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RNA interference: A powerful tool for yellow mosaic virus resistance in grain legume blackgram (Vigna *mungo* L. Hepper)

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ABSTRACT

Blackgram (*Vigna mungo* L. Hepper) is an important leguminous source of protein for a large segment of the vegetarian population in the developing countries of Asia. During the last 4-5 decades the production of blackgram is reducing or stagnating, which has resulted in escalation of its market price. Major hurdles in achieving maximum production includes its susceptibility to several fungal and viral pathogens. Out of different constraints, viral diseases mainly yellow mosaic disease caused by mungbean yellow mosaic virus (MYMV) is the major threat for huge economical losses. To engineer resistant plants against the virus Pathogen-derived resistance (PDR) is a very effective genetic engineering approach. PDR is a strategy in which resistance imparting genes are taken from the virus for which resistance is to be developed. Out of different viral genes used for PDR, invert repeat construct of MYMV-Vig *rep* gene to express ds RNA for RNA interference approach holds promise for developing geminivirus resistance. RNA interference (RNAi) is a method of blocking gene function by inserting short sequences of ribonucleic acid (RNA) that match part of the target gene's sequence. In this study, we focus on the application of RNA silencing to produce virus resistant blackgram plants. We employed RNA interference (RNAi) strategy to control blackgram-infecting MYMV. For this, we generated transgenic blackgram plants by incorporation of hp RNA of MYMV-Vig rep gene in to the blackgram genome. Morphologically normal and fertile transgenic plants from primary leaf petiole explants inoculated with *A. tumefaciens* (carrying binary vector pGD3) have been regenerated for the first time. The presence and integration of *nptII* gene into the blackgram genome was confirmed by PCR and Southern blot analysis. The transformation frequency was 1.3%. The transgenes inheritance and expression in T_1 progeny was detected by PCR and Western blotting, respectively.

Keywords: RNAi, gene silencing, MYMV, Resistance, *Vigna mungo*

INTRODUCTION

Black gram (*Vigna mungo* L. Hepper) or urd bean is a widely grown grain legume belongs to family Fabaceae and genus Vigna. Because of its high protein content and perfect combination of all nutrients(Karamany 2006), it is extensively used in Asia especially in India. Butviral 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. Legumes specially blackgram is extremely recalcitrant to *in vitro* culture and genetic transformation. Limited reports are available in connection with genetic transformation in black gram using different explants, such as cotyledonary node and shoot apex (Saini and Jaiwal, 2005). Although the previous reports showed positive results in black gram transformation, many constraints still exist to limit the improvement of black gram with desirable traits.

Among different constraints, viral diseases mainly yellow mosaic disease is the major threat for huge economical losses in the Indian subcontinent (Nene, 1973). It can cause 100% yield loss, if infection occurs at seedling stage (Varma et al., 1992; Ghafoor et al., 2000). The disease is caused by the geminivirus - MYMV (mungbean yellow mosaic virus) which has bipartite genome (Honda and Ikegami, 1986; Vanitharani et al., 1996; Mandal et al., 1997; Karthikeyan et al., 2004). The virus is transmitted by white flies (*Bemisia tabaci*). Initial symptoms of the disease appear as small yellow specks along the veins which spread over the leaf. In severe infections the entire leaf may become chlorotic which turns in to necrotic regions (Qazi et al., 2007).Attempts to

enhance the tolerance of blackgram against viruses through conventional breeding have met with limited success as it is time consuming and there is limited availability of genetic resources in the germplasm. To overcome the limitations of narrow genetic base, the conventional and traditional breeding methods are to be supplemented with biotechnological techniques.

In this context, genetic engineering has emerged as a viable option for overcoming limitations in blackgram improvement. Unfortunately, none of the known varieties of blackgram is fully resistant to mungbean yellow mosaic virus (MYMV) therefore; genetic engineering based on "pathogen-derived resistance" is viewed as a promising approach to develop MYMV resistance in blackgram. Pathogen-derived resistance is generally mediated by RNA which leads to identification of post-transcriptional gene silencing in plants. RNAi has revolutionized the possibilities for creating custom "knock-downs" of gene activity and holds immense promise in development of virus resistant transgenic plants (Wani et al., 2010). In RNA silencing, suppression of target gene expression occurs by sequence-specific interaction with RNA at the post-transcriptional level (Vanitharani et al., 2005; Almeida and Allshire, 2005). The mechanism of RNA silencing is initiated by double stranded RNA (dsRNA) whose antisense strand is complementary to the transcript of a target gene (Francisco and Faria, 2009). Double stranded (ds) RNA triggering RNA silencing could be viral replication intermediates or 'aberrant' RNAs or single- stranded RNAs that are converted in to ds RNA by host-encoded RNA-dependent RNA polymerase (RdRP) (Dalmay et al., 2000; Ahlquist et al., 2002). The long double stranded (ds) RNA is then cleaved in to short interfering (si) (21-26 nt) RNAs (waterhouse et al., 2001; Hannon, 2002; Pickford and cogoni, 2003) with the help of ribonuclease III like enzyme termed DICER (Bernstein et al., 2001; Wesley et al., 2001). These small RNA fragments serve as the specificity determinant by being incorporated in to the RNA-induced silencing complex (RISC) endonuclease (Hammond et al., 2000; Shi, 2003; Tang et al., 2003; Zou et al., 2005). This complex inhibits translation or depending on the degree of Watson-Crick complementarity, induces degradation of target mRNAs (Jones et al., 2001; Tang et al., 2007). An outline of the mechanism of RNA silencing is shown in the Fig.1

