

# Genetic transformation of a hepatoprotective plant, *Phyllanthus amarus*

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**Abstract** *Phyllanthus amarus* Schum & Thonn. is a source of various pharmacologically active compounds such as phyllanthin, hypophyllanthin, gallic acid, catechin, and nirurin, a flavone glycoside. A genetic transformation method using *Agrobacterium tumefaciens* was developed for this plant species for the first time. Shoot tips of full grown plants were used as explants for *Agrobacterium*-mediated transformation. Transgenic plants were obtained by co-cultivation of shoot tips explants and *A. tumefaciens* strain LBA4404 containing the pCAMBIA 2301 plasmid harboring neomycin phosphotransferase II (NPT II) and  $\beta$ -glucuronidase encoding (GUS) genes in the T-DNA region in the presence of 200  $\mu$ M acetosyringone. Integration of the NPT II gene into the genome of transgenic plants was verified by PCR and Southern blot analyses. Expression of the NPT II gene was confirmed by RT-PCR analysis. An average of 25 explants was used, out of which an average of 19 explants produced kanamycin-resistant shoots, which rooted to produce 13 complete transgenic plants.

**Keywords** Acetosyringone · *Agrobacterium tumefaciens* LBA4404 · GUS · NPT II · *Phyllanthus amarus* · Southern blot analysis

## Introduction

*Phyllanthus* Schum & Thonn. has been widely used by traditional medical practitioners for the treatment of jaundice

and other diseases. The Spanish name assigned to the plant is *chanca piedra*, which means “stone breaker” or “shatter stone.” It was named for its effective use to generations of Amazonian indigenous peoples in eliminating gallstones and kidney stones. In Brazil, the plant is known as *quebra-pedra* or *arranca-pedras* (which also translates to “break-stone”). The species *Phyllanthus amarus* is a small tropical herb, which occurs widely as a rainy-season weed throughout the hotter parts of India (Wealth of India 2003). The widespread usage of this herb has prompted several investigations (Calixto *et al.* 1998; Harish and Shivanandappa 2005). Various properties such as anti-HIV, anti-hepatitis B (Venkateswaran *et al.* 1987; Notka *et al.* 2004), antibacterial, and antifungal (Verpoorte and Dilhal 1987) have been associated with this plant for a long time.

Plant-based remedies have been highlighted due to their fewer side effects in comparison to synthetic drugs and antibiotics. Successful transformation technology is thought to be one of the most reasonable approaches to enhance the production of secondary metabolites through genetic manipulation of biosynthetic pathway (Mann *et al.* 2000). The genes for the key enzymes involved in the biosynthesis of lignans and its precursors, such as pinosresinol lariciresinol reductase and secoisolariciresinol dehydrogenase, have been cloned from *Forsythia intermedia* and *Podophyllum hexandrum* (Dinkova-Kostava *et al.* 1996; Xia *et al.* 2001). Genetic engineering of these enzymes may lead to high yield of the desired lignans. Hence, establishment of an efficient transgenic system is a prerequisite for genetic improvement of the valuable medicinal plant rich in therapeutically active secondary metabolites. A previous study reported the *Agrobacterium rhizogenes*-mediated transformation of *P. amarus* to obtain only transformed roots (Bhattacharyya and Bhattacharyya 2004). This paper, to the best of our knowledge, reports for the first time the successful high-

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efficiency *A. tumefaciens*-mediated genetic transformation and regeneration of complete true to mother-type plants of *P. amarus* with standardization of several factors like bacterial density, preculture period, co-cultivation time, various concentrations of plant growth regulators, kanamycin, and acetosyringone (AS). Molecular analyses confirmed the integration and expression of the transferred neomycin phosphotransferase II (NPT II) gene in transgenic plants.

## Materials and Methods

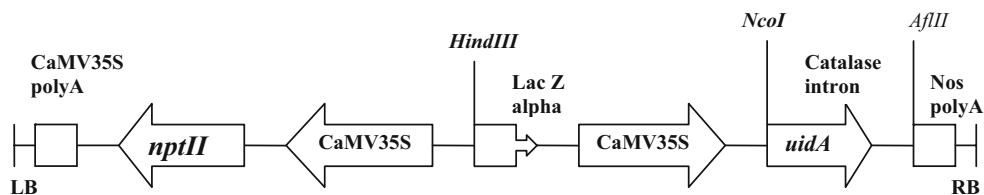
**Plant material.** *Phyllanthus amarus* Schum & Thonn. cultivated in the garden of Indian Institute of Chemical Biology was taxonomically identified from The Botanical Survey of India, Shibpur, Howrah. Voucher specimen (No. Pa 201, dated 26.06 06) was submitted there. *In vitro*-germinated seedlings of *P. amarus* were used as the source of explants for genetic transformation. Seeds were disinfected with sodium hypochlorite solution (containing 1% chlorine) with two to three drops of Tween 20 for 30 min and finally with 70% alcohol for 30 s. The seeds were washed thoroughly with sterile distilled water thrice. The disinfected seeds were germinated on MS-based medium (Murashige and Skoog 1962) with 1.5% sucrose and 0.8% agar at pH 5.8. Cultures were incubated in a plant tissue culture room with a photoperiod of 16 h (16  $\mu\text{mol m}^{-2} \text{s}^{-1}$  cool fluorescent light) at  $24 \pm 1^\circ\text{C}$  for a period of 10 wk. Shoot tips from 8-wk-old *in vitro*-raised plantlets of *P. amarus* were used as explants for *A. tumefaciens*-mediated transformation.

