Development of an efficient in vitro plant regeneration system amenable to *Agrobacterium*-mediated transformation of a recalcitrant grain legume blackgram (*Vigna mungo* L. Hepper)

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Received: 16 May 2015 / Revised: 4 July 2015 / Accepted: 7 July 2015 / Published online: 26 July 2015
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Abstract An efficient, rapid and direct multiple shoot regeneration system amenable to *Agrobacterium*-mediated transformation from primary leaf with intact petiole of blackgram (*Vigna mungo*) is established for the first time. The effect of the explant type and its age, type and concentration of cytokinin and auxin either alone or in combination and genotype on multiple shoot regeneration efficiency and frequency was optimized. The primary leaf explants with petiole excised from 4-day-old seedlings directly developed multiple shoots (an average of 10 shoots/ explant) from the cut ends of the petiole in 95 % of the cultures on MSB (MS salts and B₅ vitamins) medium containing 1.0 µM 6-benzylaminopurine. Elongated (2–3 cm) shoots were rooted on MSB medium with 2.5 µM indole-butyric acid and resulted plantlets were hardened and established in soil, where they resumed growth and reached maturity with normal seed set. The regenerated plants were morphologically similar to seed-raised plants and required 8 weeks time from initiation of culture to establish them in soil. The regeneration competent cells present at the cut ends of petiole are fully exposed and are, thus, easily accessible to *Agrobacterium*, making this plant regeneration protocol amenable for the production of transgenic plants. The protocol was further successfully used to develop fertile transgenic plants of blackgram using *Agrobacterium tumefaciens* strain EHA 105 carrying a binary vector pCambia2301 that contains a neomycin phosphotransferase gene (*nptII*) and a β-glucuronidase (GUS) gene (*uidA*) interrupted with an intron.

The presence and integration of transgenes in putative T₀ plants were confirmed by polymerase chain reaction (PCR) and Southern blot hybridization, respectively. The transgenes were inherited in Mendelian fashion in T₁ progeny and a transformation frequency of 1.3 % was obtained. This protocol can be effectively used for transferring new traits in blackgram and other legumes for their quantitative and qualitative improvements.

Keywords Direct shoot regeneration · Primary leaf · Blackgram · *Agrobacterium tumefaciens*

Introduction

Blackgram (*Vigna mungo* L. Hepper) is a key grain legume with its origin (Lukoki et al. 1980) and center of genetic diversity in India (Zeven and de Wet 1982). Because of its high protein content and perfect combination of all nutrients, including proteins (25–26 %), carbohydrates (60 %), fat (1.5 %), minerals, amino acids and vitamins (Karamany 2006), it is extensively used in Asia especially in India and now also grown in the Southern United States, the West Indies, Japan and other tropics and subtropics (Delic et al. 2009). Viral and fungal pathogens lead to severe yield losses in blackgram (Sahoo and Jaiwal 2008) and to overcome this problem, attempts have been made to obtain resistant cultivars either by conventional breeding or genetic transformation. Due to limited genetic variation among cultivated accessions and sexual incompatibility with wild relatives, genetic engineering is a good alternative for conventional breeding. However, for the production of a transgenic crop, a well-defined in vitro shoot regeneration system is required.

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Direct shoot regeneration alleviates somaclonal variation frequent in plants generated from cultured cells or tissues (Dayal et al. 2003; Mujib 2005). Direct shoot organogenesis and whole plant regeneration in blackgram has been achieved from cotyledonary node (Franklin and Ignacimuthu 2000; Saini et al. 2003; Muruganantham et al. 2005; Mony et al. 2010), cotyledon and embryonic axis (Sen and Guha-Mukherjee 1998; Ignacimuthu and Franklin 1999), shoot apex (Das et al. 2002a, b; Saini and Jaiwal 2005) and epicotyl (Saini and Jaiwal 2002) explants. Indirect shoot organogenesis *via* callus formation has been reported from various blackgram explants (Das et al. 2002a, b; Varalaxmi et al. 2007; Harisaranraj et al. 2008; Srivastava and Pandey 2011). Although, direct shoot regeneration from leaf petiole explants has been reported in a few legumes, e.g. soybean (Wright et al. 1987), cowpea (Muthukumar et al. 1995), mungbean (Mahalakshmi et al. 2006) and pigeonpea (Dayal et al. 2003, Gawali et al. 2010), there are no reports on direct multiple shoot regeneration from petioles of primary leaves in blackgram. In the present study, we report a simple one step (direct) method for successful and efficient shoot regeneration from petioles of primary leaf explants. This method has been found amenable to *Agrobacterium*-mediated genetic transformation of blackgram as evident from the transformation studies involving transient GUS expression at the regeneration site, the presence and integration of transgenes in T₀ plants and their inheritance in T₁ progeny in Mendelian fashion.

Materials and methods

Plant material, bacterial strain and vector

Seeds of commercially grown blackgram cultivars, T-9, PS-1 and PS-2 were obtained from the Pulse Research Laboratory, Division of Genetics, Indian Agriculture Research Institute, New Delhi. For detailed studies, PS-1 showing the best regeneration response was used. Binary vector, pCambia2301 (www.cambia.org) was used for optimization of parameters affecting *Agrobacterium*-mediated transformation and further transformation studies. It contains genes for neomycin phosphotransferase (NPT II) (*nptII*) and β -glucuronidase (GUS) (*uidA*), both driven by the cauliflower mosaic virus (CaMV) 35S promoter (Fig. 2). The *uidA* gene contains an intron in the coding region to ensure that it would be expressed only in plant cells and not in *Agrobacterium*. The presence of the binary vector in the *Agrobacterium* was confirmed by colony PCR using primers specific to *uidA* genes.