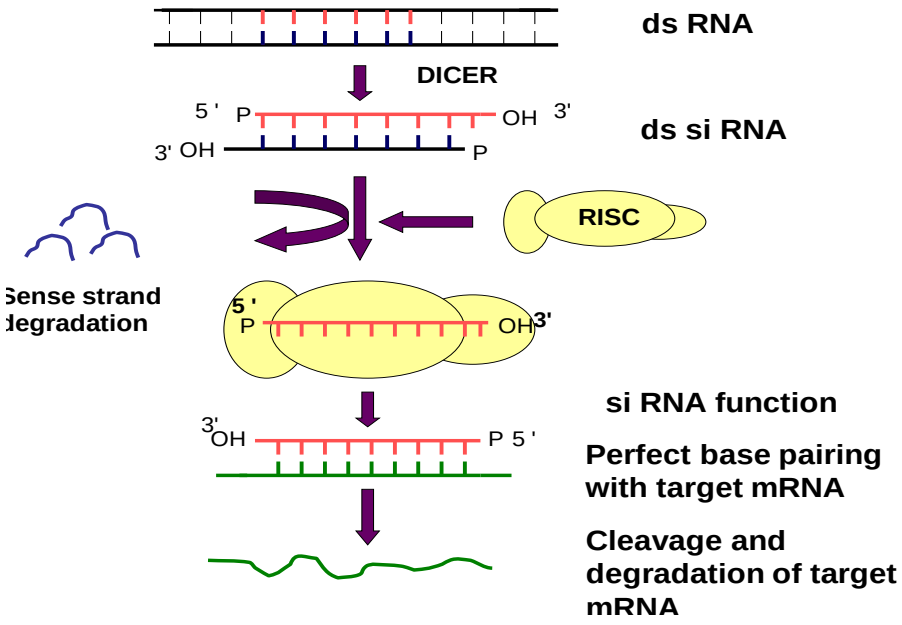


Fig. 1 Model for mechanism of RNA silencing

But availability of efficient in *vitro*regeneration system is a prerequisite for effective genetic transformation (Davis et al., 1993; Popelka et al., 2004). In the present study primary leaf explants have been used for *in vitro* regeneration of blackgram.

In blackgram, transgenes have been introduced in to genome mostly by *Agrobacterium*-mediated genetic transformation and rarely by direct gene transfer. This system offers rapid and precise mode of DNA transfer, high transformation efficiency, low or single copy integration, easy to handle and less expensive nature (veluthambi et al., 2003). The success in obtaining transgenic plants via *Agrobacterium* has been achieved in*Vigna mungo* with few explants, cotyledonary node (Saini *et al.*, 2003; Bhalla-Sarin *et al.*, 2004; Saini and Jaiwal, 2007; Bhomkar *et al.*, 2008), immature cotyledonary node (Muruganantham *et al.*, 2007) and shoot apex (Saini and Jaiwal, 2005). However only one agronomically important gene (*glyoxalase* I for alleviating salt stress) has been introduced using cotyledonary node explants (Bhomkar *et al.*, 2008). But the major problem affecting the yield of blackgram is yellow mosaic diseases. Therefore, the present investigation was undertaken to develop MYMV resistance in blackgram by

using RNA interference technology. The presence of transgenes in T_1progeny was confirmed by PCR, Southern and western blot analysis.

MATERIALS AND METHODS

Preparation of explants and multiple shoot regeneration

Seeds of fourcommercially grown cultivars of blackgram (*Vignamungo* L. Hepper) T-9, PS-1, T-27 and PU-19 were obtained from the National Bureau of Plant Genetic Resources and the Pulse Research Laboratory, Division of Genetics, Indian Agriculture Research Institute, New Delhi - 110012, India. The widely grown cultivar, PS-1 was mainly used. Sterilized seeds were cultured on MSB(Murashige and Skoog, 1962) salts and B5 (Gamborg etal., 1968) vitamins medium containing 10.0 µM BAP for 4-d at 26 ± 2°C under 16 h photoperiod of cool white-fluorescent light of intensity 80□□$Em^{-2}s^{-1}$. The primary leaf explants were excised from 4-d old seedlings raised on MSB_5medium containing 10µMBAP. The two primary leaves were separated by cutting at the nodal region and keeping the petiolar end attached to the primary leaf. Multiple shoots from primary leaf explants were induced on MSB_5 medium containing different concentrations of BAP (0-10.0 □M). The frequency of shoot regeneration and the number of multiple shoots per explant were recorded after 28 d of culture.

Rooting and plant regeneration

Well-developed shoots (2-3 cm) were excised from the proliferating explants and transferred to medium containing half-strength MS salts (Murashige and Skoog, 1962), full-strength B_5 vitamins (Gamborg et al., 1968) and different concentrations of IBA (1.0 to 5.0µM).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 leastthrice. 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).

Transformation procedure

Selection system
Development of selection system is the most important requirement for any transformation system. Blackgram seeds of cultivar PS-1 were used for the development of selection system using selective agent kanamycin for shoot organogenesis from primary leaf explants. The explants were prepared as described earlier and cultured on shoot regeneration medium (MSB_5 + 1.0 □M BAP) containing different concentrations of kanamycin (0-90 mg/l). The cultures were maintained at 26 ± 2°C under 16h photoperiod of cool-white fluorescent light of intensity 80 □$Em^{-2}s^{-1}$.The explants were subcultured on the same medium with same levels of antibiotic after every two weeks till 4 to 6 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_5+2.5 µM IBA) containing different concentrations of kanamycin (0-10 mg/l).

RNAi vector construction

The disarmed *Agrobacterium tumefaciens* strain EHA105 harboring a binary vector pCAMBIA 2301 and pGD3 were used for transformation studies.