**Bacterial strain and binary vector.** *Agrobacterium tumefaciens* strain LBA 4404 harboring the binary vector pCAMBIA 2301 (Fig. 1; 11.6 kb) was used as the vector system for transformation. The plasmid contains a catalase intron in the GUS-coding sequence. The cauliflower mosaic virus 35S promoter (CaMV35S) and nopaline synthase terminator (NOS) drive the reporter gene. The selective marker gene NPT II is driven by CaMV35S promoter and terminated by the CaMV35S poly A signal. For infection, a single colony was grown overnight in liquid LB (Sambrook *et al.* 1989) supplemented with 100  $\text{mg l}^{-1}$  kanamycin

(Sigma, St. Louis, Missouri), 50  $\text{mg l}^{-1}$  of rifampicin (Sigma, St. Louis, Missouri), and 200  $\mu\text{M}$  AS (Sigma, St. Louis, Missouri) at  $28^\circ\text{C}$  on a shaker until the final O.D. of the culture reached to 0.8–1.0 at 600 nm. The bacterial culture was centrifuged, and the pellet was resuspended in liquid MSo medium supplemented with 0.1  $\text{mg l}^{-1}$  kinetin and 0.1  $\text{mg l}^{-1}$  indole 3-acetic acid (IAA) at concentrations of 0.1  $\text{mg l}^{-1}$  and 200  $\mu\text{M}$  AS. MSo is defined as medium containing inorganic and organic MS salts, 3% sucrose, with pH adjusted to 5.8. The culture was agitated on a shaker till the growth of the bacterium reached an O.D. of 0.8, which was used for infection.

**Transformation and regeneration.** A number of transformation parameters were studied in order to achieve the maximum transformation efficiency in *P. amarus*. The parameters included bacterial culture O.D., period of preculture, co-cultivation period, concentration of AS, concentration of kanamycin, and type of plant growth regulators used for regeneration. Each of these experiments was repeated thrice on independent days, keeping two replicates per experiment. Each experiment included 25 explants to study the effects.

Shoot tip explants taken from 8-wk-old plantlets were used for preculture or directly for transformation. Preculturing of explants was studied for a period of 0, 1, 2, and 3 d on MS medium with B<sub>5</sub> vitamins (Satyavathi *et al.* 2002) with or without plant growth regulators. The explants were injured with a hypodermic needle, shaken gently in the bacterial culture (O.D.) 0.8 (O.D. 0.8) for about 30 min, and blotted dry on a sterile filter paper. After infection, they were co-cultivated in the dark for 1, 2, 3, and 4 d at  $25 \pm 2^\circ\text{C}$ . The co-cultivation medium consisted of solidified MSo with or without plant growth regulators. The beneficial effects of AS were studied during co-cultivation by using AS at doses of 100, 200, and 300  $\mu\text{M}$ . The concentration of kanamycin in the regeneration medium was standardized using doses of 25, 50, 75, 100, and 125  $\text{mg l}^{-1}$  and measuring transformation efficiency of transgenic plants. Cefotaxime studied at doses of 62.5, 125, and 250  $\text{mg l}^{-1}$  was used to kill the adhering *Agrobacterium* after co-cultivation. Following co-cultivation, the explants were washed with sterile distilled water containing 125  $\text{mg l}^{-1}$  cefotaxime, blotted dry, and



**Figure 1.** Schematic representation of the T-DNA region of pCAMBIA2301. The plasmid contains a catalase intron in the GUS-coding sequence. The CaMV 35S and NOS terminator drive the GUS reporter

gene. The selective marker gene NPT II is driven by CaMV35S promoter and terminated by the CaMV35S poly A signal.

**Table 1.** Effect of preculture and co-cultivation period on transformation efficiency of *Phyllanthus amarus*

Transformation parameters <sup>a</sup>	No. of explants regenerating shoots on kanamycin-containing medium	Transformation efficiency (%) <sup>b</sup>
Preculture period (d) <sup>c</sup>		
0	3.4±2.5	11.6 a
1	8.0±3.0	30.4 b
2	15.0±2.5	60.0 c
3	9.0±1.2	33.3 b
Co-cultivation period (d) <sup>d</sup>		
0	6.0±4.0	30.4 b
1	8.0±1.5	22.2 ab
2	11.0±2.5	45.0 bc
3	15.0±3.2	58.7 c
4	4.0±0.2	14.2 a

<sup>a</sup> Each period was tested using 25 explants, keeping two replicates for three independent days.

<sup>b</sup> Percent of explants producing kanamycin-resistant and GUS-positive plants. Means with different letters were significantly different ( $p < 0.05$ ).

<sup>c</sup> Preculture was carried out using MSo medium supplemented with 0.1 mg l<sup>-1</sup> kinetin and 0.1 mg l<sup>-1</sup> IAA.

