Journal of Medicinal Plants Research Vol. 5(11), pp. 2316-2321, 4 June, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals

Full Length Research Paper

Agrobacterium- Mediated transformation of pharmaceutically important Indian medicinal herb *Bacopa monnieri* (L.)

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Accepted 21 February, 2011

An efficient, rapid and simple *Agrobacterium* -mediated transformation protocol was developed for ayurvedic and pharmaceutically valuable Indian medicinal herb *Bacopa monnieri* (L.) using EHA 105 strain harboring the binary vector pCAMBIA 1301 containing *hpt* and *gus* genes. *In vitro* derived node explants were immersed in *Agrobacterium* suspension (OD600 = 0.8) for 10 min, and co-cultured on Murashige and Skoog (MS) co-cultivation medium supplemented with 0.1 mg/l NAA, 1.0 mg/l BAP and 0.1 mg/L GA_3 at 25 ± 2°C for 3 days. Putatively transformed plants were selected by the ability of node segments to produce hygromycin resistant plantlets with intermittent callus after 45 days of culture on selection medium containing 10 mg/l hygromycin and 250 mg/l cefotaxime. Gus histochemical analysis of putatively transformed plants and transgenic tissues confirmed transformation event. Polymerase chain reaction (PCR) analysis was performed to confirm the stable integration of *gus* transgene. A transformation frequency of 68.8% was achieved.

Key words: Brahmi, node segments, *gus* (β – glucuronidase), *hpt* (hygromycin phosphotransferase).

INTRODUCTION

In a report by the World Health Organization (WHO, Geneva), nearly 80% of the populations of developing countries rely on traditional medicine, mostly plant based drugs for their primary health care needs. One fifth of all the plants found in India are used for medicinal purpose (Schippmann et al., 2002), about 10% of the known medicinal plants of India are restricted to non-forest habitats, and *Bacopa monnieri* is one among the medicinal herb with multipurpose therapeutic uses. The rapid progress in the area of crop biotechnology is mainly because of the development of efficient regeneration and suitable *Agrobacterium* -mediated transformation protocols for different crop species (Hiei et al., 1997). Similar success can be achieved in medicinal plants also by developing efficient regeneration and *Agrobacterium* -mediated transformation protocols, which in turn could be used for the enhancement of their secondary metabolite content through metabolic engineering (Mahmoud and Croteau, 2001).

B. monnieri L. commonly known as 'brahmi' is a member of Scrophulariaceae family. The plant is an important medicinal herb in Indian subcontinent grows up to 1320 m elevation. It grows in damp and marshy places and on the banks of slow flowing rivers and lakes. The perennial creeper is a glabrous, sprawling succulent herb, with ayurvedic medicinal importance (Tiwari et al., 2001). In traditional medicine, the plant is widely used for the treatment of insomnia, mental instability, infertility in females, and also enhancing power of speech. Bacoside A and B are the two principal steroidal saponins in the plant, in addition to herpestine, brahmine, hersaponin,

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Abbreviations: MS, Murashige and Skoog; **BAP**, N_6 – Benzylaminopurine; **GA_3**, Gibberellic acid; **NAA,** α-Naphthalene acetic acid.

nictotine, luteolin and its glucosides, stigmasterol, stigmaotanol B-sitosterol, monnierini, betulic acid, and phytosterols (Jain and Kulshrestha, 1993). On the basis of medicinal importance, common value and potential for future research and development, the herb is placed second in a priority species list of the most important medicinal plants (Rajani, 2008), and identified as one among 32 medicinal plants identified for cultivation, conservation and development by the National Medicinal Plants Board (NMPB), Government of India (NMPB, 2004). Brahmi is recommended for immediate attention by NMPB, and Technology Information Forecasting and Assessment Council (TIFAC) and categorized in the list of highly endangered plants in India (http://www.nmpb.nic.in/prioritisemedicinalplants.htm).

The annual market demand for this ayurvedic important herb is estimated around 1000 tons during 2007 and it is likely to be more in the coming years due to its multipurpose medicinal uses (NMPB, 2008).

Our earlier reports on *in vitro* micropropagation (Ramesh et al., 2006), synthetic seed mediated plant conversion after storage at different temperature profiles (Ramesh et al., 2008) and genetic stability analysis in micropropagated and hardened plants (Ramesh et al., 2011) widened the scope for research in this important medicinal herb. Though *Agrobacterium* -mediated transformation from leaf explants were reported earlier (Nisha et al., 2003), high frequency regeneration of transgenic plants from *in vitro* derived node explants of *B. monnieri* using hygromycin as plant selection marker have not yet been reported.