Explant preparation, multiple shoot induction and plant regeneration

Healthy and uniform mature seeds were rinsed with 70 % alcohol (v/v) for 1 min and then surface sterilized in 0.2 % (w/v) aqueous solution of mercuric chloride for 5 min. The seeds were subsequently rinsed with autoclaved distilled water for 4–5 times. Sterilized seeds were germinated on MSB medium [MS (Murashige and Skoog 1962) salts, B₅ (Gamborg et al. 1968) vitamins, 3 % sucrose and 0.8 % agar as a gelling agent] supplemented with 10.0 μ M BA. The primary leaves with petiole attached were excised from in vitro raised 4-day-old seedlings and were cultured with petiolar cut end slightly embedded in MSB medium containing different concentrations of BA (0–10.0 μ M) for multiple shoot induction.

The shoot multiplication response of BA was compared with other cytokinins, by culturing the explants on MSB basal medium containing kinetin, zeatin, 2-iP and TDZ at an equimolar concentration of 1.0 μ M. The effect of auxins (IAA or NAA) on shoot regeneration response of BA was studied by culturing the explants on MSB basal medium containing either 0.5 or 1.0 μ M of IAA or NAA along with 1.0 μ M BA. The effect of the presence or absence of lamina on the primary leaf petiole (excised at node) was studied by culturing the primary leaf petiole with whole lamina or without lamina or transverse proximal or longitudinal half of lamina on MSB basal medium supplemented with 1.0 μ M BA. The effect of the age of explant on multiple shoot induction was determined by culturing the primary leaf explants excised from 2 to 10-day-old seedlings. In order to study the effect of genotype on shoot regeneration, explants were excised from seedlings of different cultivars, T-9, PS-1 and PS-2, and cultured on MSB medium supplemented with 1.0 μ M BA.

All the culture work was performed under aseptic conditions in laminar air flow cabinet. In all the cases, the pH of the medium was adjusted to 5.8 with 1 N NaOH and 1 N HCl prior to autoclaving at 121 °C under a pressure of 15 psi for 20 min. All the cultures were maintained at 26 $\pm$ 2 °C under 16 h photoperiod of cool -white fluorescent light of intensity 80 μ Em⁻² s⁻¹. The well-developed shoots (2–3 cm) were excised from the proliferating explants and transferred to medium containing half-strength MS salts, full-strength B₅ vitamins, 3 % sucrose and 2.5 μ M IBA for rooting. The rooted shoots were established in pots containing soil as per the method described by Saini and Jaiwal (2005).

For each treatment, 24 explants were cultured and each experiment was repeated at least thrice. Cultures were observed every day and the data pertaining to the number of explants forming shoots, the number of shoots and the length of shoots per explant were recorded after 28 days of culture. The data was subjected to the analyses of variance (ANOVA) and significant

treatment differences were selected by Newman-Keul's multiple range test (Bruning and Kintz 1977).

Selection system

To determine the concentration of the selective agent for the selection of transformed shoots, the primary leaf explants were cultured on shoot regeneration medium (MSB+1.0 μM BA) containing different concentrations of kanamycin (0–90 mg l^{-1}) as a selective agent. The explants were subcultured on the same fresh medium with same levels of antibiotic after every 2 weeks till 4 weeks. The data on the number of explants forming shoots and the number of shoots per explant was recorded after 4 weeks of culture. The shoots regenerated from untransformed (control) explants were transferred to rooting medium (MSB+2.5 μM IBA) containing different concentrations of kanamycin (0–10 mg l^{-1}) to determine the kanamycin concentration that suppressed the root induction.

Optimization of transformation protocol and regeneration of transformants

Various factors influencing transformation efficiency of blackgram were optimized using transient GUS expression. *A. tumefaciens* strain EHA105 (pCambia2301) was grown in liquid YEP (yeast extract 10.0 g l^{-1} , peptone 10.0 g l^{-1} , NaCl 5.0 g l^{-1} , pH 7.2) medium containing 10 mg l^{-1} rifampicin (for bacterial selection) and 5 mg l^{-1} tetracycline (for plasmid selection) for overnight at 28 °C on an orbital shaker (Orbitek, India) at 220 rpm until the O.D. of bacterial suspension reached 0.7 at 600 nm. The bacterial culture was centrifuged at 6000 rpm in a 15 ml tube (Tarson, India) for 5 min. The pellet was resuspended in liquid MSB medium containing 1.0 μM BA and 100 μM acetosyringone. The primary leaf explants with petiole excised from 4-day-old in vitro raised seedlings were inoculated by immersing in *Agrobacterium* suspension (containing 10^6 to 10^9 cells/ml of bacteria) for 10–40 min with gentle shaking at 80 rpm. The explants were blotted dry on sterile filter paper and co-cultivated on filter paper moistened with liquid MSB co-cultivation medium containing 1.0 μM BA adjusted to pH (5.0–5.8) for 1–4 days at 22–28 °C under light conditions. The effect of phenolic-acetosyringone (30–130 μM) and thiol compounds, L-cysteine (0–7 mM) and dithiothreitol (DTT) (0.5–2.0 mM) in bacterial inoculation and co-cultivation media were also studied. After co-cultivation, the explants were washed with sterile distilled water, blotted dry on sterile filter paper and immersed in freshly prepared X-gluc (5-bromo-4-chloro-3-indolyl- β -glucuronide) solution at 37 °C overnight in darkness according to Jefferson (1987). The staining solution was removed the following day and the tissues were decolorized using 70 % alcohol and examined under a stereo-microscope. For each variable in the experiment, 24 explants were used

and each experiment was repeated thrice. The frequency of transient GUS expression was calculated as the number of explants showing blue sectors (due to the large cut surface area) at the site of regeneration out of the total number of agro-inoculated explants.

