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Agrobacterium tumefaciens-Mediated Genetic Transformation of *Vigna aconitifolia* and Stable Transmission of the Genes to Somatic Seedlings

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Abstract: This investigation highlights a reproducible method of *Agrobacterium*-mediated genetic transformation developed for *V. aconitifolia* cv BMB-43. Efficient plant regeneration via somatic embryogenesis has been developed using leaf explants of *in vitro* derived seedlings. The plasmid contained a bi-functional fusion gene conferring both neomycin phosphotransferase gene (*nptII*) and β-glucuronidase (GUS) gene (*gus A)* interrupted with an intron, both driven by the Cauliflower Mosaic Virus (CAMV) 35S promoter. Using a series of tissue culture media for the establishment of embryogenic cultures, co-cultivation, selection, maturation and rooting, we recovered transgenic mothbean plants. Expression of positive histochemical GUS activity (51.0%) in the transgenic seedlings was observed. The integration of the stable transgenes (65.3%) in the plant genomes was shown by means of PCR amplification of these genes from plant genomic DNA and Southern blot hybridization with gene-specific probes. This method allows high-efficiency production of transgenic plants in mothbean.

Key words: *Agrobacterium*, genetic transformation, legumes, mothbean

INTRODUCTION

Agrobacterium-mediated transformation has been used successfully in grain legumes for over a decade. Biotechnological approaches such as gene transfer for enhanced disease and pest resistance offer opportunities for rapid improvement of mothbean. However, the availability of an *in vitro* regeneration system is a prerequisite for effective genetic transformation (Davis *et al*., 1993; Popelka *et al*., 2004). *Vigna aconitifolia* (Jacq.) commonly called as mothbean, is an important food legume of substantial economic importance in arid and semiarid areas (Malabadi and Nataraja, 2003). It is widely adapted, nutritious and an important energy and protein source for both human and animal consumption. It also improves soil fertility through the symbiotic nitrogen fixation. It is the most drought resistant crop among kharif pulses (Malabadi, 2002). On account of its mat like growth habit, it is useful against wind erosion in sandy areas and its pods are consumed as green vegetables. Its green as well as dry plants make a good fodder (Malabadi, 2002; Malabadi and Nataraja, 2003). Abiotic and biotic factors limit the yield in mothbean. Insects are the major constraint to mothbean production and attack virtually every developmental stage of the crop. Unfortunately, interspecific hybridization with mothbean has been largely unsuccessful and the wild species have not responded well to introgression through conventional breeding techniques for the yield improvement. A reproducible, reliable transformation system, combined with traditional breeding techniques, could aid in improving both the quality and yield of this crop. To meet the needs of both functional genomics and cultivar improvement, a simple, inexpensive, rapid and efficient transformation system successful with a range of cultivars is ideally required (Sharma and Ortiz, 2000).

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Legumes are generally considered recalcitrant to regeneration in tissue culture and thereby hampering the genetic transformation (Somers *et al*., 2003; Popelka *et al*., 2004). Somatic embryogenesis and organogenesis has been reported in several grain legumes including mothbean (Malabadi, 2002; Malabadi and Nataraja, 2002, 2003). Prolific literature is also available on genetic transformation of grain legumes *viz*., *Vigna unguiculata* (cowpea) (Popelka *et al*., 2006; Garcia *et al*., 1986, 1987; Ikea *et al*., 2003; Muthukumar *et al*., 1996; Penza *et al*., 2004), *Cicer arietinum* (chickpea) (Polowick *et al*., 2000, 2004; Fontana *et al*., 1993; Jaiwal *et al*., 2001; Kar *et al*., 1996, 1997; Krishnamurthy *et al*., 2004; van Rheenen *et al*., 1993; Senthil *et al*., 2004; Tewari-Singh *et al*., 2004), *Cajanus cajan* (pigeonpea) (Dayal *et al*., 2003; Geetha *et al*., 1999; Lawrence and Koundal, 2001), *Arachis hypogea* (peanut) (Eapen and George, 1994; Brar *et al*., 1994; McKently *et al*., 1995; Rohini and Rao, 2000; Sharma and Anjaiah, 2000), *Vigna mungo* (black gram) (Saini and Jaiwal, 2005; Saini *et al*., 2003), *Pisum sativum* (pea) (Schroeder *et al*., 1993; Grant *et al*., 1995), *Glycine max* (soybean) (Aragao *et al*., 2000; Christou *et al*., 1989; Hinchee *et al*., 1988; Zhang *et al*., 1999) and *Phaseolus vulgaris* (beans) (Aragao *et al*., 1998).