<sup>d</sup> Co-cultivation was carried out using MSo medium supplemented with 0.1 mg l<sup>-1</sup> kinetin and 0.1 mg l<sup>-1</sup> IAA and 200 μM AS.

transferred to nine kinds of regeneration medium containing MSo with thidiazuron (TDZ) at concentrations of 0.22, 0.44, 0.88, and 1.5 mg l<sup>-1</sup>; 6, benzyl amino purine (BAP) at 0.225, 0.44, 0.88, and 1.54 mg l<sup>-1</sup>; and MSo with kinetin and IAA at a concentration of 0.1 mg l<sup>-1</sup> each. All of the nine regeneration media contained kanamycin 50 mg l<sup>-1</sup> and cefotaxime 125 mg l<sup>-1</sup>. Following shoot regeneration, the shoots of 3–4 cm in length, the shoots were transferred to rooting media comprised of half-strength MS liquid basal medium supplemented with 0.7 mg l<sup>-1</sup> indole 4-butyric acid (IBA) and 5 mg l<sup>-1</sup> kanamycin. The regenerated transgenic

**Table 2.** Effect of composition of co-cultivation medium on transformation efficiency of *Phyllanthus amarus*

Composition of co-cultivation medium <sup>a</sup>	No. of explants regenerating shoots on kanamycin-containing medium	Transformation efficiency (%) <sup>b</sup>
MSo	5.2±1.3	20.9 a
MSo+Kn+IAA <sup>c</sup>	10.0±3.8	40.9 b
MSo+Kn+IAA+AS <sup>d</sup>	16.0±3.8	59.8 c

<sup>a</sup> Each medium was tested by using 27 explants, keeping two replicates per treatment for three independent days.

<sup>b</sup> Percent of explants producing kanamycin-resistant and GUS-positive plants. Means with different letters were significantly different ( $p < 0.05$ ).

<sup>c</sup> MSo medium supplemented with 0.1 mg l<sup>-1</sup> kinetin and 0.1 mg l<sup>-1</sup> IAA.

<sup>d</sup> MSo medium supplemented with 0.1 mg l<sup>-1</sup> kinetin and 0.1 mg l<sup>-1</sup> IAA and 200 μM AS.

**Table 3.** Effect of acetosyringone concentration in co-cultivation on transformation efficiency of *Phyllanthus amarus*

Concentration of AS <sup>a</sup> (μM)	No. of explants regenerating shoots on kanamycin-containing medium	Transformation efficiency (%) <sup>b</sup>
100	8.5±1.3	32.4 a
200	16.0±1.2	64.5 b
300	11.4±1.7	44.8 c

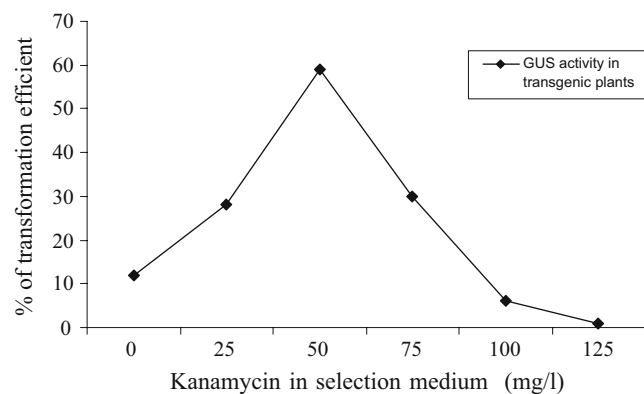
<sup>a</sup> AS was added to MSo medium supplemented with 0.1 mg l<sup>-1</sup> kinetin and 0.1 mg l<sup>-1</sup> IAA. Each concentration was tested using 25 explants, keeping two replicates for three independent days.

<sup>b</sup> Percent of producing kanamycin-resistant and GUS-positive plants. Means with different letters were significantly different ( $p < 0.05$ ).

plants were transferred to pots containing a mixture of vermiculite and soil (1:1) for acclimatization.

Explant mortality, number of explants expressing GUS, and GUS spots/explant were examined while standardizing the transformation parameters. The assay for the GUS activity in transgenic tissues was carried out as previously described (Jefferson *et al.* 1987).

**Molecular analyses.** The presence of the NPT II gene in the transformed shoots was analyzed through PCR with genomic DNA of five different plants obtained from transformations events. The NPT II-specific primer sequences were 5'-GAGGCTATT CCGCTATGACTG-3' and 5'-ATCGGGAGGGGCGATACCGTA-3'. Each PCR reaction was performed in 50 μl (total volume) of a reaction mixture consisting of 1× reaction buffer, 100 ng DNA, 200 μM dNTPs, 2.5 mM MgCl<sub>2</sub>, 66 ng of each primer, and 2.5 U of Taq DNA polymerase (Invitrogen, Carlsbad, CA). PCR was carried out in an Eppendorf Mastercycler personal (Eppendorf, Westbury, NY) under the following conditions: 94°C for 3 min as preheating, then 35 cycles of 94°C denaturing for 30 s, 55°C annealing for 45 s, 72°C synthesis for 1 min, and 10 min at



**Figure 2.** Effects of kanamycin concentration on transformation efficiency of *P. amarus*. Transformation efficiency is defined as the percentage of explants producing kanamycin-resistant and GUS-positive plants.