To generate transgenic plants of blackgram, primary leaf explants with petiole excised from 4-day-old seedlings raised in vitro on MSB medium containing 10.0 μM BA were immersed in *Agrobacterium* suspension prepared in liquid MSB+1.0 μM BAP+5.0 mM L-cysteine+1.5 mM DTT+acetosyringone (100 μM) (adjusted to pH 5.5) for 20 min with occasional shaking at 24 °C. Agro-inoculated explants were blotted on sterile filter paper and co-cultured in Petri dish lined with filter paper moistened with liquid MSB+1.0 μM BA, 5.0 mM L-cysteine and 1.5 mM DTT for 3 days under 16 h photoperiod at 24 °C. After co-cultivation, explants were washed 3–4 times with sterile distilled water with vigorous stirring and blotted dry on sterile filter paper. The explants were cultured on semi-solid MSB medium containing 1.0 μM BA, 70 mg l^{-1} kanamycin and 500 mg l^{-1} cefotaxime for shoot regeneration. The explants were sub-cultured on to a fresh medium containing same levels of antibiotics after every 2 weeks for a total of 4 weeks. Green shoots recovered on selection medium were rooted on medium containing half-strength MS salts, full-strength B₅ vitamins, 3 % sucrose, 2.5 μM IBA, 10.0 mg l^{-1} kanamycin and 500 mg l^{-1} cefotaxime. The putative transformed plants were established in soil and grown to maturity to collect seeds. The seeds were sown in soil to raise the T₁ plants.

DNA extraction

The total plant genomic DNA was extracted from fresh young leaves of putative transformed (T₀) and non-transformed (control) plants by the CTAB (cetyl trimethyl ammonium bromide) method (Rogers and Bendich 1988).

Polymerase chain reaction analysis

Putative transgenic plants (T₀) were screened by PCR for the presence of the *nptII* gene. Plant genomic DNA isolated from the leaf tissue was used as a template to amplify 680-bp coding region of *nptII* gene by using specific primers 5' CTGG GCACAACAGACAATCG 3' and 5' GCGATACCGTAAAG CACGAG 3'. PCR was carried out with Taq DNA polymerase (MBI Fermentas). Twenty five microlitre (μl) reaction mixture (total volume) consisted of ~100 ng of plant DNA, 0.25 μl of each primer (forward and reverse each of *nptII* gene), 2.5 μl of 10 $\times$ PCR buffer, 1.5 μl of 15 mM MgCl₂, 0.5 μl of 200 mM dNTPs mix and 1 Unit of Taq DNA polymerase. The amplification reaction was carried out using a thermal cycler (Applied Biosystem) under following conditions: 1 cycle of 94 °C for 5 min, 30 cycles of 94 °C for 40 s, 56 °C for 40 s and 72 °C

for 1 min followed by a final extension at 72 °C for 5 min for detection of *nptII* gene amplification. PCR amplified DNA fragments were analyzed by electrophoresis on 1 % agarose gel and visualized with ethidium bromide (Sambrook et al. 1989). DNA from non-transformed (control) plant was used as a negative control while the plasmid (pCAMBIA2301) was used as a positive control.

Southern blot hybridization

10 microgram (μg) of genomic DNA from transformed and non transformed (control) plants were digested with *HindIII* (10 U/ μl) restriction enzyme in 100 μl reaction volume, separated on a 0.9 % agarose gel, blotted on positively charged nylon membrane (Amersham) and fixed by ultraviolet (UV) cross-linking. The blot was hybridized with dCT-[P^{32}]-labeled probe following the supplier's instructions (Bio-Rad, USA). The membrane was washed in 2x SSC (0.3 M NaCl, 0.03 M trisodium citrate 2.H₂O, pH-7.0) twice for 15 min each at room temperature in 1x SSC for 1 min at 65 °C and finally in 0.5x SSC for 5 min at room temperature. The membrane was processed for autoradiography.

PCR analysis of T₁ transgenic plants

Plants from T₁ progeny were screened by polymerase chain reaction (PCR) for the presence of *nptII* gene. DNA from non-transformed (control) plant was used as a negative control while the plasmid (pCAMBIA2301) was used as a positive control.

Results and discussion

Plant regeneration through multiple shoot induction

The regeneration potential of primary leaf explants with petioles excised from in vitro raised seedlings on BA (10.0 μM) was evaluated further on MSB medium with various concentrations of BA (0.0–10.0 μM) (Table 1). The explants on MSB basal medium alone showed expansion of leaf lamina in size and swelling of the petiolar region within a week of culture. Subsequently, the petiolar cut end developed an average of 2.0 shoots in 90 % of the cultures within 4 weeks. This is in contrast to earlier reports, where no shoot regeneration was noticed on MSB basal medium from leaf petioles of *V. radiata* (Mahalakshmi et al. 2006). This may be due to the pretreatment of the initial explants during seed germination with cytokinin (BA) which has resulted in significant increase in the regeneration frequency (data not shown). Presumably, pretreatment on cytokinin activates and/or induces proliferation of pre-existing competent cells in the tissue that results in enhanced regeneration process. Addition of BA in MSB

medium further improved the regeneration frequency and shoots per explant. BA induced direct multiple shoots from the cut end of the explants (Figs. 1 and 2). BA at 1.0 μM concentration was optimal inducing 10 shoots per explant in 95 % of the cultures.