Binary vector, pCAMBIA 2301 was used for optimization of parameters affecting *Agrobacterium*-mediated transformation. It contains genes for neomycin phosphotransferase (NPT II) (*npt*II) and β-glucuronidase (GUS) (*uidA*), both driven by the Cauliflower mosaic virus (CaMV) 35S promoter (Fig.2a). The *uidA* gene contained 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 *uid*A genes.

Binary vector pGD3 was used for MYMV resistance. It contains a neomycin phosphotransferase gene (*nptII*)as a plant selectable marker and hpRNA for AC1 (replicase) gene of MYMV. Neomycin phosphotransferase gene was driven by nos promoter while replicase gene was driven by CaMV 35S promoters (Fig.2b). The construct was procured from Prof. K. Veluthambi, Madurai Kamraj University, Madurai, India. The presence of the binary vector in the *Agrobacterium* was confirmed by colony PCR using primers specific to *nptII* genes.

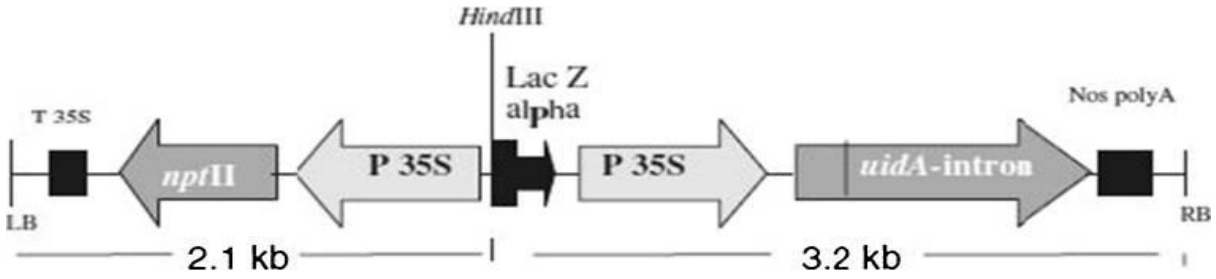


Fig. 2a A schematic representation of the T-DNA of pCAMBIA2301 containing the *uid A* (GUS) and *nptII* genes (not drawn to scale). The position of HindIII is indicated on the T-DNA. No other HindIII sites are present on pCAMBIA2301 (total size 11.6 kb). LB/RB: left and right TDNA border sequences

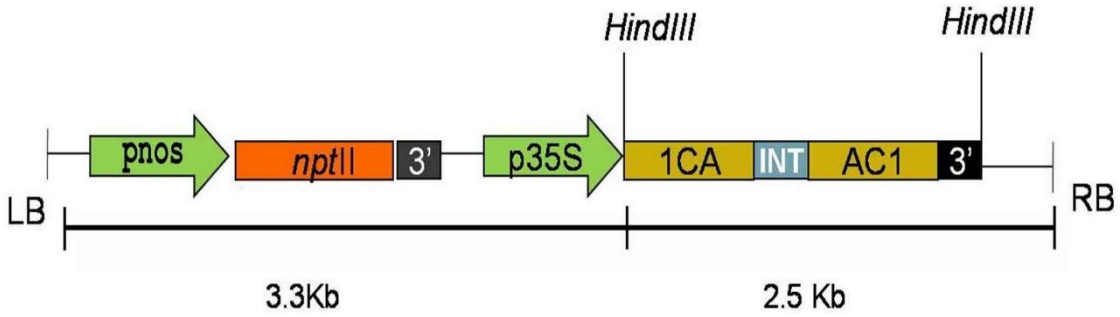


Fig. 2b Linear map of T-DNA (5.8 kb) of pGD3. It contains MYMVhpAC1 (replicase) and *npt*II genes. P5'- nos promoter, p 35S- CaMV 35S promoter, 3'- CaMV 35S terminator. The position of *Hind*III sites are indicated on the T-DNA

Optimization of *Agrobacterium*-mediated transformation protocol

Various factors influencing transformation efficiency of blackgram were optimized using transient GUS expression. For GUS expression studies, *Agrobacterium tumefaciens* strain EHA105 (pCAMBIA2301) was used. The primary leaf explants excised from 4-d-old *in vitro* raised seedlingswere inoculated by immersing in *Agrobacterial* suspension (containing 10^6 to 10^9 cells/ml of bacteria) for 10-40 min with gentle shaking at 80 rpm. The explants were co-cultivated on filter paper moistened with liquid MSBco-cultivation medium containing 1.0 µM BAP adjusted to pH (5.5) for 1-3 days at 22-28°C under light conditions. The effect of phenolic - acetosyringone (100 µM) and thiol compounds, L-cysteine (5 mM), DTT (1.5 mM) in bacterial inoculation and co-cultivation media were also tested. 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 *et al.* (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, 60 explants were used and each experiment was repeated thrice. The frequency of transient GUS expression was calculated as the number of explants showing blue spots at the site of regeneration out of the total number of explants treated.

Agroinfiltration and regeneration of putative transformed plants

A. tumefaciens strain EHA105 (pGD3) culture was resuspended in liquid MSB medium containing 1.0 µM BAP and 100µM acetosyringone. The primary leaf explants excised from 4-d-old seedlings raised on MSB + 10 µM BAP medium, were immersed in bacterial suspension for 25 min with occasional shaking at 25°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 BAP medium for 3 days under 16 h photoperiod at 26 ± 2°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 BAP, 70 mgl^{-1} kanamycin and 500 mgl^{-1} cefotaxime for shoot regeneration. The explants were sub-cultured on to a fresh medium containing same levels of antibiotics after every two weeks for a total 4-6 weeks. Green shoots recovered on selection medium were rooted on MSB medium containing 2.5 □M IBA, 10.0 mgl^{-1} kanamycin and 500mgl^{-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 T_1 plants.