**Table 4.** Effect of plant growth regulators in kanamycin-containing regeneration medium on transformation efficiency of *Phyllanthus amarus*

Plant growth regulators (mg l <sup>-1</sup> ) <sup>a</sup>				No. of explants regenerating shoots on kanamycin-containing medium	Transformation efficiency (%) <sup>b</sup>
TDZ	BAP	Kinetin	IAA		
0.22	–	–	–	1.2±4.5	6.2 a
0.44	–	–	–	3.4±1.3	14.2 b
0.88	–	–	–	9.7±2.2	36.7 c
1.54	–	–	–	10.4±3.2	41.6 cd
–	0.22	–	–	1.5±1.2	4.7 a
–	0.44	–	–	2.8±2.8	10.5 ab
–	0.88	–	–	5.0±0.8	21.2 bc
–	1.54	–	–	6.2±1.8	24.7 bc
–	–	0.1	0.1	15.1±1.5	60.5 d

<sup>a</sup> Plant growth regulators were added to MSo medium. Each medium was tested using 25 explants, keeping two replicates for three independent days. All media contained 50 mg l<sup>-1</sup> kanamycin.

<sup>b</sup> Percent of producing kanamycin-resistant and GUS-positive plants. Means with different *letters* were significantly different ( $p < 0.05$ ).

72°C as final extension. Amplified DNA fragments were electrophoresed on 1.0% agarose gels detected by ethidium bromide staining and photographed through the Bio-Rad gel documentation instrument (Bio-Rad, Alfred Nobel Drive, Hercules, CA).

For Southern blot analysis, genomic DNA (10 µg) from five different transgenic plants obtained from independent transformation events was digested overnight with *Bam*H1. The restriction fragments resolved by electrophoresis on 0.8% agarose gels were blotted by the upward capillary method into Immobilon NY<sup>+</sup> membrane (Millipore, Billerica, Massachusetts) using 20× SSC transfer buffer. Membranes were probed with an α-<sup>32</sup>P-labeled NPT II fragment generated through the Fermentas (Fermentas, Hanover, Maryland) random priming method following manufacturer's instructions. Following 16 h of hybridization at 68°C, the membranes were washed with 2× SSC, 0.1% SDS at 68°C for 20 min followed by washing with 1× SSC, 0.1% SDS and finally with 0.1× SSC, 0.1% SDS for 10 min at room temperature. The washed membranes were wrapped in plastic wrap and subjected to autoradiography at -70°C for 72 h (Sambrook *et al.* 1989).

For RT-PCR analysis, total RNA was isolated from young shoots of *P. amarus* through the monophasic lysis reagent, TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol and treated with DNase (Invitrogen, Carlsbad, CA). RT-PCR was conducted using the Fermentas Revert First strand complementary DNA (cDNA) synthesis kit (Fermentas, Hanover, Maryland) following the manufacturer's instructions. PCR of the NPT II gene was carried out according to the conditions as described above. The house keeping gene actin was used as a control to indicate the amount of starting RNA. The sequences were designed from the *Nicotiana tabacum* actin gene (Accession no: X63603). The primers were 5'-CGCGAAAAGATGACTCAAATC-3' and 5'-AGATCCTTTCTGATATCCACG-3', which gave a 533-bp product with cDNA. The PCR products were electrophoresed on 1.0% agarose gels, detected by ethidium bromide staining, and photographed through the Bio-Rad gel documentation instrument.

*Statistical methods.* Standard statistical methods were followed. All data are the mean of values of three independent experiments. Statistical comparison between groups was performed with one-way ANOVA with the help

**Table 5.** Comparative profile of transgenic plants of *Phyllanthus amarus*

Experiment	No. of explants co-cultivated	No. of explants regenerating shoots on kanamycin-containing medium (%) <sup>a</sup>	No. of regenerated plants			No. of explants regenerating GUS-positive plants (%) <sup>d</sup>
			Kanamycin resistant	GUS positive <sup>b</sup>	PCR positive <sup>c</sup>	
1	25	20 (80.0)	17	13	11	13 (52.0)
2	25	21 (84.0)	18	14	12	14 (56.0)
3	25	19 (76.0)	17	14	12	14 (56.0)
Total	75	60 (80.0)	52	41	35	41 (54.7)

<sup>a</sup> Regeneration medium containing 50 mg l<sup>-1</sup> kanamycin was used.

<sup>b</sup> Plants showing GUS gene expression at the fully matured stage.

<sup>c</sup> Plants showing PCR amplification of the NPT II gene.

<sup>d</sup> Number of explants that regenerated at least one transgenic plant confirmed by GUS gene expression.



of Sigmapstat, version 3.01. Differences were considered significant at  $p < 0.05$ .