However, BA at higher concentration (5.0 μM) induced shoot buds along with callus at the cut end of the petiole. The shoot length showed inverse correlation with the increase in BA concentration. These results point towards higher requirement of cytokinin (10.0 μM) for induction and differentiation of multiple shoots from leaf petiole. However, greatly reduced BA concentration (1.0 μM) proved to be crucial for development of shoots. The shoot regeneration from leaf petiole explants has also been achieved either directly on BA containing medium in *V. radiata* (Mahalakshmi et al. 2006) or indirectly through callus formation on 2,4-5-T and then transferring callus to 5.0 μM BA in *V. unguiculata* (Muthukumar et al. 1995). Multiple shoot regeneration from leaf petiole explants of pigeon pea has also been obtained on medium containing same levels of dual cytokinins, BA (5.0 μM) and kinetin (5.0 μM) (Dayal et al. 2003; Villiers et al. 2008). In pigeon pea, an additional step of elongation of multiple shoot is required on medium containing GA₃ (Dayal et al. 2003; Villiers et al. 2008). However, regeneration frequency in these studies is far from optimum. The maximum shoot regeneration from leaf petiole explants was only 46 % in *Cajanus cajan* (Villiers et al. 2008) and 84 % in *V. radiata* (Mahalakshmi et al. 2006). However, in the present study 95 % shoot regeneration response was achieved on MSB medium containing BA as a sole growth regulator. Other cytokinins like kinetin, zeatin, 2-ip and TDZ reduced the regeneration response and shoot number when used at equimolar concentration (1.0 μM) (Table 1).

Among cytokinins, BA was found to be the most effective for induction of higher regeneration frequency and the maximum number of multiple shoots (10) in *V. mungo*. Similar morphogenetic response was obtained in *V. radiata* but with a higher concentration of BA (Mahalakshmi et al. 2006). Addition of NAA or IAA to BA enriched medium decreased the number of shoots regenerated from leaf petiole explants. However, the regeneration frequency of the explant was not much affected. Thus shoot forming response of BA (1.0 μM) was not improved when it was supplemented with auxins either NAA or IAA each at 0.5 or 1.0 μM concentration. Both the auxins at higher concentration (1.0 μM) in combination with 1.0 μM BA induced callus at the cut end of petioles. The nature of callus differed with the type of auxin. NAA induced yellow creamish friable callus whereas IAA induced light greenish callus which did not regenerate shoots even on transfer to shoot induction medium (Table 1). In contrast to present study, the maximum shoot regeneration response was achieved on 0.5 μM NAA and 22 μM BA in *Cajanus cajan* (Srinivasan et al. 2004). Explants comprising

Table 1 Influence of plant growth regulators [μM] on induction of multiple shoots from 4-days- old primary leaf with petiole explants of *Vigna mungo* cv. PS-1

Kinetin (μM)	Zeatin (μM)	2-ip (μM)	TDZ (μM)	BA (μM)	NAA (μM)	IAA (μM)	Frequency of regenerating explants (%)	Mean number of shoots per explants (Mean $\pm$ S.E.) ²	Mean shoot length per explants (cm) (Mean $\pm$ S.E.) ²
1.0	—	—	—	—	—	—	88	3.1 $\pm$ 0.4a	5.2 $\pm$ 0.6a
—	1.0	—	—	—	—	—	80	1.6 $\pm$ 0.2b	6.4 $\pm$ 0.8a
—	—	1.0	—	—	—	—	50	1.8 $\pm$ 0.6b	2.1 $\pm$ 0.2b
—	—	—	1.0	—	—	—	87	2.1 $\pm$ 0.7b	1.2 $\pm$ 0.1b
—	—	—	—	0	—	—	90	2.0 $\pm$ 0.2b	1.6 $\pm$ 0.3b
—	—	—	—	0.5	—	—	95	6.5 $\pm$ 0.3c	1.3 $\pm$ 0.1b
—	—	—	—	1.0	—	—	95	10.0 $\pm$ 0.8d	1.1 $\pm$ 0.3b
—	—	—	—	2.0	—	—	95	8.0 $\pm$ 0.4e	0.9 $\pm$ 0.1b
—	—	—	—	5.0	—	—	95	Shoot buds and callusing of tissue	No shoot formation
—	—	—	—	1.0	0.5	—	90	4.3 $\pm$ 0.6f	1.2 $\pm$ 0.6b
—	—	—	—	1.0	1.0	—	90	Friable yellowish callus	No shoot formation
—	—	—	—	1.0	—	0.5	95	3.6 $\pm$ 0.5af	1.1 $\pm$ 0.4b
—	—	—	—	1.0	—	1.0	95	Green compact callus	No shoot formation

Culture medium: MSB (MS salts + B₅ vitamins)

Data based on 24 explants per treatment and each experiment repeated thrice. Data scored after 28 days of culture

Means followed by the same letter are not significantly different according to Newman-Keuls multiple-range test ($P=0.05$)

Fig. 1 a-f In vitro regeneration of multiple shoots from 4-day-old primary leaf explants of *Vigna mungo* cv. PS1 **a** Primary leaf explant with petiole excised from 4-day-old in vitro-raised seedlings on MSB medium containing 10.0 μM BA **b** Direct shoot regeneration from primary leaf petiole on MSB medium supplemented with 1.0 μM BA **c** Multiple shoot regeneration on MSB medium supplemented with 1.0 μM BA after 4 weeks of culture **d** Induction of roots on in vitro regenerated shoot on MSB basal medium supplemented with 2.5 μM IBA **e** Establishment of an *in vitro* regenerated plant in pot containing soil **f** A regenerated plant with flower and pods

