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Agrobacterium tumefaciens-Mediated Genetic Transformation of Green Gram [*Vigna radiata* (L.) Wilczek] - A Recalcitrant Grain Legume

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ABSTRACT

Background: Green gram is grown in many parts of India as a source of dietary protein (21-25%). It is an important nitrogen fixing crop which fixes atmospheric nitrogen (119-140 kg/ha) to soil and enhance the soil productivity. In the present investigation, efficient *Agrobacterium*-mediated genetic transformation of *Vigna radiata* L. (Wilczek) has been achieved with *VrTIP1* gene for abiotic stress resistance *i.e.* moisture and salinity stress.

Methods: Four days old shoot tip and cotyledonary node were used for *in vitro* regeneration with MS medium supplemented with BAP 2.0 mg/l, kinetin 0.5 mg/l and 50 mg/l kanamycin for co-cultivation with *Agrobacterium tumefaciens* strains, LBA 4404. The modified binary vector pCXSN, EHA105 containing hygromycin phosphotransferase II (*hpt II*) marker genes and a synthetic TIP1 gene under a constitutive CaMV35S promoter were used for transformation of *Vigna radiata* L. cotyledonary node explants. Putative transformants selected from hygromycin resistant shoots were subsequently rooted on MS medium supplemented with 1.0 mg/l NAA and later transferred to sterile vermiculite followed by transfer to the transgenic green house.

Result: The T_1 plants were produced from PCR positive T_0 plants and analysed for presence and integration of transgenes in putative T_1 plants were confirmed by polymerase chain reaction (PCR) amplification of 752 bp of *hpt II* fragment. This protocol can be effectively used for transferring new traits in greengram and other legumes for their quantitative and qualitative improvements.

Key words: Abiotic stress, *Agrobacterium tumefaciens*, Green gram (*Vigna radiata* L.) Genetic transformation, TIP1 gene.

INTRODUCTION

Green gram [*Vigna radiata* (L.) Wilczek] is an important edible grain legume grown for its protein-rich edible seeds in many tropical and sub tropical countries. It is an important nitrogen fixing crop and has a short life cycle therefore is widely grown as mixed, inter crop or in rotation to improve nitrogen status of soil. It is grown mainly in arid and semi-arid situations across the country during *Kharif* and *Rabi* season and contributes nearly 15% of total production (Patra *et al.,* 2018).

The reasons for low productivity in green gram are its susceptibility to biotic and abiotic stresses (Jaiwal and Gulati, 1995). Conventional breeding has limited scope due to less variability in germplasm. Genetic transformation through *Agrobacterium* and regeneration of transgenic plants has been reported in black gram (Das *et al.,* 2016), green gram (Mekala *et al.,* 2016).

In the past few years, some progress has been made regarding the *in vitro* regeneration of green gram (Mahalakshmi *et al.,* 2006), but very little success has been reported regarding genetic transformation of green gram through *Agrobacterium* (Hoque *et al.,* 2007). Though the genetic transformation of grain legumes have been difficult and challenging till now, but significant progress has been made in the recovery of transformed plants via *Agrobacterium* in soybean, pea, chickpea, pigeon pea (Bhatnagar- Mathur and Sharma 2016). The members of genus *Vigna* such as *V. mungo* and *V. radiata* have been shown to be susceptible to *Agrobacterium tumefaciens* and only transgenic callus lines have been generated. The present study describes the establishment of efficient *A. tumefaciens* based transformation protocol for production of transgenic of *V. Radiata* plant.

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MATERIALS AND METHODS

Seeds of green gram [*Vigna radiata* (L.) Wilczek] cv. IPM-02-03

were procured from Regional Research and Technology Transfer Station, Odisha University of Agriculture and Technology (OUAT), Berhampur, Ganjam, Odisha were used in the present study. The experiment was conducted in Dept of Agril. Biotechnology, OUAT, Bhubaneswar in 2018.

Explant preparation

Seeds of *Vigna radiata* L. were washed thoroughly with water for 10-20 min and then treated with a solution of the tween 20 (5% v/v) for 10 min and surface sterilized with $HgCl_2$ (0.1% w/v) for 5 min. The seeds were rinsed thrice with autoclaved distilled water to remove any trace of contaminants. Seeds were sown in MS medium (Murashige and Skoog, 1962) supplemented with BAP 2 mg/l (8.5 µM). After germination the cotyledonary node and shoot tip explants were excised from 4 day old seedling under asceptic condition as described by Patra *et al.* (2018).