Molecular characterization of transformants

Total genomic DNA was isolated from fresh leaves of non-transformed (control),putative transformant plants (T_0) and their progeny using the CTAB (Cetyl trimethyl ammonium bromide) method (Rogers and Bendich, 1988).

PCR analysis of putative transgenic plants

Putative transgenic plants (T_0) were screened by the PCR for the presence of *nptII* gene. Plant genomic DNA isolated from the leaf tissue was used as a template to amplify *nptII* gene by using specific primers. PCR was carried out with Taq DNA polymerase (MBI Fermentas). 25µl reaction mixtures consisted of ~ 100 ng of plant DNA, 0.25 µl of each primer (forward and reverse each of nptIIgene), 2.5 µl of PCR buffer, 1.5 µl of MgCl2, 0.5 µl of dNTP mix and 1 Unit of Taq DNA polymerase.

The amplification reaction was carried out using a thermal cycler (Perkin Elmer, Foster city, USA) with initial denaturation at 94°C for 5 min, followed by, 30 cycles of 94°C for 40 sec (denaturation), 56°C for 40 sec (annealing) and 72°C for 1 min (extension) followed by a final extension at 72°C for 5 min for detection of *nptII* gene amplification.

*npt*II primers sequence	Forward I	5^1 CTGGGCACAACAGACAATCG 3^1
	Reverse II	5^1 GCGATACCGTAAAGCACGAG3^1
Rep primers sequence	Forward I	5^1 AAGTTCATACGAGTCTGTCG 3^1
	Reverse II	5^1 ACCAAGCATCGTTCGAATAG3^1

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 from pGD3 was used as a positive control.

Southern blot analysis

7-9 µg of genomic DNA from transformed and non-transformed (control) plants was digested with 10 units of HindIII (10 U/µl) restriction enzyme in 100 µl reaction volume, incubated at 37°C in shaking water bath (Heto, Denmark) overnight, blotted on positively charged nylon membrane (Amersham) and fixed by ultraviolet (UV) cross-linking. The blot was hybridized with randomly primed (α^{32}P) labelednptII probe following the supplier's instructions (Bio-Rad, USA). The membrane hybridized with nptII probe was washed following stringent conditions to remove non-specific binding of labeled probe and any trace of unincorporated (α^{32}P) dCTP. Initial washing step was carried out in 2x SSC (0.3 M NaCl, 0.03 M trisodium citrate dihydrate, pH - 7.0) twice for 15 min, at 25°C. This was followed by washing in 1x SSC for min at 65°C and finally in 0.5x SSC for 5 min. at room temperature or when radio activity counts per min declined to 25-40 per min, washing step was stopped and the membrane was processed for autoradiography.

Analysis of T_1 progeny
PCR analysis of T_1 transgenic plants

Plants from T_1 progeny were screened by polymerase chain reaction (PCR) for the presence of *nptII*gene as described above.

Protein extraction from plant (T_1) material

Leaf tissue (1.0g fresh weight) from transformed as well as from the (untransformed) control plants were homogenized with protein extraction buffer containing 40 mM Tris HCL (pH7.2) 5M EDTA and 10 mM β–mercaptoethanol. The homogenate was centrifuged at 18000 g for 15 min at 4°C. The supernatant was used for Western blot analysis.

SDS PAGE and Western analysis

40µg protein was separated on 12% sodium dodecyl sulphate poly acrylamide gel (SDS-PAGE) at 25 mA constant current in the Tris-glycine buffer (25 mM Tris, 250 mM glycine, pH 8.3, 0.1% SDS). The separated proteins were blotted on to a PVDF membrane pre wetted in 100% methanol and then equilibrated in protein transfer buffer, using an electro blotter at 100 mA current overnight. Subsequently, the membrane was incubated in blocking solution (5% nonfat dry milk prepared in PBST buffer (10 mM Tris pH 7.5, 500 mM NaCl, 0.05% tween-20) for 1h at 40 rpm at room temperature. The membrane was given three

wash with PBST buffer for 5 min each. The blot was incubated in PBST buffer containing 1% nonfat dry milk and 1:1000 dilutions of anti-rabbit antisera of *npt*II for 1h at 40 rpm. The blot was washed thrice with PBST buffer (5 min/wash) and incubated with secondary antibody (anti rabbit IgG, conjugated with horse radish peroxidase) for 1h. The membrane was incubated with NBT-BCIP (Nitroblue tetrazolium-bromo chloro indolyl phosphate) till color developed.

RESULTS AND DISCUSSION

Plant regeneration through multiple shoot formation

The primary requirement for an efficient plant regeneration system is the selection of an appropriate explant. In the present study, the seedling (primary leaf) explants which are easily available throughout the year and are relatively free from microbial contamination have been used for *in vitro* plant regeneration. In blackgram, regeneration from the primary leaf explants with petiole via direct organogenesis has not been achieved so far. However, primary leaves via indirect organogenesis through callus formation regenerated limited number of shoots with low regeneration frequency (Srivastava and Pandey, 2011).

Development of selection system

Development of an efficient selection system is primary requirement for plant transformation. It allows growth or proliferation of only transformed cells and eliminates most, preferably all non-transformed cells.Neomycin phosphotransferase (*npt*II) gene is widely used selectable marker gene which is isolated from Tn5 of the *E.coli* K_{12}. It provides resistance to certain aminoglycoside antibiotics such as kanamycin, neomycin, geniticin and paramomycin by detoxifying them via phosphorylation.