Fig. 2 A schematic representation of the T-DNA of pCambia2301 containing the *uidA* (GUS) and *nptII* genes (not drawn to scale). The position of HindIII is indicated on the T-DNA. No other HindIII sites are

present on pCambia2301 (total size 11.6 kb). LB/RB: left and right T-DNA border sequences

of the petiole with basal half of the lamina or the petiole with complete lamina proved to be efficient for shoot regeneration (95 %) than the explants without lamina (60 %) (Table 2). The explants with lamina produced 10 shoots per explant while those without lamina produced a single shoot per explant. This indicates that the presence of lamina was essential to elicit full regeneration potential of petiole and induction of multiple shoots. Similar beneficial role of the lamina on shoot induction has also been demonstrated in *Cajanus cajan* (Dayal et al. 2003) and *V. radiata* (Mahalakshmi et al. 2006). The primary leaves with petiole excised from seedlings of different ages on MSB medium containing 1.0 μ M BA showed increase in the regeneration frequency and the number of shoots per explant up to 4-days, thereafter, both the parameters decreased. The decrease in number of shoots per explant was more than the

decrease in frequency of regeneration. The explants excised from 4-day-old seedlings were more responsive for shoot regeneration than those from 10-day-old seedlings (Table 3). These results are in agreement with those obtained in *Cajanus cajan* (Srinivasan et al. 2004), and are in contrast to those obtained in *V. radiata* (Mahalakshmi et al. 2006).

The shoot regeneration response of 4-day-old leaf petiole explants with half lamina on 1.0 μ M BA was tested to determine its applicability to other blackgram cultivars, PS-1, PS-2 and T-9. Genotype had a marked effect on regeneration frequency and shoots per explant. Highest regeneration frequency (95 %) was obtained with PS-1 with ten shoots per explant with an average length of 1.1 cm (Table 4). Similar genotypic differences in shoot regeneration from other explants have also been reported in previous studies in *Vigna mungo* (Saini and Jaiwal 2002; Saini et al. 2003; Saini and Jaiwal 2005).

Table 2 Effect of explant type on in vitro shoot regeneration from *Vigna mungo* cv. PS-1

Explant type	Frequency of regenerating explants (%)	Mean number of shoots per explants (Mean $\pm$ S.E.) ²	Mean shoot length per explants (cm) (Mean $\pm$ S.E.) ²
Entire primary leaf with petiole	90	10.0 $\pm$ 0.8a	1.6 $\pm$ 0.3a
Transverse basal half of primary leaf with petiole	95	10.0 $\pm$ 0.6a	1.1 $\pm$ 0.3a
Longitudinal half of primary leaf with petiole	90	3.3 $\pm$ 0.3b	1.3 $\pm$ 0.4a
Leaf petiole without lamina	60	1.3 $\pm$ 0.0c	1.1 $\pm$ 0.5a
Primary leaf without petiole	0	0	0

Culture medium: MSB (MS salts + B₅ vitamins) + BA (1.0 μ M)

² Data based on 24 explants per treatment and each experiment repeated thrice. Data scored after 28 days of culture

Means followed by the same letter are not significantly different according to Newman-Keuls multiple-range test (P=0.05)

Selection

Kanamycin has been successfully employed as a selectable marker in transformation of various legume crops like *Vigna mungo* (Karthikeyan et al. 1996; Saini et al. 2003; Saini and Jaiwal 2005; Bhomkar et al. 2008),

Table 3 Effect of age of the donor seedling on regeneration from primary leaf with petiole explants of *Vigna mungo* cv. PS-1

Age in days	Frequency of regenerating explants (%)	Mean number of shoots per explant (Mean $\pm$ S.E.) ²	Mean shoot length per explants(cm) (Mean $\pm$ S.E.) ²
2	70	3.1 $\pm$ 0.8a	1.6 $\pm$ 0.3a
3	85	7.2 $\pm$ 0.3b	1.3 $\pm$ 0.1a
4	95	10.0 $\pm$ 0.8c	1.1 $\pm$ 0.3a
5	95	8.0 $\pm$ 0.4d	1.0 $\pm$ 0.3a
6	90	6.5 $\pm$ 0.3e	1.5 $\pm$ 0.1a
7	85	4.0 $\pm$ 0.2f	1.3 $\pm$ 0.3a
10	70	3.8 $\pm$ 0.3f	1.4 $\pm$ 0.4a

Culture medium: MSB (MS salts + B₅ vitamins) + BA (1.0 μ M)

² Data based on 24 explants per treatment and each experiment repeated thrice. Data scored after 28 days of culture

Means followed by the same letter are not significantly different according to Newman-Keuls multiple-range test (P=0.05)

Table 4 In vitro shoot regeneration of 4-day-old primary leaf with petiole explants obtained from different cultivars of *Vigna mungo*

Cultivar	Frequency of regenerating explants (%)	Mean number of shoots per explant (Mean $\pm$ S.E.) ²	Mean shoot length per explant (cm) (Mean $\pm$ S.E.) ²
PS-1	95	10.0 $\pm$ 0.8a	1.1 $\pm$ 0.3a
T-9	90	6.6 $\pm$ 0.7b	1.2 $\pm$ 0.4a
PS-2	80	7.0 $\pm$ 0.3b	1.0 $\pm$ 0.3a

Culture medium: MSB (MS salts + B₅ Vitamins) + BA (1.0 μ M)

² Data based on 24 explants per treatment and each experiment repeated thrice. Data scored after 28 days of culture

Means followed by the same letter are not significantly different according to Newman-Keuls multiple-range test (P=0.05)