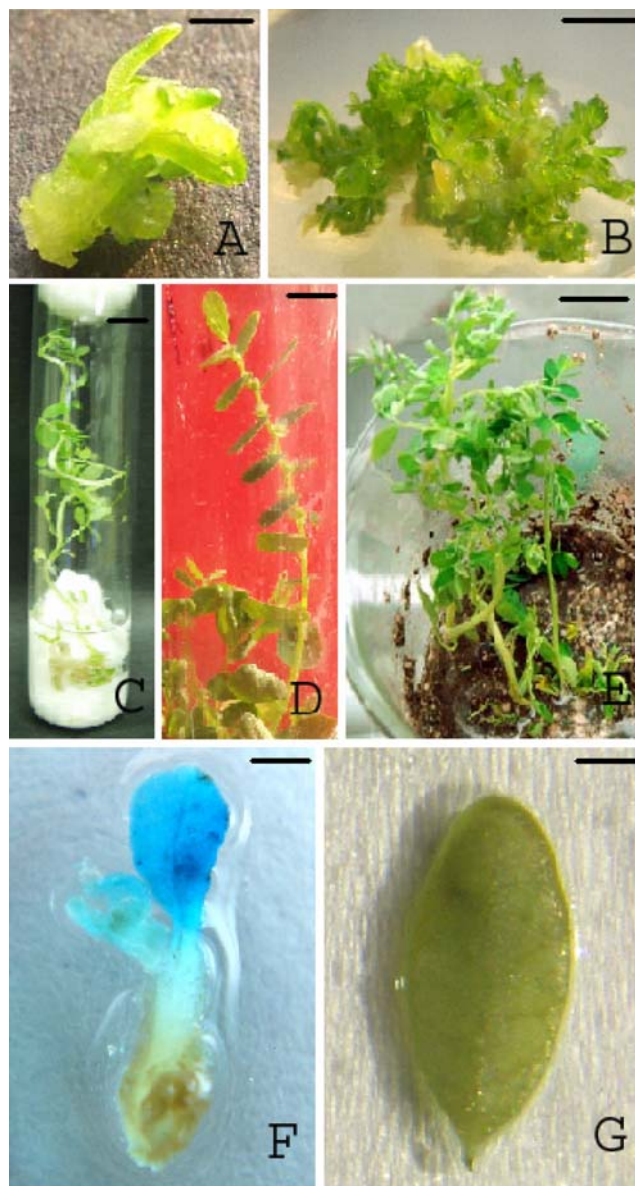
## Results and Discussion

**Agrobacterium strain and bacterial density.** In the present study, we used the *A. tumefaciens* strain LBA4404 harboring pCAMBIA2301, known to be an efficient transformation system for several dicotyledonous plants. Previous studies have shown that LBA4404 gave rise to a higher transformation frequency than the EHA101 or C58 strain (Tohidfar *et al.* 2005). Overnight grown culture of LBA4404 in LB resuspended in liquid MSo supplemented with  $0.1 \text{ mg l}^{-1}$  kinetin,  $0.1 \text{ mg l}^{-1}$  IAA, and  $200 \text{ }\mu\text{M}$  AS led to an efficient infection medium (data not shown). In our preliminary studies, it was found that usage of bacterial cultures at O.D. 0.8 was most suitable for infection (data not shown). An O.D. of more than 1 resulted in severe necrosis of the plant tissue during the co-cultivation period, as was found by Hu *et al.* (2006).

**Preculture period.** Preculturing of explants can have positive effects on transformation efficiency (Hu *et al.* 2006). Our results revealed that a preculture period of 2 d was effective for *P. amarus* (Table 1), producing a 60% of transformation efficiency. Preculture for longer periods of time decreased the transformation efficiency. The beneficial effect of a preculture period on transformation frequency in the presence of plant growth regulators could be attributed to promotive effects of plant growth regulators on cell division, and actively dividing cells are more vulnerable to delivery and integration of T-DNA (An 1985).

**Effects of plant growth regulators and AS during co-cultivation on transformation efficiency.** Co-cultivation is one of the most important steps for *Agrobacterium*-mediated transformation of plants (James *et al.* 1993). During this period, T-DNA is incorporated into plant genomic DNA. A period of 3 d co-cultivation at  $25^\circ\text{C}$  in the dark was optimal for *P. amarus* transformation efficiency (Table 1). Co-cultivation media supplemented with various plant growth regulators were examined to enhance the transformation efficiency, but only kinetin and IAA had a positive effect (data not shown). Further, it was found that co-cultivation medium without plant growth regulators did not increase the transformation efficiency compared to that supplemented with kinetin  $0.1$  and  $0.1 \text{ mg l}^{-1}$  IAA (Table 2). The presence of plant growth regulators in the co-cultivation medium provided stimuli to the cells and helped in high-frequency regeneration of transgenic shoots as compared to the usage of co-cultivation medium without plant growth regulators (Wang and Fang 1998). Further, AS at  $200 \text{ }\mu\text{M}$  in the co-cultivation medium was optimum in comparison to  $100$  and  $300 \text{ }\mu\text{M}$  (Table 3). The

stimulating effect of AS on transformation efficiency was also seen in Table 2. Co-cultivation medium supplemented with  $200 \text{ }\mu\text{M}$  AS produced an efficiency of 59.8% in comparison to that containing only kinetin and IAA (Table 2). This is in good agreement with previous studies