Culture media and conditions

Modified MS media used in this investigation were supplemented with 3% sucrose and adjusted to pH 5.8 before autoclaving at 15lb pressure at 121°C for 15 min**.** To prepare semisolid media, 6 g/l agar (Himedia, India) was added before autoclaving. All the cultures were maintained under continuous white light (fluorescence density of 60 µmol/m^2/s) at 25°C ± 2°C.

Binary vector and *agrobacterium*

The disarmed *Agrobacterium tumefaciens* strain EHA105 harboring a binary vector pCXSN, which contains TIP1 gene and a hygromycin phosphotransferase gene (*hptII*) both driven by CaMV35S promoter, was used for transformation studies (Fig 1).

Transformation and plant regeneration

Agrobacterium tumefaciens strain EHA105 harbouring plasmid vector pCXSN was cultured on 20 ml of YEM medium containing 50 mg/l kanamycin and 50 mg/l rifampicin and kept in an incubator for overnight at 28°C on a rotary shaker at 120 rpm. Bacteria were pelleted at 5000 rpm for 10 min and resuspended in liquid shoot regeneration medium (SR) containing MS salts, B5 vitamins, 3% sucrose and 2 mg/l BAP. The density of bacterial suspension was checked at 600 nm and dilutions were made for different concentrations of bacterial cells. Optical density 0.6-0.8 was found to be optimum for transformation. The cotyledonary node explants excised from 2 days old germinated seedling were inoculated for 10 - 60 min with different concentrations of bacterial cells (10^6 - 10^9 cells/ml) and co-cultured on liquid MS (Shoot Regeneration) medium for 1 - 4 d under 16-h photoperiod (cool white fluorescent tubes, irradiance of 80 µmol /m^2/ s) and temperature of 25 ± 2°C. To increase the transformation efficiency, acetosyringone (100 µM) was added to inoculation medium and efficiency was evaluated. The effect of pre-culture of explants, on SR medium for 0 - 3 d and mechanical injury, both the factors either alone or in combination prior to inoculation with bacterium, was also investigated. After co-cultivation, the explants were washed 3-4 times with liquid SR medium containing cefotaxim 250 mg/l to check the bacterial growth for further shoot development. For each treatment, 40 explants were used and each experiment was repeated thrice. Hygromycin sensitivity test was carried out to find out the minimum concentration of hygromycin required to inhibit the growth of untransformed explants in order to design the selection medium. The test was carried out by culturing the explants on regeneration medium along with different levels of hygromycin concentration (0, 5.0, 10.0, 15.0, 20.0 and 25.0 mg/l). Putative transformants and percent of putative transformants were calculated on the basis of hygromycin selection.

DNA extraction

The total genomic DNA was extracted from young fresh leaves of the putative transformants (T_0) and non-transformed (control) plants by CTAB method (Doyle and Doyle, 1990).

Polymerase chain reaction analysis

Putative transformants (T_0) were screened by the polymerase chain reaction (PCR) for the presence of the TIP1 gene. The genomic DNA of plants obtained from co-cultivated explants, control plants and plasmid DNA (positive control) were used as template for PCR confirmation of the targeted transgene with primers TIP1gene.

Nucleotide sequence of specific primer TIP1geneis as follows;

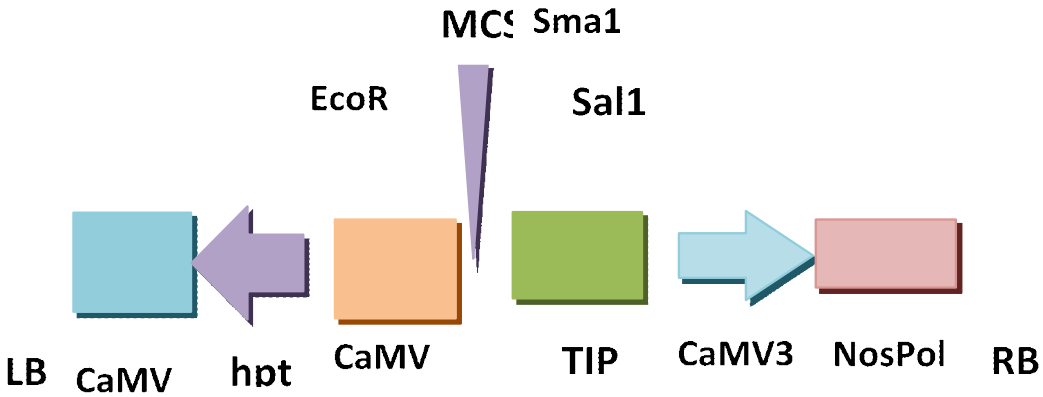

Fig 1: Linear map of T DNAregion of vector pCXSN.