MATERIALS AND METHODS

In vitro micropropagation and hardening

In vitro micropropagation of *B. monnieri* was achieved using the previously optimized protocol from our laboratory (Ramesh et al., 2006, 2008) using Murashige and Skoog (1962) medium supplemented with NAA (0.1 mg/L), BAP (1.0 mg/l), GA_3 (0.1 mg/l), 3% (w/v) sucrose, and solidified using 0.8% (w/v) phyta agar. The pH of the micropropagation medium was adjusted to 5.75 to 5.8 with 0.5 N NaOH and autoclaved at 121°C for 20 min. For *in vitro* micropropagation, node cuttings were cultured in phyta jars plant tissue culture container (Hi-Media, India) containing 50 ml medium. The cultures were maintained in a culture room at 25 ± 2°C under a photoperiod of 16:8, with a light intensity of 80 to 100 µmol m^{-2} s^{-1}. Micropropagated plantlets with profuse root system were hardened (Ramesh et al., 2008) and maintained in the shade house.

Agrobacterium –mediated transformation of *B. monnieri* nodes

The general protocol followed for *Agrobacterium* -mediated transformation after optimizing the conditions was as follows. The uninodal segments aseptically excised from axenic cultures of *B. monnieri* were preincubated for two days in shoot regeneration medium, which comprised of basal MS medium supplemented with 0.1 mg/l NAA, 1.0 mg/l BAP and 0.1 mg/l GA_3 (Figure 1A). *Agrobacterium* -mediated transformation was performed following the protocol described by Hiei et al. (1994) with a few modifications. A single colony of *Agrobacterium tumefaciens* strain EHA 105 harboring the binary vector pCAMBIA 1301 was grown overnight at 28°C with shaking (200 rpm) in liquid YEP medium (5 ml) containing 10 mg/l rifampicin and 100 mg/l kanamycin. An aliquot of the bacterial suspension (25 µl) was transferred to 50 ml fresh YEP medium containing the same antibiotics and again incubated under the same condition. When the culture was at density of OD600 = 0.8, cells were pelleted by centrifugation at room temperature, 5000 rpm for 10 min, and the resulting pellet was resuspended in 5 ml liquid MS medium. Preincubated nodal explants were swirled in bacterial suspension for 10 min, blotted dry on sterile Whatman No. 1 filter paper in order to remove the residual cells on the explants and placed on solid co-cultivation medium. After 3 days of co-culture at 25°C, co-cultivated explants were washed with 50 ml MS medium supplemented with 250 mg/l of cefotaxime, blot dried on sterile filter paper.

Selection of transformants

After co-cultivation explants were transferred to MS-SEL medium (MS salts, B5 vitamins, 0.1 mg/l NAA, 1.0 mg/L BAP, 0.1 mg/L GA_3, 250 mg/L cefotaxime, hygromycin 10 mg/L , 3% (w/v) sucrose and solidified using 0.8% (w/v) phyta agar, pH 5.8). Cultures were incubated in the culture room at 25°C. Transformed explants were passed through two selection cycles of 15 days each, and at the end of second selection, only the hygromycin resistant node segments with intermittent calli and small shoot buds were transferred to fresh medium for plant regeneration. Putatively transformed plantlets were transferred to Magenta boxes containing sterile sand and garden soil, covered with plastic bags to maintain humidity and maintained in the growth chamber for two weeks. Acclimatized plantlets were later established to pot containing autoclaved mixture of red soil, sand and vermiculite in a shade house.

Histochemical gus assay

Hygromycin resistant node, leaf and whole putatively transformed plants were immersed overnight in a reaction mixture containing X-gluc as substrate, and gus activity was analyzed histochemically as described by Jefferson (1987). Gus expression was visually observed and photographed. The frequency of transient transformation is expressed as the ratio between the number of explants showing blue coloration and the total number of explants kept for staining.