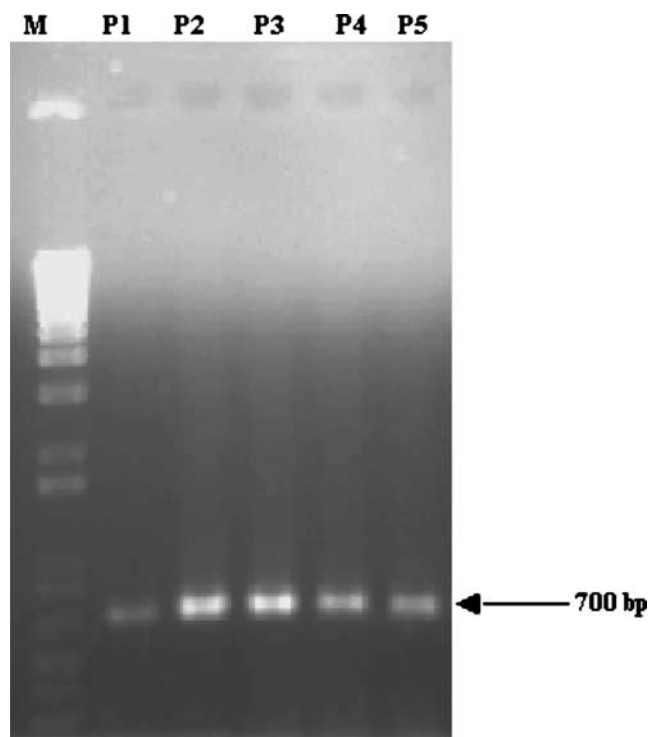


**Figure 3.** Regeneration of independent transgenic plants of *P. amarus* from shoot tip explants on MSo medium supplemented with  $0.1 \text{ mg l}^{-1}$  kinetin and  $0.1 \text{ mg l}^{-1}$  IAA  $50 \text{ mg l}^{-1}$  kanamycin and  $125 \text{ mg l}^{-1}$  cefotaxime. **A**, Initiation of multiple shoots from a shoot tip explant; *bar*= $0.5 \text{ cm}$ . **B**, Development of multiple shoots after 30 d of culture; *bar*= $1 \text{ cm}$ . **C**, Elongation of shoots in half-strength MSo liquid medium containing  $0.7 \text{ mg l}^{-1}$  IBA; *bar*= $2 \text{ cm}$ . **D**, *In vitro* flowering of transgenic shoots in half-strength MSo liquid medium containing  $0.7 \text{ mg l}^{-1}$  IBA; *bar*= $1 \text{ cm}$ . **E**, Acclimatization of a transgenic plant in 1:1 sand and soil mixture; *bar*= $1 \text{ cm}$ . **F**, GUS activity as evidenced by development of blue color in the lamina of a leaf of one of the transgenic plant; *bar*= $0.1 \text{ cm}$ . **G**, Control, non-transgenic leaf showing no development of blue color; *bar*= $0.1 \text{ cm}$ .

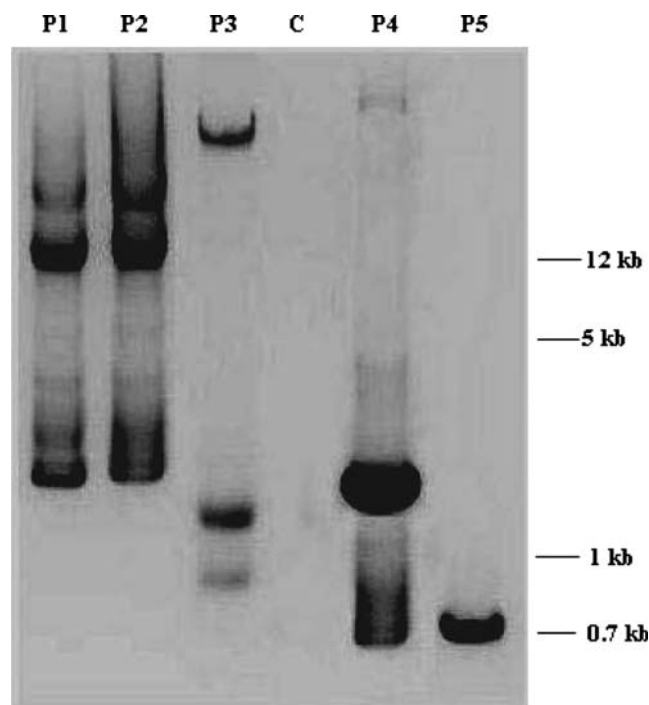
such as in *Malus domestica* (James *et al.* 1993) and *Oryza sativa* subsp. *indica* (Rashid *et al.* 1996, etc.). The phenolic compound AS has been known to induce vir genes and increase transformation frequency (Shimoda *et al.* 1990).

**Sensitivity to kanamycin.** Kanamycin, a widely selective agent used for plant transformation, can be phytotoxic and retards the growth of the transformed tissue if used at high concentrations. The ability to regenerate transgenic shoots in different concentrations of kanamycin was studied (Fig. 2). It was seen that transformation efficiency reached to 28% at 25 mg l<sup>-1</sup> kanamycin, as reflected by GUS histochemical assays. Kanamycin at 50 mg l<sup>-1</sup> was used for selection, and maximum GUS activity was noted (Fig. 2). A high dose of kanamycin, such as 75, 100, and 125 mg l<sup>-1</sup> decreased transformation efficiency (Fig. 2). To suppress the growth of *A. tumefaciens*, cefotaxime was used at a concentration of 125 mg l<sup>-1</sup> after screening with different concentrations of cefotaxime (data not shown).