However, there are only few reports are available to date on genetic transformation in mothbean (Kamble *et al*., 2003; Eapen *et al*., 1987; Kohler *et al*., 1987a,b; Lee *et al*., 1993). The goal of the investigation reported here was to develop a reliable transformation method for *Vigna aconitifolia* cv BMB-43. We described herein a reproducible method of transformation, recovery of transgenic plants and the subsequent testing of both expression and inheritance of the introduced gene.

MATERIALS AND METHODS

Plant Material and Establishment of Embryogenic Tissue

Fresh seeds of *Vigna aconitifolia* cv. BMB-43 were procured from the University of Agricultural Sciences, Dharwad, Karnataka state, India. Seeds were surface sterilized with 70% alcohol and 0.1% mercuric chloride for 5 min, rinsed three times with sterile double distilled water. Seeds were germinated aseptically on MS medium (Murashige and Skoog, 1962) with 2.0% sucrose (Hi media, Mumbai) and 0.7% agar (Sigma St. Louis, USA) under cool white fluorescent light (100 μmol m^{-2} s^{-1}) at 25± 2°C with a relative humidity of 55-60%. Explants were taken from the leaves of 10-day-old aseptically grown seedlings. Leaf segments of approximately 5.0 mm^2 were cut and placed on callus induction medium containing MS medium supplemented with 5.0 μM IBA, 9.3 μM KN and 150 mg L^{-1} adenine sulphate (Induction medium) in 150 mmx25 mm culture tubes (Malabadi and Nataraja, 2002, 2003). The pH of the medium was adjusted to 5.8 prior to autoclaving at 1.04 kg cm^{-2} for 15 min. The cultures were incubated for 4 to 6 weeks under cool white fluorescent light (100 μmol m^{-2} s^{-1}) at 25±2°C with a 16 h photoperiod. Embryogenic structures such as globular, oval and heart shaped embryos were identified through the routine microscopic observation of cultures and the cultures were again subcultured on induction medium supplemented containing 9.3 μM KN and 10% v/v coconut water (Malabadi and Nataraja, 2002, 2003) for another 2 to 4 weeks for the bulking of embryogenic tissue for the following transformation experiments.

Agrobacterium Strain and Culture Medium

Agrobacterium-tumefaciens strain EHA105 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}; $MgSO_4$. $7H_2O$ 0.2 g L^{-1}; KH_2PO_4 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. Bacteria were pelleted at 4, 000 rpm for 10 min and suspended in a liquid MS basal medium

supplemented containing 9.3 μM KN and 10% v/v coconut water (induction medium) (Malabadi and Nataraja, 2002).

Genetic Transformation

Embryogenic tissue showing globular, oval and heart shaped embryos precultured in a liquid MS medium supplemented with 9.3 μM KN and 10% v/v coconut water (induction medium) (Malabadi and Nataraja, 2002) were infected by the bacteria for 3 h (infection period) on rotating shaker at 300 rpm. After 3 h of infection, embryogenic tissue was blotted out on sterile filter paper and co-cultured on semi-solid MS medium containing 9.3 μM KN and 10% v/v coconut water (induction medium) under a 16 h photoperiod at 25±2°C for 3 days. After 3 days (co-cultivation period), co-cultured embryogenic tissue on filter papers were transferred on semi-solid MS medium containing 9.3 μM KN and 10% v/v coconut water (induction medium), 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. The cultures were maintained for at least 4 weeks with 2 subcultures.