Vigna unguiculata (Muthukumar et al. 1995; Chaudhary et al. 2007), *Cicer arietinum* (Krishnamurthy et al. 2000), *Pisum sativum* (Grant et al. 1998), *Arachis hypogea* (Sharma and Anjaiah 2000), *Vigna radiata* (Jaiwal et al. 2001) and *Vigna angularis* (Yamada et al. 2001). The sensitivity of plant cells to the selective agent depends upon the genotype, the explant type, size and developmental stage of tissue(s). So it is compulsory to determine the lowest concentration of the selective agent that suppresses growth and proliferation of untransformed cells. The survival (determined by necrosis of explants), regeneration frequency and the average number of shoots per explant decreased with increasing kanamycin concentration. Kanamycin at 70 mg l⁻¹ in shoot regeneration medium drastically reduced the survival as well as the regeneration frequency of the explants and completely bleached the non-transformed shoots. Kanamycin concentrations higher than 70 mg l⁻¹ were lethal causing necrosis of explants and complete inhibition of regeneration. Therefore, 70 mg l⁻¹ kanamycin was chosen for the selection of the transformed shoots in the transformation experiments. Root induction was completely inhibited in non-transformed (control) shoots cultured on MSB + 2.5 μ M IBA medium containing 10.0 mg l⁻¹ kanamycin.

Optimization of transformation protocol

Several chemical and physical parameters are pre-requisites for efficient transformation by *A. tumefaciens*. Bacterial concentration (cells/ml), inoculation time, co-cultivation period, addition of phenolic compound (acetosyringone) and various antioxidants in co-cultivation medium are significant factors affecting competence of tissue(s) and *Agrobacterium* virulence for achieving the maximum transformation frequencies. These factors were optimized to improve the transformation

efficiency on the basis of transient *uidA* expression using primary leaf explants (Table 5).

Of the four different bacterial concentrations (10⁶, 10⁷, 10⁸, 10⁹ cells/ml), the maximum transformation frequency was observed at a concentration of 10⁷ cells/ml with a constant increase up to 10⁷ cells/ml and decrease thereafter, as reported in tobacco, *Arabidopsis thaliana* (Lin et al. 1994) and other grain legumes (Bean et al. 1997). Too much bacteria results in *Agrobacterium* aggregation or trouble in eliminating them after co-cultivation or oversensitive reply of explants with less regeneration potential. So the concentration of bacteria used for transformation is an important parameter that may influence the number of transformed cells/explant in various species (Fillati et al. 1987; Saini and Jaiwal 2007).

Variable transient GUS expression were found when explants were incubated with bacterial suspension for 10 to 40 min with utmost transformation frequency in explants incubated for 20 min with gentle shaking at 90 rpm. Further increase caused difficulty in bacterial elimination instead of any increase in transformation frequency which remains unaffected.

For primary leaf explants, 3-days of co-cultivation period was optimal and any further increase had negative effect on regeneration potential of explants due to bacterial overgrowth, decreasing the transformation frequency. Most of the *Vigna* species, *Vigna unguiculata* (Chaudhary et al. 2007) and *Vigna radiata* (Jaiwal et al. 2001; Sonia et al. 2007) required 2–3 days co-cultivation period for the maximum transformation frequency.

Addition of acetosyringone (100 μ M), to the bacterial-suspension medium and co-cultivation medium increased transformation rate of *Vigna mungo* with greater GUS activity. Similar affirmative effects has been reported in several leguminous species such as *Pisum sativum* (Svabova and Griga 2008), *Glycine max* (Santarem et al. 1998; Ko and Korban 2004), *Vigna angularis* (Yamada et al. 2001), *Phaseolus vulgaris* (De Clercq et al. 2002), *Vigna mungo* (Saini et al. 2003; Muruganantham et al. 2007), *Vigna unguiculata* (Popelka et al. 2006) and *Vigna radiata* (Sonia et al. 2007). Acetosyringone increases transformation potential of *Agrobacterium* strain by enhancing *vir* functions during transformation (Stachel et al. 1986) and has been used in several other plant species also (Atkinson and Gardner 1991; Kaneyashi et al. 1994).

Maximum frequency of transient GUS expression was recorded at pH-5.5, in the presence of acetosyringone (100 μ M). The *vir* gene induction and transformation are influenced by the pH of co-cultivation medium. Low pH and acetosyringone together may be crucial to *vir* gene induction (Kapila et al. 1997). They can affect the *Agrobacterium* virulence to a long extent however, variations have been found across species (Godwin et al. 1991). Our results were in accordance with these observations. Higher transformation frequency has been reported at low pH of bacterial inoculation and co-culture media (Popelka et al. 2006; Sonia et al. 2007; Solleti et al. 2008).

Table 5 Effect of different transformation parameters on transient GUS activity in primary leaf petiole explants of *Vigna mungo* co-cultivated with *Agrobacterium tumefaciens* strain EHA105 harboring binary vector pCAMBIA2301

Factor	Variable	% explant showing GUS activity
Bacterial concentration (cells/ml)	10 ⁶	67a
	10 ⁷	88b
	10 ⁸	80b
	10 ⁹	76b
Inoculation time (minutes)	10	60a
	20	85b
	30	79b
	40	61a
Co-cultivation time (days)	1	58a
	2	76b
	3	89c
	4	81bc
Acetosyringone (μM)	30	60a
	50	69b
	70	76b
	100	90c
	130	82bc
pH	5.0	59a
	5.2	71b
	5.5	82c
	5.8	66b
Co-cultivation temperature (°C)	22	55a
	24	73b
	26	68b
	28	48c
L- cysteine (mM)	0.0	58a
	2.0	69b
	5.0	85c
	7.0	79c
DTT (mM)	0.5	59a
	1.0	66b
	1.5	81c
	2.0	74c

Data based on 24 explants per treatment and each experiment repeated thrice

Mean values within column for each factor separately followed by same letter are not significantly different according to Newman-Keuls multiple-range test ($p=0.05$)