Forward- 5'- GATTGA AGA TGC CGA TCA GAA ACA TCG CCA-3'
Reverse- 5'- CTG GGT CTA GTAGTCAGT GGT TGG GAG CTG-3'

PCR was carried out with Taq DNA polymerase following modified PCR protocol. To ensure that reagents were not contaminated, DNA from non-transformed (control) plants was included in the experiments. The amplified products were separated by electrophoresis on a 1% agarose gel and visualized with ethidium bromide in Gel Documentation Unit.

RESULTS AND DISCUSSION

Cotyledonary node explants excised from four days old *in-vitro* raised seedlings were tested on MS medium and transformed by infecting with *Agrobacterium tumefaciens* strain LBA4404 (Fig 2A) containing the binary vector pCXSN-TIP1(Fig 1). The multiple shoots (Fig 2B and Fig 2C), developed from cotyledonary node were transferred to fresh MS semi-solid culture with 50 mg/l kanamycin after 4 -6 weeks under standard cultural conditions. In the present study, transformation frequency was highest when co-cultivation was performed at 24°C. These shoot buds were transferred into MS basal medium supplemented with 0.5 mg/l NAA ad developed roots in 4 weeks from (Fig 2D) basal part of shoots. These roots elongated (10 - 15 cm) and became sturdy and branched (Fig 2E) in 4 weeks and developed plantlets. Significant survivability was found from the explants co-cultivated for 72 hours 81.45%. Survivability percentage was reduced to 61.92% in co-cultivation period