Histochemical Test

GUS activity was determined immediately after co-cultivation of embryogenic tissue by histochemical GUS assay according to Jefferson (1987). Embryogenic tissue 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 embryogenic tissue on filter paper was subcultured on MS medium supplemented with 10 μM ABA and 5% (v/v) coconut water (maturation medium). The cultures were incubated in dark condition for at least 6 to 9 weeks for the maturation of somatic embryos. After 9 weeks, individual somatic embryos were isolated and placed on half-strength MS medium without growth regulators. Fully germinated somatic embryos matured into transgenic plantlets within 2 to 3 weeks.

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 of *nptII*, 2.0 μL of 10xPCR buffer (Roche, 100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3), 0.5 μL of 10 mM dNTP stock (Operon Technologies). The negative control mixture contained all reagents except 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 *et al.* (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* (530bp or 5.3kb) was labeled with digoxigenin-11-dUTP in the PCR conditions according to Saini and Jaiwal (2005). 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.

Analysis of Transgene Inheritance

The levels of transgene integration were analyzed for the presence of *nptII* gene using PCR and Southern blot analysis as described above.

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 ($a = 0.05$) following ANOVA. All statistical analysis was performed using the SPSS statistical software package.

RESULTS AND DISCUSSION

A current trend in genetic transformation of recalcitrant grain legumes has been to target meristems as a source of totipotent cells. In the investigation reported here, rapid and efficient regeneration methods for such target cells were based on regeneration of plantlets via somatic embryogenesis. Somatic embryogenesis would be an ideal regeneration procedure for any transformation system, due to the single cell origin of embryoids. In our study, the leaf explants cultured on MS medium supplemented with 5.0 μM IBA, 9.3 μM KN and 150 mg L^{-1} adenine sulphate produced a mass of embryogenic tissue. Our results showed an average of 34.6% of somatic embryogenesis using leaf as the best source of explants at least in case of mothbean variety BMB-43 (Table 1). This will also confirm our previous studies on the establishment of embryogenic system in mothbean (Malabadi and Nataraja, 2002, 2003). Legumes in general have been particularly recalcitrant to *in vitro* regeneration and this is a bottleneck for genetic transformation. After co-culture of embryogenic tissue with *Agrobacterium*, additional subculture on MS medium containing 9.3 μM with 10% v/v coconut water induced further development and maturation of somatic embryos. Further development of embryos and complete transgenic plantlets occurred when somatic embryos were transferred to half strength MS medium without growth regulators. We therefore opted for regeneration via somatic embryogenesis, which has been a successful platform for transformation protocols at least

Table 1: Recovery of transgenic seedlings following *Agrobacterium*-mediated genetic transformation of *V. aconitifolia* cv. BMB-43

Experiment details	Somatic embryogenesis	GUS (%)	Transformation (%)	Total No. of somatic embryos recovered per gram fresh wt. of callus	Total No. of transgenic seedlings recovered per gram fresh wt. of callus
I	36.0±2.6a	46.0±3.1a	68.0±2.8a	7.0±0.1b	6.0±0.2b
II	42.0±1.7a	53.0±2.5a	63.0±2.5a	9.0±0.4b	5.0±0.1b
III	26.0±1.2a	54.0±2.9a	65.0±3.7a	17.0±0.6b	10.0±0.8b
Mean	34.6±2.7a	51.0±3.4a	65.3±3.1a	11.0±0.4b	7.0±0.1b

I, II, III represents 3 independent experiments conducted separately. Mean ±SE followed by the same letter (s) in each column were not significantly different at p = 0.05

in case of mothbean cv. BMB-43. The success of *Agrobacterium*-mediated transformation depends in part on the efficient interaction of *Agrobacterium* with the host plant cells. 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. In case of previous studies with *Agrobacterium*-mediated transformation and direct DNA transfer using PEG-mediated transformation of protoplasts and electroporation in mothbean has shown that *V. aconitifolia* protoplasts could be transformed and regenerated into plants (Kamble *et al*., 2003; Eapen *et al*., 1987). This is also in conformity with the findings of the present study. However, the frequency of transformation by using particle bomabardment was low. The method developed in this investigation which combines the somatic embryogenesis, optimized co-culture conditions and *Agrobacterium* strain selection, yields efficiencies of transformation about an order of magnitude better than most other systems (Popelka *et al*., 2006; Polowick *et al*., 2000, 2004; Dayal *et al*., 2003; Rohini and Rao, 2000; Zhang *et al*., 1999).