Effect of kanamycin on shoot regeneration and root induction

Prior to transformation, an optimal concentration of the kanamycin for the selection of transformed shoots was determined by culturing the non-transformed (control) primary leaf explants on shoot regeneration medium (MS salts +B_5 vitamins +1.0 µM BAP) containing different concentrations of kanamycin (0-90 mgl^{-1}). The survival (determined by necrosis of explants), regeneration frequency and the average number of shoots per explant decreased with increase in kanamycin concentration. Kanamycin at 70 mgl^{-1} in shoot regeneration medium drastically reduced the survival as well as the regeneration frequency of the explants and completely bleached the shoots regenerated from non-transformed (control) explants (Fig.4 d-f).

For rootingcomplete, inhibition of root induction was observed in non-transformed (control) shoots cultured on MSB + 2.5 µM IBA medium containing 10.0 mgl^{-1}kanamycin . This indicates that root induction is much more sensitive to kanamycin than shoot organogenesis as the shoot induction was inhibited at 70 mgl^{-1} and root induction at10 mgl^{-1}.

Genetic transformation of *Vigna mungo* via *Agrobacterium tumefaciens* EHA 105 (pCAMBIA 2301) using optimized conditions

Primary leaf excised from 4-d-old seedlings raised *in vitro* on MSBmedium containing 10.0 µM BAP were immersed in *Agrobacterium* suspension (10^7 cells/ml) prepared in liquid MSB + 1.0 µM BAP + 5 mM L- cysteine + 1.5 mM DTT + acetosyringone (100µM) (adjusted to pH 5.4) for 20 min at 24ºC with occasional shaking. The agro inoculated explants were co-cultured on semisolid MSB medium supplemented with 1.0 µM BAP, 5.0 mM L-cyteine and 1.5 mM DTT for 3 days under 16h photoperiod at 25ºC. After co-cultivation, the explants were washed and immersed in GUS solution to detect transient GUS expression in the explants. Eighty percent of the explants showed GUS activity after 3 days of co-cultivation. GUS activity was predominantly confined at detachment sites of both the primary leaves (Fig.4i). Transformation with pCAMBIA 2301 was performed to check transient GUS expression in explants as GUS gene was not present in the binary vector pGD3.

Regeneration of transformants

From 230 explants inoculated with *Agrobacterium* a total of 79 green shoots produced on kanamycin selection medium (Table 1, Fig.4 a-h). Out of these, 66 shoots formed roots in the presence of kanamycin (10 mgl^{-1}) and cefotaxime (500 mgl^{-1}). Sixty-six rooted shoots (plantlets) were transferred to soil, where 55 plants survived, grew to maturity and produced transgenic seeds in the green house. The seeds were collected and sown in soil to raise the T_1 plants. The total time required from the inoculation of explants to establish plants in the green house was approximately 2 months. Molecular analysis of these plants was carried out to confirm their transgenic nature.

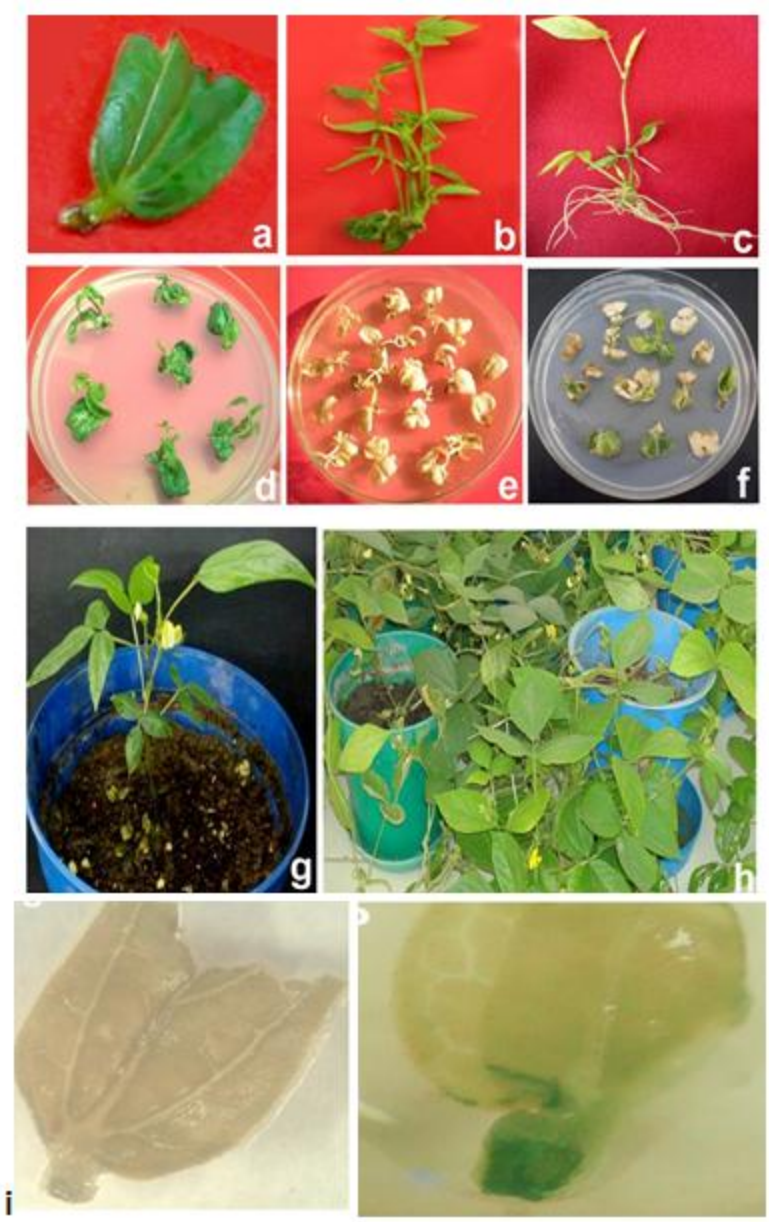


Fig. 4 Genetic transformation of primary leaf explants of *Vigna mungo*L. Hepper cv. PS1 with *Agrobacterium tumefaciens* strain EHA105 harboring a binary vector pGD3, that contained hp *rep* and *npt*II genes.