Inclusion of antioxidants like L-cysteine (5.0 mM) and DTT (1.5 mM) completely inhibited the browning and necrosis of explants cells and increased the number of explants showing intense GUS activity. They have been used for co-cultivation in order to enhance the final efficiency of the transformation protocol. L-cystein acts as a nutritional supplement and is involved in pathogen-defense response. Its has thiol group which acts as an antioxidant or wound inhibitor. DTT is also an efficient antioxidant with potential anti-necrotic property and is least toxic for plant cells (Svabova and Griga 2008). The beneficial role of thiol compounds on greater

survival of explants during co-cultivation has been reported in soybean (Olhoft et al. 2001, 2003; Zeng et al. 2004), *Vigna unguiculata* (Popelka et al. 2006), *Zea mays* (Frame et al. 2002) and *Vitis vinifera* (Das et al. 2002a, b).

During co-cultivation, temperature is an important factor affecting T-DNA transfer. In the present study, transformation frequency was highest when co-cultivation was performed at 24 °C. Co-cultivation at low temperature has been reported in several instances (Fullner and Nester 1996; Kapila et al. 1997; Dillen et al. 1997; Popelka et al. 2006). It has also been found that the temperature dependence profile of T-DNA transfer does not

Fig. 3 a–b Transient GUS expression in primary leaf explants inoculated with *Agrobacterium tumefaciens* strain EHA 105 harboring a binary vector pCambia2301 that contained *uidA* (GUS) and *nptII* genes a. The non-transformed (control) primary leaf with petiole explant showing no GUS activity b. Primary leaf explant with petiole showing transient GUS activity at the regeneration site after 3 days of co-cultivation with *Agrobacterium tumefaciens* EHA105 (pCambia2301)

parallel with that of *vir* gene induction, indicating that another factor possibly the formation of a conjugal pilus is causing temperature sensitivity of T-DNA transfer (Fullner et al. 1996).

Transformation and GUS activity

The applicability of the present regeneration protocol for *Agrobacterium*-mediated transformation and the competency of the regeneration competent meristematic cells at cut end of primary leaves were determined by inoculating the explants with *A. tumefaciens*. The *uidA* gene contained an intron in the coding region so that it would not be expressed in *Agrobacterium*. Eighty five percent of the explants showed endogenous GUS activity immediately after co-cultivation at regenerating sites of the primary leaves indicating the amenability of explants to *Agrobacterium*-mediated transformation. The endogenous GUS activity was not found in non transformed (control) explants (Fig. 3). Histochemical GUS assay has also been used to confirm the presence of the GUS gene and hence transformation of chrysanthemum ‘Orlando’ petal explants (Song et al. 2012)

Regeneration of transformants

A total of 230 explants produced 79 green shoots on kanamycin selection medium and 66 were rooted (28.69 %) in the presence of kanamycin (10 mg l⁻¹) and cefotaxime (500 mg l⁻¹). Sixty six rooted shoots when transferred to soil, 55 of them survived (23.91 %), matured and produced transgenic seeds in the green house (Table 6). The seeds were sown to raise the T₁ plants. Establishment of plants required approximately 2 months. Molecular analysis confirmed their transgenic nature.

Molecular analysis of transgenic plants

PCR analysis for *nptII* and *virA* genes

PCR analysis showed amplification of an expected fragment of 680 bp which corresponds to the coding region of *nptII* gene and shows the presence of transgene in nine out of 55 putative transformed plants (Fig. 4). Untransformed (control) plants did not show any amplification. The negative results

Table 6 Summary of the transformation of 4-day-old primary leaf explants of *Vigna mungo* cv. PS-1

Expt. no.	No. of explants inoculated with <i>Agrobacterium</i> suspension	No. of shoots recovered on selection medium ^a	No. of shoots rooted on selection medium ^b	No. of putative transgenic plants established in soil	No. of plants positive for <i>nptII</i> gene by PCR	No. of plants positive for <i>nptII</i> by Southern blot hybridization
1	85	31	27	25	9	3
2	75	27	22	20		
3	70	21	17	10		
Total	230	79	66	55		

Cocultured with *Agrobacterium tumefaciens* strain EHA105 containing a binary vector pCambia2301

^a Selection medium for shoot regeneration: MSB + BA (1.0 μM) + kanamycin (70 mg l⁻¹) + cefotaxime (500 mg l⁻¹)

^b Selection medium for root induction: MSB + IBA (2.5 μM) + kanamycin (10 mg l⁻¹) + cefotaxime (500 mg l⁻¹)

Fig. 4 Molecular analysis of primary transformants of *Vigna mungo* transformed with *Agrobacterium tumefaciens* strain EHA105 harboring a binary vector pCambia2301 that contained *uid A* (GUS) and *nptII* genes **a** PCR analysis of putative transformants with primers specific to the coding region of *nptII* gene Lane M: DNA Molecular weight marker (1 kb) Lane C: DNA from non-transformed (control) plant Lane P: positive plasmid DNA Lanes 1 to 10: DNA from transformed plants (T_0) **b**. PCR analysis of putative transformants with primers specific to the coding region of *vir A* gene Lane M: DNA Molecular weight marker (1 kb) Lane C: DNA from non-transformed (control) plant Lane P: Plasmid DNA Lanes 1 to 9: DNA from transformed plants (T_0)

could be due to the survival of non-transformed shoots on the selection medium. Since most of the legumes including *Vigna* species show high tolerance to kanamycin (Saini and Jaiwal 2005) hence the high number of escaped shoots on selection may be a possibility. PCR analysis of *nptII* positive plants with *vir* primers did not show any amplification thus ruling out the possibility of *Agrobacterium* contamination in the transformed plants.