Genomic DNA extraction and PCR analysis

To confirm the stable integration of *gus* gene, genomic DNA was extracted from the transformed and non-transformed leaves by cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson, 1980). PCR analysis was carried out to confirm the presence of transgenes in the primary transformant (T_0). The primer sequences were: *gus*, 5'-GGT GGG AAA GCG CGT TAC AAG-3' and 5'-GTT TAC GCG TTG CTT CCG CCA-3'. PCR was performed for *gus* in a 25 µl reaction volume containing 1X PCR buffer, 1.5 mM $MgCl_2$, 20 pmole of each primers, 1.25 mM dNTPs, 1.0 U of Taq polymerase and ~50 ng of template. Amplification was performed in a programmable Thermal cycler (Eppendorf, Germany) under following conditions, 1X (94°C for 5 min), 40X (94°C for 1 min, 55°C for 1 min, 72°C for 2 min), 1X (72°C for 10 min). Five microlitres of the PCR product was resolved on ethidium-bromide-stained 1% agarose gel and visualized using a UV transilluminator.

RESULTS AND DISCUSSION

In vitro micropropagation

In two of our earlier reports, we optimized various concentrations of auxins (NAA and IAA) and cytokinins (BAP and adenine sulphate) for high frequency multiple shoot induction from *in vitro* derived nodal explants (Ramesh et al., 2006) and encapsulation of node segments of *B. monnieri* (Ramesh et al., 2008). Plantlets with multiple shoots and roots were transferred to shade house and maintained.

Regeneration of transformants

In recent years, plant transformation mediated by *A. tumefaciens* has become the most used method for the introduction of foreign genes into plant cells and the subsequent regeneration of genetically modified plants due to its simplicity and efficiency. The approach has been reported to be successful in several food crops, but limited in medicinal plants. In this work, an improved *Agrobacterium* –mediated transformation approach was achieved using *in vitro* micropropagated *B. monnieri* plants derived node segments as target explants.

A total of 90 node segments were co-cultivated in 3 independent transformation experiments and 45 of them were transferred to selection medium. Node segments co-cultivated for 10 min showed signs of survival after two selection cycles of 15 days each. All the co-cultivated explants developed intervening callus tissues during the second selection cycle. While the control uninfected explants inoculated onto the selection medium showed browning and senescence. Hygromycin-resistant node segments transferred to fresh medium proliferated into shoots within two weeks and most of the regenerated plantlets were with tiny shoots and leaves (Figure 1B). A total of 31 putatively transformed hygromycin resistant plantlets were recovered with an average frequency of 68.8% (Table 1). Since there are no other reports on this important ayurvedic medicinal plant using hygromycin as selection marker and development of transgenics using specific genes, extensive research in this field is required. In the present study, all the *gus* assayed leaves from five randomly selected putatively transformed plants showed strong and stable blue coloration along the midrib regions of the leaves and nodes (Figures 1C to E). The general distribution and different levels of *gus* activity obtained in different explants of *B. monneri* indicate the potential of pCAMBIA 1301 vector for stable transformation of medicinal plants. Similar observation has been previously reported in kanamycin resistant *B. monnieri* plants transformed with *A. tumefaciens* strain EHA 105 harboring the binary vector pBE2113 (Nisha et al., 2003).

The presence of *hpt* gene encoding *hygromycin phosphotransferase* enzyme facilitates the survival of only transformed explants and plants in selection medium. The vector pCAMBIA 1301 used in the present study has been proven ideal for transformation as reported earlier (Saharan et al., 2004). The inhibitory effect of hygromycin (10 mg/l) on the growth of non-transformed explants was seen within 8 to 10 days. None of the untransformed node segments are able to survive in selection medium with hygromycin and inclusion of appropriate concentration of selective agents in the regeneration medium is proved to be a critical factor in distinguishing true resistant plants from the non-transformed plants also called as escapes. Regenerated plantlets with well-developed shoots and roots transferred to Magenta boxes containing sterile sand and garden soil survived in the growth chamber and after 4 to 6 weeks, only 50% of putatively transformed plants were successfully acclimatized in shade house (Figure 1F).