In the present study, susceptibility of embryogenic tissue to agro-infection may be developmentally regulated, as the plant derived factors recognized by *Agrobacterium* may be missing in the tissue at the development stage inoculated. 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 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, 65 contained positive neomycin phosphotransferease gene (*nptII*) resulting in higher transformation efficiency (65.3%) than GUS (51.0%). These values are the mean of 3 independent experiments tested with the genotype BMB-43 (Table 1). Further, the average number of somatic embryos recovered per gram fresh wt of embryogenic tissue on maturation medium after co-culture with *Agrobacterium* was found to be 11.0 (Table 1). This has resulted in the formation of 7.0 transgenic seedlings per gram fresh wt of embryogenic tissue (Table 1). The expression of the downstream Gene *UidA* (GUS) suggested that the neomycin phosphotransferase gene (*nptII*) must have transcribed. However, the efficient translation of transgenes of prokaryotic origin to eukaryotes needs to be investigated. 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 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 (5.3kb) contains a single *KpnI* site at the multiple cloning site located in the *lacZ* alpha region. The number of hybridization signals indicated

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 and 4 = Genomic samples of transgenic mothbean seedlings showing the integration of *nptII* gene at 530bp (5.3 kb). Lane 3 = Genomic samples of control (non-transgenic) plants of mothbean

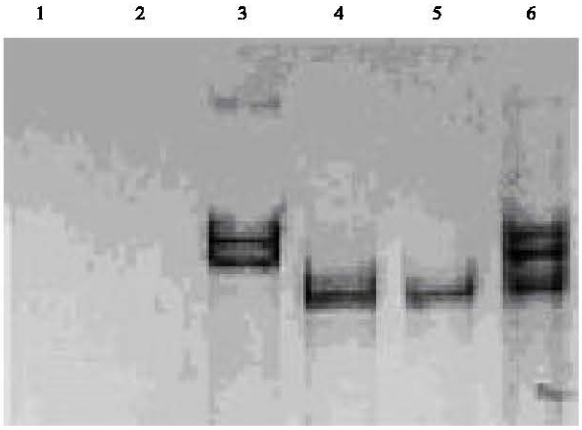

Fig. 2: Southern blot analysis of DNA of 4 independent transformed mothben plantlets and non-transformed (control) plants. Lanes 1 and 2 from non-transformed control plants. Lanes 3, 4, 5 and 6 from transformed mothbean plants showing single and multiple copies of genes integrated into the plant genome following successful transformation

that few plants have a single copy and others have a 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 legumes (Ikea *et al*., 2003; Aragao *et al*., 1998; Grant *et al*., 1995; Krishnamurthy *et al*., 2004).

The devised transformation protocol is rapid, reproducible and efficient and might be applicable to several genotypes of mothbean. The method of mothbean transformation described herein is not laborious in the initial preparation of the explants. However, the use of mature seed is advantageous, as it does not require a continuous supply of developing material. In order to considered truly viable and effective, the method of transformation should be reproducible with a broad range of mothbean genotypes. The cultivar used in this study *viz*., BMB-43 was selected because it is locally grown and also we have pre-determined responses in tissue culture. Therefore, success with other cultivars can not be anticipated and it needs the standardization of tissue culture protocols. These results pave the way for the introduction of new traits into mothbean. Therefore, the next step is to use this transformation system to introduce insect protection and other genes into mothbean germplasm to complement existing breeding programs. This will help guarantee food availability by increasing and stabilizing yields of this important legume crop.

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