a. The non-transformed (control) primary leaf explant excised from 4- d- old seedlings.
b. Direct multiple shoot regeneration from primary leaf explant on MSB medium supplemented with 1.0 µM BAP after 4 weeks of culture
c. Induction of roots on *in vitro* regenerated shoot cultured on MS basal medium supplemented with IBA (2.5 µM) + kanamycin (10 mgl^{-1}) + cefotaxime (500 mgl^{-1}).
d-f. Recovery of shoots from non-transformed (control) and transformed explants in the presence or absence of kanamycin.*In vitro* regeneration of shoots from the non-transformed (control) explants cultured on MSB +1.0 µM BAP medium without kanamycin (d), Non-transformed (control) explant cultured on MS+1.0 µM BAP medium containing 70 mgl^{-1} kanamycin, showing completely bleached shoots (e), *Agrobacterium tumefaciens* strain EHA105 (pGD3) inoculated explants cultured on MS + 1.0 µM BAP medium containing 70.0 mgl^{-1} kanamycin and 500 mgl^{-1} cefotaxime showing regeneration of green transformed shoots (f).
g. A fertile transgenic plant growing in pot containing soil.

h. Well established transgenic plants in pots containing soil in the green house.
i Transient GUS expression in primary leaf explant inoculated with *Agrobacterium tumefaciens* strain EHA 105 harboring a binary vector pCAMBIA2301 that contained *uid* A (GUS) and *npt*ll genes. (The non-transformed (control) primary leaf explant showing no GUS activity and explant showing transient GUS activity after 3 days of co-cultivation with *Agrobacterium tumefaciens* EHA105 (pCAMBIA2301).

Table 1: Summary of transformation of primary leaf explants of *Vigna mungo* (cv. PS-1) following inoculation with *Agrobacterium tumefaciens* strain EHA 105 containing a binary vector pGD3

Exp. No.	No. of explants inoculated with *Agrobacterium*	No. of shoots recovered on selection medium[1]	No. of shoots rooted on selection medium[2]	No. of putative transgenic plants established in soil	No. of plants +ve for *npt*II by PCR	No. of plants +ve for *npt*II by Southern hybridization
1	85	31	27	25	2	1
2	75	27	22	20	4	1
3	70	21	17	10	3	1
Total	230	79	66	55	9	3

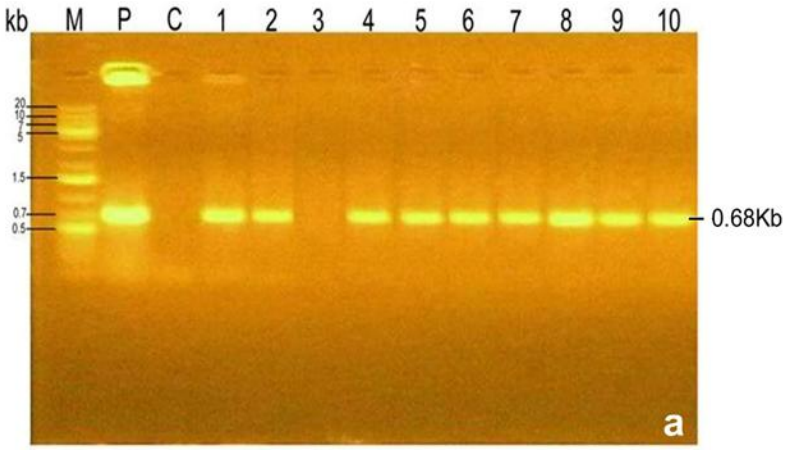
1) Selection medium for shoot regeneration : MSB + BAP (1.0 µM) + kanamycin (70 mgl^{-1}) + cefotaxime (500 mgl^{-1})
2) Selection medium for root induction : MSB + IBA (2.5 µM) + kanamycin (10 mgl^{-1}) + cefotaxime (500 mgl^{-1})

PCR analysis for *npt*II and *vir*A gene

PCR analysis showed amplification of an expected fragment of 685 bp corresponding to the coding region of *npt*II gene indicating the presence of transgene in 9 out of 55 putative transformed plants established in soil. No amplification was detected in the untransformed (control) plants (Fig.5a). The negative results could be due to non-transformed shoots surviving on the selection medium, incomplete insertion, or the transferred gene was not stably integrated into plant genome and was lost (Hess *et al*., 1990; Langridge *et al*., 1992). PCR analysis of *npt*II positive plants with *vir* primers did not show any amplification thus rule out the possibility of *Agrobacterium* contamination in the transformed plants (Fig. 5 b).

Southern hybridization

Southern analysis of *npt*II PCR positive T_0 plants was performed to confirm the integration of *npt*II gene into their genome and to determine its copy number. The genomic DNA of PCR positive plants was digested with *Hind*III and hybridized with 680 bp amplicon of *npt*II gene (probe). Since *npt*II probe hybridized to digested DNA from transgenic plants but not to digested DNA from untransformed (control) plants, the result indicated that *npt*II gene was incorporated into blackgram genome. The first *Hind*III site in pGD3 is next to right T-DNA border, in downstream of *npt*II gene (Fig.2b). Digestion with *Hind*III and subsequent probing with *npt*II coding region identifies border fragments between the T-DNA and plant DNA.