Transformation efficiency

Further, in this study we have shown that the T-DNA is stably maintained in the genome of primary transformant (T_0). Genomic DNA obtained from five independent hygromycin and *gus* positive plants revealed the presence of amplification product of 1200 bp *gus* transgene (Figure 2). In our three independent experiments, we achieved a transformation frequency of 68.8% and on average; we recovered 10 plants per 15 co-cultivated node segments (Table 1). The final transformation frequency reported here for *Agrobacterium* -mediated transformation of *B. monnieri* node segments using hygromycin phosphotransferase as selection marker was higher than that reported by Nisha et al. (2003) with a final transformation frequency of 63.50% using neomycin phosphotransferase as selection marker and leaf segments as explants. This improved final transformation efficiency might be due to the enhanced infection given at the cut ends of freshly obtained young node segments. The reported final transformation efficiency was also higher when compared with other medicinal plants; for example, Tae et al. (2005) achieved 1 to 3% efficiency for the medicinal plant *Taraxacum platycarpum* using EHA105/pCAMBIA vector and GV3101/ pBI121. Norwati et al. (2007) used pCAMSB1 for the biolistic transformation of *Tectona grandis* and obtained final transformation efficiency of 59%. Whereas, Nanditha et al. (2008) and obtained an efficiency of 33.33% for *Centella asiatica* using EHA105/pCAMBIA2301. Similarly Franklin et al. (2007) obtained a transformation frequency of 55% for the medicinal plant *Hypericum perforatum* using *A. tumefaciens* strains LBA4404 and EHA105, and *A. rhizogenes* strains A4 and LBA9402 harboring pCAMBIA 1301. Only in few medicinal plants, transformation frequency higher than 68.8% has been reported. For example, Yan and Wang (2007) obtained an efficiency of 86.7% for *Salvia miltiorrhiza;* Khawar et al. (2003)