**Regeneration of transgenic plants.** After co-cultivation, the shoot tip explants were placed on nine kinds of regeneration medium, and transformation efficiency was determined (Table 4). All of these media contained 50 mg l<sup>-1</sup> kanamycin and 125 mg l<sup>-1</sup> cefotaxime. After 2 wk of selection, numerous shoots regenerated from the shoot tips on all these media. The data reveal that 1.54 mg l<sup>-1</sup> TDZ was



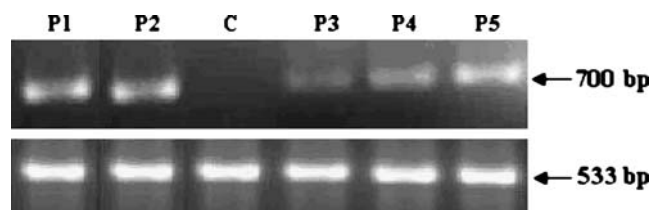
**Figure 4.** PCR analysis for detecting the NPT II gene in five transgenic plants of *P. amarus*. Lane M, 250 bp DNA ladder (Invitrogen); lanes P1–P5, independent transgenic plants. Transgenic plants show the amplification of the NPT II gene as visualized by the appearance of a 700-bp fragment.



**Figure 5.** Southern blot analysis showing of five transgenic plants of *P. amarus*. BamHI-digested genomic DNA was hybridized with the NPT II gene probe. Lane C, control, non-transgenic plant; lanes P1–P5, independent transgenic plants. Transgenic plants show independent T-DNA incorporation into the genomic DNA.

better in regenerating transgenic clusters (41.6% transformation efficiency) compared to the same dose of BAP (24% transformation efficiency). However, both produced stunted shoots. Out of the nine regeneration media, that containing 0.1 mg l<sup>-1</sup> kinetin and 0.1 mg l<sup>-1</sup> IAA was optimal with 60.5% transformation efficiency. Elongation of shoots also took place in this medium. In accordance with previous reports, multiple transgenic shoots with normal morphology regenerated very well with the lower dose of plant growth regulators (Bhattacharyya and Bhattacharyya 2001; Ghanti *et al.* 2004). The elongated shoots were transferred to the rooting medium to obtain complete plants.

A total of 75 shoot tip explants were used in three independent transformation experiments, of which 52 explants



**Figure 6.** RT-PCR analysis of the expression of the NPT II gene in five transgenic plants of *P. amarus*. Lane C, control, non-transgenic plant; lanes P1–P5, independent transgenic plants. Transgenic plants show an amplified band of 700 bp. The lower panel shows the amplification of actin used as a loading control.

produced transgenic shoots on regeneration medium containing  $0.1 \text{ mg l}^{-1}$  kinetin,  $0.1 \text{ mg l}^{-1}$  IAA,  $50 \text{ mg l}^{-1}$  kanamycin, and  $125 \text{ mg l}^{-1}$  cefotaxime (Table 5). After two to three rounds of subculture using the same medium, these transgenic plants were transferred to rooting medium to produce complete plantlets. A total of 41 complete transgenic plantlets were obtained from three independent transformation experiments. The transformation efficiency was found to be 54.6%. The transgenic plants had the same morphology as that of normal plants (Fig. 3E).

**GUS gene expression.** GUS activity was checked after 30 d of growth on kanamycin-containing regeneration media. GUS gene expression was primarily detected at the lamina and petiole of the leaves, as evidenced by the development of a blue color (Fig. 3F). On the other hand, leaves from the control plants, when treated with the same method, developed no color. Eighty percent of the kanamycin resistant was GUS positive.

**Molecular analyses.** To confirm the incorporation of T-DNA into the plants, molecular analysis was carried out with genomic DNA from five regenerated plants through PCR and Southern blot analyses. PCR amplification with NPT II primers and transgenic plant genomic DNA revealed the presence of a 700-bp fragment (Fig. 4). PCR amplification using the control plant genomic DNA showed no bands, indicating thereby the absence of NPT II gene. To confirm the incorporation of T-DNA into genomic DNA and to check the fidelity of PCR products, Southern blot analyses revealed the presence of several strong hybridization signals (Fig. 5), which were observed in all five transgenic plants used for analysis. Different band sizes of different intensities suggested variable integration into the genomic DNA of the transgenic lines. Further, to confirm the nature of transgenic plants growing on the kanamycin-containing media, expression studies of NPT II gene were carried out. cDNA isolated from the independent transgenic plants was subjected to RT-PCR analysis. The gel photograph showed the presence of a band of 700 bp amplified from the cDNA products of five transgenic plants, while the control RNA showed no amplification (Fig. 6).

Plant regeneration is usually a bottleneck for the development of a highly reproducible transformation protocol (Anuradha *et al.* 2006). The present study reveals that it is possible to obtain transgenic *P. amarus* within 4–5 mo by using shoot tips as a target for *Agrobacterium*-mediated transformation. In conclusion, the development of an efficient transformation protocol for *P. amarus*, a medicinal plant that produces plenty of useful secondary compounds, can lead to the genetic improvement of the herb.

