS activity. The highlights of this study include the three-step selection strategy and the use of acetosyringone, which allowed high frequency of transformed plantlets. This methodology can now be used to transfer other useful genes into vanilla.

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