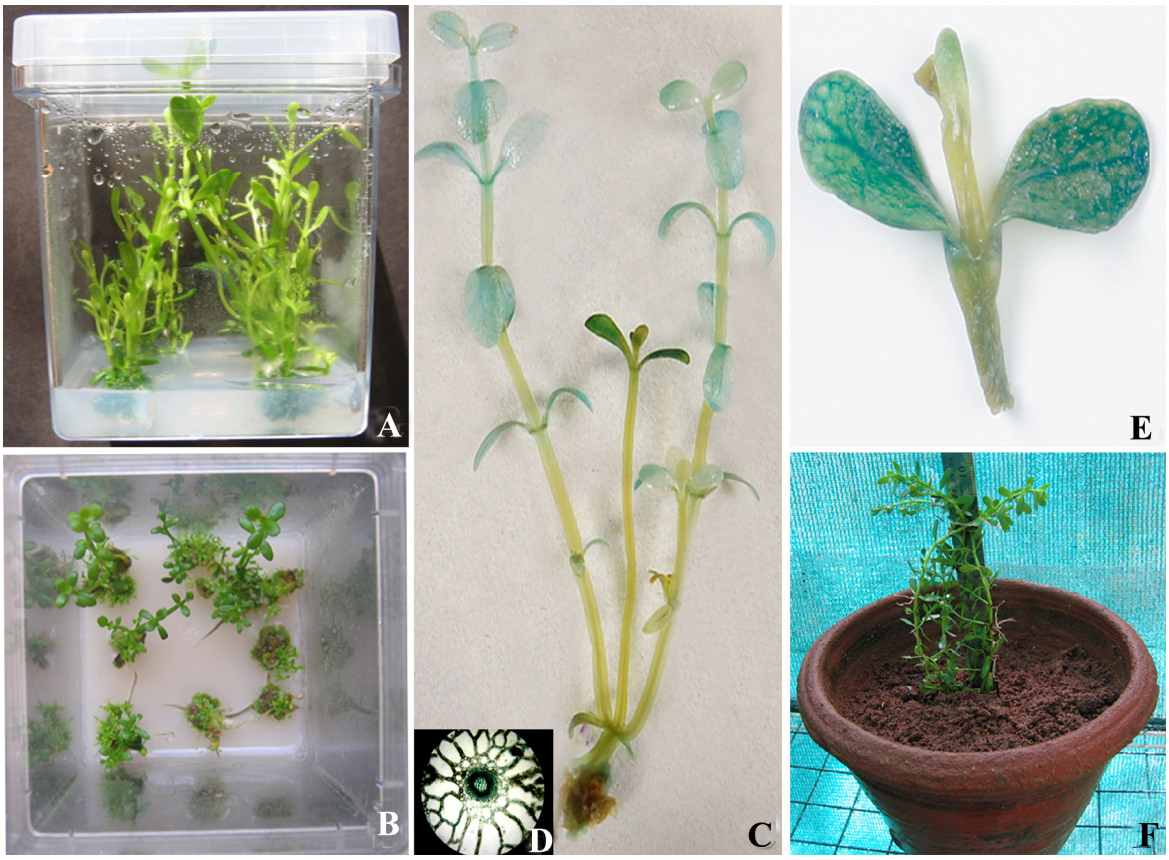

Figure 1. *Agrobacterium* - mediated transformation of *B. monnieri* using node explants. (A) Direct induction of adventitious shoots from node explants not subjected to *A. tumefaciens* treatment and cultivated on hygromycin-free medium (control); (B) multiplication of hygromycin-resistant regenerated adventitious shoots; (C) Primary transformant (T_0) showing GUS expression; (D) Cross section of node segments showing *gus* expression (40x); (E) Blue *gus* spot in transformed leaf explants; (F) acclimatized transformed plant.

Table 1. Stable transformation frequency of *B. monnieri* node segments co-cultivated with *Agrobacterium* strain EHA105 harbouring pCAMBIA 1301.

S/No.	Number of node segments co-cultivated	Number of node segments transferred to selection medium	Number of hygromycin resistant plantlets regenerated	Stable transformation frequency (%)
1.	30	15	10	66.6
2.	30	15	11	73.3
3.	30	15	10	66.6
Mean±SE			10.33 ± 0.51	68.8 ± 1.99

produced 100% transformation efficiency for *Salvia sclarea* and *S. pratense* using *Agrobacterium* strain A281 harboring pTiBo542 and pBI121.1. Therefore, it could be concluded that this improved protocol using node segments and hygromycin phosphotransferase as selection marker would be a better choice for other related medicinal herbs to improve the good agronomic quality and higher content of pharmaceutical compounds or a modified secondary metabolites profile in future.

ACKNOWLEDGEMENT

The authors gratefully acknowledge the use of the Bioinformatics Infrastructure Facility, Alagappa University funded by the Department of Biotechnology, Ministry of

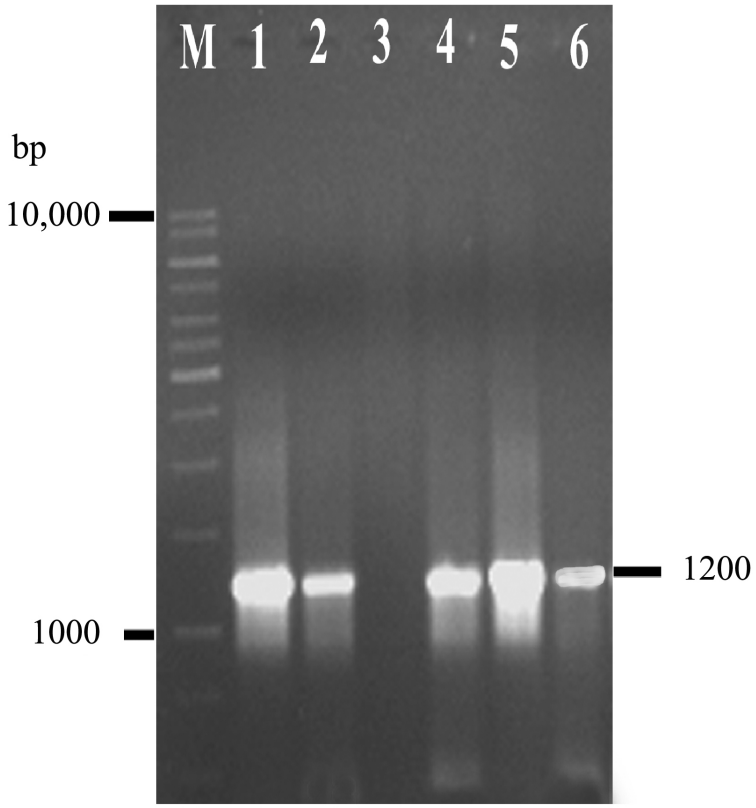

Figure 2. Polymerase chain reaction analysis of five randomly selected transgenic T_0 lines showing integration of the coding region of *gus* gene (1200 bp) in the genome of randomly selected putatively transformed plants. M, 1 kb ladder; 1, *gus* amplified pCAMBIA 1301 as a positive control; 2, 4-6, transgenic plants showing the *gus* amplification; 3, untransformed plant.

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