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## References

- An G. High-efficiency transformation of cultured tobacco cells. *Plant Physiol.* 79: 568–570; 1985.
- Anuradha T. S.; Jami S. K.; Datla R. S.; Kirti P. B. Genetic transformation of peanut (*Arachis hypogea* L.) using cotyledonary node as explant and a promoter less gus:nptII fusion gene based vector. *J. Biosci.* 31: 235–246; 2006.
- Bhattacharyya R.; Bhattacharyya S. High frequency *in vitro* propagation of *Phyllanthus amarus* Schum & Thonn. by shoot tip culture. *Ind. J. Exp. Biol.* 39: 1184–1187; 2001.
- Bhattacharyya S.; Bhattacharyya R. Development of a potent *in vitro* source of *Phyllanthus amarus* roots with pronounced activity against surface antigen of the hepatitis B virus. *In Vitro Cell. Dev. Biol. Plant* 40: 504–508; 2004. doi:10.1079/IVP2004560.
- Calixto J. B.; Santos A. R. S.; Filho V. C.; Yunes R. A. A review of the plants of the genus *Phyllanthus*: their chemistry, pharmacology, and therapeutic potential. *Med. Res. Rev.* 18: 225–258; 1998. doi:10.1002/(SICI)1098-1128(199807)18:4<225::AID-MED2>3.0.CO;2-X.
- Dinkova-Kostava A. L.; Gang D. R.; Davin L. B.; Bedgar D. L.; Chu A.; Lewis N. G. (+)-Pinoresinol/(+)-lariciresinol reductase from *Forsythia intermedia*. *J. Biol. Chem.* 271: 29473–29482; 1996. doi:10.1074/jbc.271.46.29473.
- Ghanti K. S.; Govindaraju B.; Venugopal R. B.; Ramgopal Rao S.; Kaviraj S. P.; Jabeen F. T. Z.; Barad A.; Rao S. High frequency shoot regeneration from *Phyllanthus amarus* Schum & Thonn. *Ind. J. Biotechnol.* 3: 103–107; 2004.
- Harish R.; Shivanandappa T. Antioxidant activity and hepatoprotective potential of *Phyllanthus niruri*. *Food. Chem.* 95: 180–185; 2005. doi:10.1016/j.foodchem.2004.11.049.
- Hu Z.; Wu Y. R.; Li W.; Gao H. Factors affecting *Agrobacterium tumefaciens*-mediated genetic transformation of *Lycium barbarum* L. *In Vitro Cell. Dev. Biol. Plant* 42: 461–466; 2006.
- James D. J.; Uratsu S.; Cheng J.; Negri P.; Viss P.; Dandekar A. M. Acetosyringone and osmoprotectants like betaine or proline synergistically enhance *Agrobacterium*-mediated transformation of apple. *Plant Cell Rep.* 12: 559–563; 1993. doi:10.1007/BF00233060.
- Jefferson R. A.; Kavanagh T. A.; Bevan M. W. GUS fusions:  $\beta$ -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* 6: 3901–3907; 1987.
- Mann V.; Harker M.; Pecker I.; Hirschberg J. Metabolic engineering of astaxanthin production in tobacco flowers. *Nature Biotechnol.* 18: 888–892; 2000. doi:10.1038/78515.
- Murashige T.; Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15: 473–497; 1962. doi:10.1111/j.1399-3054.1962.tb08052.x.
- Notka F.; Meier G.; Wagner R. Concerted inhibitory activities of *Phyllanthus amarus* on HIV replication *in vitro* and *ex vivo*. *Antiviral Res.* 64: 93–102; 2004.
- Rashid H.; Yokoi S.; Toriyama K.; Hinata K. Transgenic plant production mediated by *Agrobacterium* in indica rice. *Plant Cell Rep.* 20: 701–705; 1996.
- Sambrook J.; Fritish E. F.; Maniatis T. Molecular—cloning a laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor 1989.
- Satyavathi V. V.; Prasad V.; Gita Lakshmi B.; Lakshmi Sita G. High efficiency transformation protocol for three Indian cotton



- varieties via *Agrobacterium tumefaciens*. *Plant Sci.* 162: 215–223; 2002. doi:10.1016/S0168-9452(01)00547-7.
- Shimoda N.; Toyoda-Yamamoto A.; Nagamine J.; Usami S.; Katayama M.; Sakagami Y.; Michida Y. Control of expression of *Agrobacterium vir* genes by synergistic action of phenolic signal molecules and monosaccharides. *Proc. Natl. Acad. Sci. U S A* 87: 6684–6688; 1990. doi:10.1073/pnas.87.17.6684.
- The Wealth of India A dictionary of Indian raw materials and industrial product. CSIR, New Delhi, India, pp 34–36; 2003.
- Tohidfar M.; Mohammadi M.; Ghareyazie B. *Agrobacterium*-mediated transformation of cotton (*Gossypium hirsutum*) using a heterologous bean chitinase activity. *Plant Cell Tiss. Org. Cult.* 83: 83–96; 2005. doi:10.1007/s11240-004-6155-2.
- Venkateswaran P. S.; Millman I.; Blumberg B. S. Effect of an extract from *Phyllanthus niruri* on hepatitis B Virus and woodchuck hepatitis viruses: *in vitro* and *in vivo* studies. *Proc. Natl. Acad. Sci. USA* 87: 274–278; 1987. doi:10.1073/pnas.84.1.274.
- Verpoorte R.; Dilhal P. P. Medicinal plants of Surinam—IV. Antimicrobial activity of some medicinal plants. *J. Ethnopharmacol.* 21: 315–318; 1987. doi:10.1016/0378-8741(87)90107-3.
- Wang G. L.; Fang H. L. Mechanism and technology of plant genetic engineering. Science Publisher, Beijing; 1998.
- Xia X.; Costa M. A.; Pelissier H. C.; Davin L. B.; Lewis N. G. Secoisolariciresinol dehydrogenase purification, cloning, and functional expression. *J. Biol. Chem.* 276: 2614–12623; 2001.