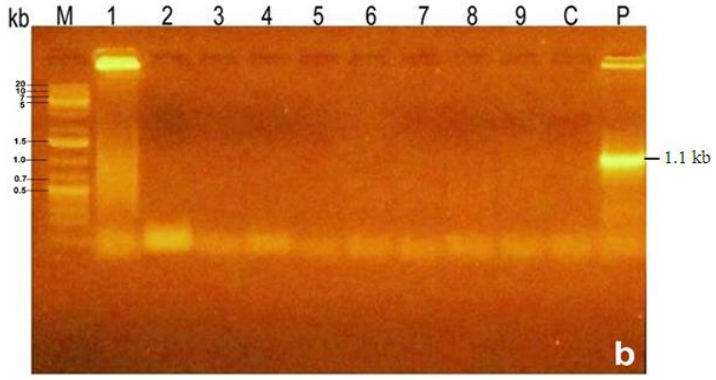


Fig. 5 Molecular analysis of primary transformants of *Vigna mungo* recoveredfrom primary leaf explants transformed with *Agrobacterium tumefaciens* strain EHA105 harboring a binary vector pGD3, that contained hp *rep* and *npt*II (a) **PCR analysis of putative transformants with primers specific to the coding region of *npt*II *gene*.** Lane M: DNA Molecular weight marker (1kb),Lane C: DNA from non-transformed (control) plant Lane P: positive plasmid DNA, Lanes 1 to 10: DNA fromtransformed 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 (1kb),Lane C: DNA from non-transformed (control) plant, Lane P: Plasmid DNA, Lanes 1 to 9: DNA fromtransformed plants (T_0)

*Hind*III also cleaves the entire AC1 as an internal fragment of about 1.2 kb. The sizes of the bands detected with the *npt*II probe were greater than that of *npt*II- containing *Hind*III frangment (3.3 kb) confirms the integration of the *npt*II gene into plant genome (Fig. 6). DNA from untransformed (control) plant failed to hybridize to the *npt*II probe. The hybridization pattern showed that three plants had a single copy of T-DNA integrated in the genome. Hybridization signals in all the three plants were of the same size indicating that these plants are derived from a single transformation event. Thus, Southern analysis confirmed the presence and integration of the transgene in the transformants. An overall stable transformation frequency of 1.3% (number of the Southern positive plants / total number of explants inoculated with *Agrobacterium* x 100) was obtained.

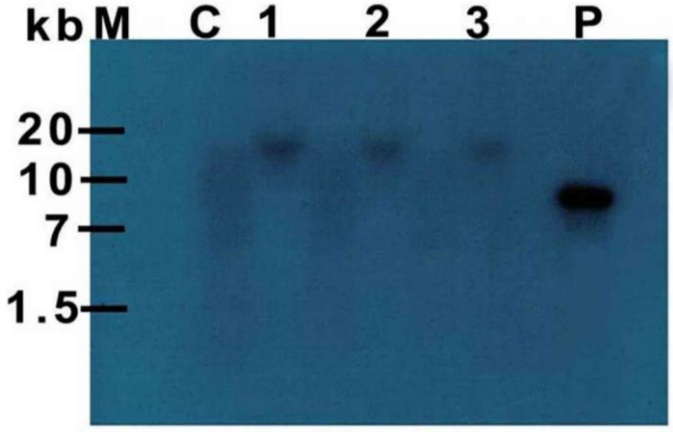


Fig.6: Southern hybridization of genomic DNA from primary transformants of *Vigna mungo* recoveredfrom primary leaf explants with *Agrobacterium tumefaciens* strain EHA105 harboring a binary vector pGD3, that contained hp *rep* and *npt*II genes.Southern blot analysis of genomic DNA of transformed and non- transformed control plants. The DNA was digested with *Hind*III, and blot was probed with the PCR amplified fragment (685 bp) of *npt*II gene. Lane M: DNA Molecular weight marker (1kb) ,Lane C: DNA from untransformed (control) plant, Lanes 1-3: DNA from transformed plants , Lane P: Plasmid DNA

Analysis of T_1 progeny
PCR analysis of T_1 progeny

All the three Southern positive T_0 plants were self-fertilized to produce seeds. The progeny of the T_0 plants was analyzed by PCR using *npt*II- specific primers (Fig. 7). The progeny showed segregation for the presence and absence of *npt*II-amplified band in a simple Mendelian 3:1 ratio suggesting a simple integration site (Fig.7). Statistical analysis of the progeny also confirmed inheritance and segregation of the transgene in 3:1 ratio at 0.05% significance level (Table 2).