Southern hybridization

Southern analysis of T_0 plants positive for *nptII* gene by PCR was performed to confirm the integration of *nptII* gene into their genome and to determine its copy number. The genomic DNA of PCR positive plants was isolated and digested with *HindIII* and hybridized with 680 bp amplicon of *nptII* gene (probe). *nptII* probe was hybridized to digested DNA from transgenic plants but not to untransformed (control) plants, indicating that *nptII* gene was integrated into blackgram genome. Digestion with

HindIII and subsequent probing with *nptII* coding region identifies border fragments between the T-DNA and plant DNA. Untransformed (control) plant DNA did not hybridize to the *nptII* probe. However, the bands size with the *nptII* probe were greater than *nptII* containing *HindIII* fragment (2.1 kb) establishing the integration of the *nptII* gene into genome of the plant. The hybridization pattern showed that three plants had a single copy of T-DNA integrated in the genome (Fig. 5) Hybridization signals in all the three plants were of the same size indicating that these plants are derived from a single transformation event. However, six plants that were PCR positive were negative after southern analyses which may be due to the incomplete insertion, unstable integration of the transferred gene into plant genome and hence its loss (Hess et al. 1990; Langridge et al. 1992). Thus, southern analysis established the existence and integration of the transgene in the transformants. A stable transformation frequency of 1.3 % (number of the southern positive plants / total number of explants inoculated with *Agrobacterium* $\times$ 100) was obtained.

PCR analysis of T_1 progeny

All the three Southern positive T_0 plants were self fertilized to produce seeds. PCR was done by using *nptII* specific primers to analyze the progeny of the T_0 plants and segregation for the presence and absence of *nptII*-amplified band in a simple Mendelian 3:1 ratio was observed suggesting a single integration site (Table 7), (Fig. 6). Statistical analysis of the progeny also confirmed inheritance and segregation of the transgene in 3:1 ratio at 0.05 % significance level.

In conclusion, morphologically normal and fertile plants of *V. mungo* have been regenerated via direct shoot organogenesis from the leaf petiole explants on MSB medium containing BA as sole growth regulator for the first time. The type and

Fig. 5 Southern hybridization of genomic DNA from primary leaf explants with *Agrobacterium tumefaciens* strain EHA105 harboring a binary vector pCambia2301 that contained *uid A* (GUS) and *nptII* genes. **a**. Southern blot analysis of genomic DNA of transformed and non-transformed control plants. The DNA was digested with *HindIII*, and blot was probed with the PCR amplified fragment (680 bp) of *nptII* gene. Lane M: DNA Molecular weight marker (1 kb) Lane C: DNA from untransformed (control) plant Lanes 1–3: DNA from transformed plants Lane P: Plasmid DNA

Table 7 Segregation ratios of the *nptII* gene in selfed progenies of T₀ transformed *V. mungo* cv. PS-1

Transformants	Total number of T ₀ seeds collected	Number of T ₁ plants raised in pots	Number of plants positive for <i>nptII</i> by PCR	Number of plants negative for <i>nptII</i> by PCR	(<i>p</i> value) χ^2 –Test	Expected segregation ratio
1	40	20	15	5	0.49 (<i>p</i> <0.0-5)	3:1
2	32	14	10	4	0.56 (<i>p</i> <0.0-5)	3:1
3	35	16	12	4	0.82 (<i>p</i> <0.0-5)	3:1

concentration of cytokinin, type and age of explant, preconditioning of explants and genotype significantly influenced the regeneration response. Earlier less number of shoots per explant from various explants were reported (Saini and Jaiwal 2002; Saini et al. 2003; Saini and Jaiwal 2005). Similarly, the previous studies on plant regeneration via indirect organogenesis also reported a very low regeneration frequency with a few shoots (Srivastava and Pandey 2011). Because of direct regeneration, the somaclonal variations are low and the time required for establishing a plant in pot is only 8 weeks. Moreover, regeneration site, i.e. cut end of the petiole is fully exposed to *Agrobacterium*, therefore such explants are more suitable for *Agrobacterium*-mediated genetic transformation for the production of transgenic plants. Since the plant is recalcitrant to transformation, the various factors affecting the efficiency of gene transfer were optimized. The effect of inclusion of antioxidants (L-cysteine and DTT) in co-cultivation medium was not reported previously in blackgram. The presence of 5 mM L-cysteine and 1.5 mM DTT in the co-cultivation medium inhibited the browning/necrosis of explant cells and increased the number of explants showing intense GUS activity. Similar results were observed in several plant species (Dan 2008). The presence and integration of

transgenes in T₀ plants was confirmed by PCR and Southern blot analysis. The T₀ plants transmitted transgenes to progeny in Mendelian fashion as revealed by PCR.

Acknowledgments Authors are thankful to the Center for Application of Molecular Biology to International Agriculture (CAMBIA) for plasmid pCAMBIA2301 and Dr. P. A. Kumar, NRC on Plant Biotechnology, IARI, New Delhi for providing laboratory facilities for the Southern blot hybridization. PKJ is grateful to Department of Biotechnology, New Delhi for financial support to his laboratory for improvement of grain legumes. MS is thankful to Council of Science and Industrial Research, Department of Biotechnology and Department of Science and Technology, New Delhi for research fellowships.

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Fig. 6 PCR analysis of T₁ plants using primers specific to *nptII* gene. Lane M: DNA Molecular weight marker (1 kb) Lane C: DNA from non-transformed (control) plant Lane P: Positive plasmid DNA Lanes 1 to 6: DNA from T₁ transformed plants Lane N: Negative control (water)

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