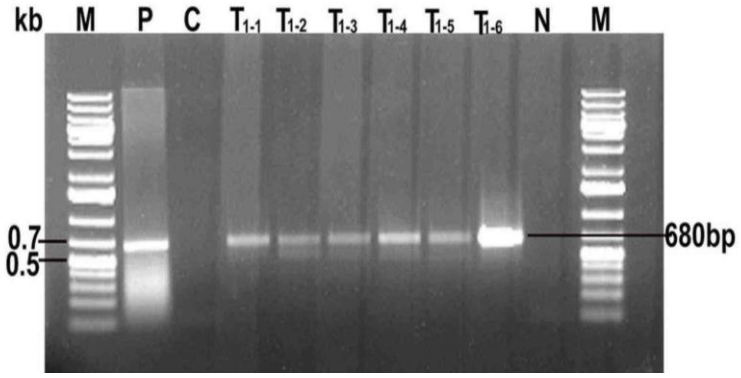


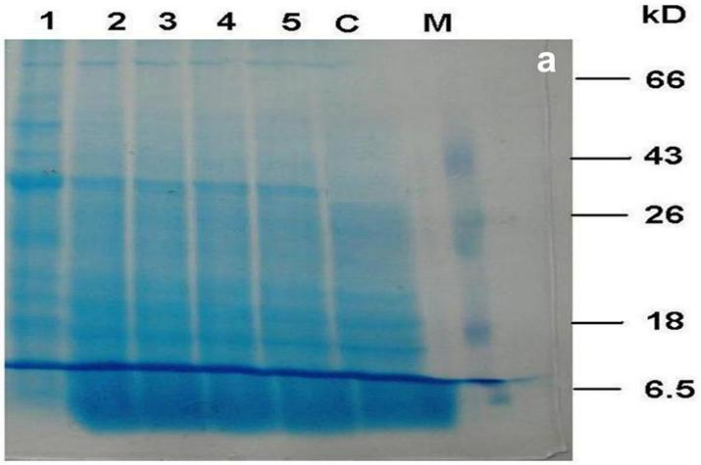
Fig. 7: PCR analysis of T_1 plants using primers specific to *npt*II gene-Lane M: DNA Molecular weight marker (1kb),Lane C: DNA from non-transformed (control) plant, Lane P: positive plasmid DNA, Lanes1 to 6: DNA from T_1 transformed plants

Table2: Segregation ratios of the *npt*II gene in selfed progenies of transformed *V. mungo* cv. PS1

Transfo-rmants	Total number of T_0 seeds collected	Number of T_1 plants raised in pots	Number of plants positive for *npt*II by PCR	Number of plants negative for *npt*II by PCR	(p value) χ^2 –Test	Expected segreg-ation ratio
1	40	20	15	5	0.49 (p>0.05)	3:1
2	32	14	10	4	0.56 (p>0.05)	3:1
3	35	16	12	4	0.82 (p>0.05)	3:1

Western analysis of T_1 plants

The expression of *npt*II gene in T_1 transgenic plants was confirmed by Western blotting.The total soluble protein from transgenic and untransformed (control) plants were run on 12% SDS polyacrylamide gel electrophoresis (Fig. 8a) and subjected to immuno-blotting using antibodies specific to NPTII.All the PCR positive T_1 plants analyzed for western blotting showed the cross reaction of *npt*II antibodies with protein (of 30 kDa which corresponds to the *npt*II) from all of them (Fig. 8b). The untransformed (control) plants showed no cross - reactivity with anti- *npt*II antibodies.



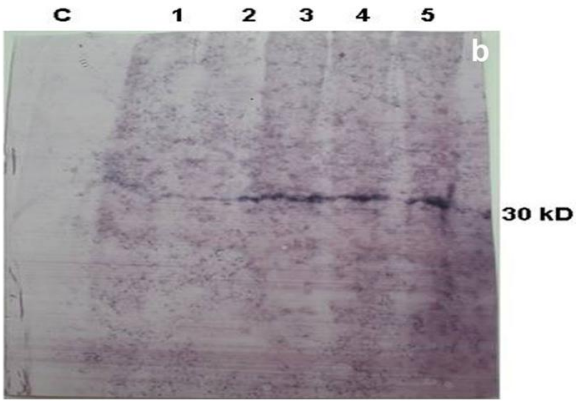


Fig. 8: Size fractionation of neomycin phosohotransferase (NPTII) protein from T_1 transformed and untransformed plants by SDS-PAGE and Western analysis. a) SDS-PAGE analysis of T_1 transgenic plants- Lanes 1-5: Protein from transformed plants, Lane C: Protein from untransformed plants, Lane M: Protein molecular weight marker **b)** Western blot analysis of T_1 plants for neomycin phosphotransferase using *npt*II antibodies**.** Lanes 1-5: Protein from transformed plants, Lane C: Protein from untransformed plants

In conclusion, morphologically normal and fertile transgenic plants of blackgram have been obtained for the first time using primary leaf explants, *Agrobacterium tumefaciens*and RNAi technology. Most of the earlier reports of blackgram transformation used cotyledonary node as explants (Saini *et al.*,2003; Bhalla-Sarin *et al*., 2004; Saini and Jaiwal, 2007; Muruganantham *et al.*, 2007; Bhomkar *et al*., 2008). Maximum transformation frequency obtained was 1% with mature and 7.6% with immature cotyledonary node explants (Saini *et al*., 2003; Muruganantham *et al.*, 2007). These explants were mechanically injured with a fine needle at nodal region prior to their inoculation with *Agrobacterium* which results in damage of the regenerable cells present at the node during injury. However, the primary leaf petiole explants have meristematic regenerable cells at the cut end of explants which are easily accessible to *Agrobacterium* and there is no need of injury.

Since the plant is recalcitrant to transformation. The various factors affecting the efficiency of gene transfer were optimized. Bacterial strains differ in their virulence and the choice of the appropriate strain is an important factor for successful transformation. In present study, *Agrobacterium* super virtulent strain EHA 105 has been used for transformation as reported earlier. Addition of acetosyringone (100 μM) to the bacterial inoculation and/or co-cultivation media and co-cultivation for 3 d have transformation rates similar to earlier reports (Saini and Jaiwal, 2007).

The effect of inclusion of antioxidants (L-cysteine and dithiothreitol) in co-cultivation medium was not reported previously in blackgram. The presence of 5mM L-cysteine and 1.5 mM dithiothreitol 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 T_0 plants was confirmed by PCR and Southern blot analysis. The T_0 plants transmitted transgenes toprogeny in Mendelian fashion as revealed by PCR. The expression of transgene in T_0 progeny was confirmed by Western blot analysis.

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