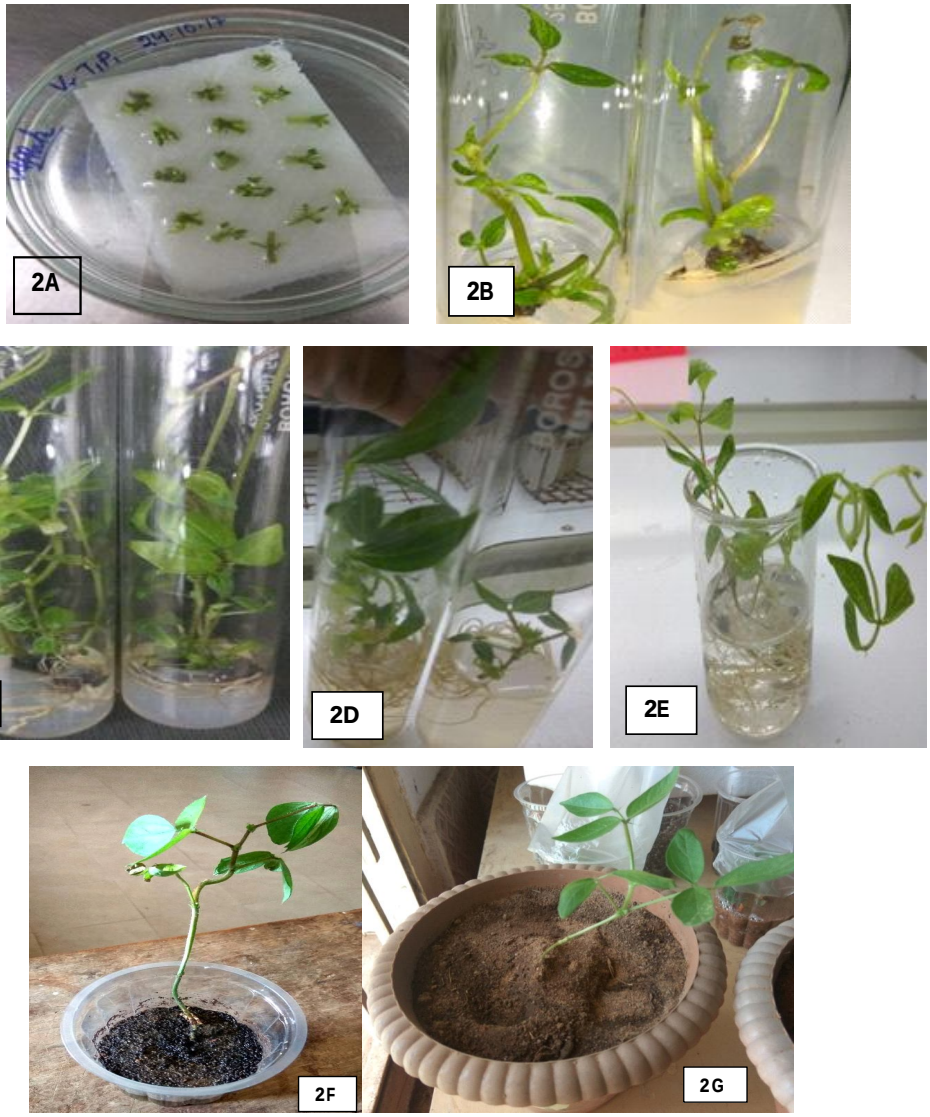

Fig 2A: Explants co cultivated with *Agrobacterium*. **B:** Multiple shoots of *V. radiata* L. after co cultivation. **2C:** Development of Multiple shoots after 20 days of co cultivation. **2D:** Development of roots with 0.5 mg/L NAA. **2E:** Acclimatisation of plantlets in normal water in culture room. **2F:** Pre hardening of rooted shoots with soil: sand: FYM (1:1:1). **2G:** Rooted shoots were transferred to pots containing soil: sand: FYM (1:1:1) mixture for hardening in green house.

of 96 hours. Hence 72 hours found to be suitable for co-cultivation period.

Selection of transformants

Kanamycin and hygromycin have been successfully employed as a selectable marker in transformation of various legume crops. It is compulsory to determine the highest concentration of the selective agent that suppresses growth and proliferation of untransformed cells. Hygromycin at 25 mg/l in shoot regeneration medium drastically reduced the survival as well as the regeneration frequency of the explants and completely bleached the non transformed shoots. Hygromycin concentrations higher than 25 mg/l were lethal causing necrosis of explants and complete inhibition of regeneration. Therefore, 25 mg/l Hygromycin 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 MS medium containing 0.5 mg/l IAA medium containing 25.0 mg/l hygromycin.

The stable transformed shoot buds were selected by periodic increase in hygromycin concentration. Initially after 20 days on medium containing 5.0 mg/l hygromycin, a high number of shoot buds were obtained which later developed roots and develop plantlets (Fig 2F). When transferred to medium containing higher concentrations of the antibiotic (15.0, 20.0, 25.0 mg/l), many of the shoots became yellowish, and were discarded after 45 days. Out of the developing hygromycin resistant transgenic plants, about 20% showed normal shoot and root development compared to a conversion frequency of 67% in the control plantlets. The putative transgenic plants had no phenotypic abnormalities in comparison to the untransformed control plants (Fig 2G). The results of the current work showed that the use of cotyledonary node explants is more efficient compared for genetic transformation of green gram (*Vigna radiata* L.) varieties.

Optimization of transformation protocol

Optimization of transformation protocol is required for efficient transformation by *A. tumefaciens*. Bacterial concentration (cells/ml), inoculation time, co-cultivation period, addition of acetosyringone, temperature and pH 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 TIP1 expression using cotyledonary node explants (Table 1).

Molecular analysis of putative transformants

Putative transformants were screened by the PCR in presence of *hptII* genes. For this purpose the total genomic DNA was isolated from the leaf tissue of transformants survived on the selection medium (Hygromycin B 25 mg/l) as well as control. Then the PCR reaction was carried out using *hptII g*ene specific primer and plasmid DNA used as a positive control.

The PCR amplified product was electrophoresed on 1.2% agarose gel. Expected band of about 752 bp was obtained only in six samples out of 17 samples tested. The transformation efficiency found to be about 4.41% (Table 3 and Fig 4). Six T_0 plants produced 44 seeds which on germination produced T_1 plants were tested for presence of the TIP1 gene.

From the PCR analysis, 28 numbers of plants were found to be positive for presence of TIP1 transgene (Fig 3, 4 and Table 3).

The expression of TIP1 gene through *Agrobacterium*-mediated transformation and tolerant to moisture and salinity stress was detected in *Arabidopsis* (Li *et al.,* 2015). The natural ability of *A. tumefaciens* to introduce DNA into plant cells is being widely exploited for the genetic transformation of plants (VanWordragen and Dons, 1992). Therefore, this system is considered to be suitable for the transformation of green gram. Transformation and successful regeneration

Table 1: Effect of different transformation parameters on % of explants survived in Selection media in cotyledonary node explants of *Vigna radiata* co-cultivated with *Agrobacterium tumefaciens* strain EHA105 harboring binary vector pCXSN.

Factors	Variables	% of explants survivedin Selection media
Bacterial concentration (cells/ml)	10^6	65
	10^7	85
	10^8	79
	10^9	72
Innoculation time (min)	10	55
	20	84
	30	76
	40	62
Co cultivation time (hr)	24	57
	48	75
	72	88
	96	78
Acetosyringone conc (µm)	30	58
	50	67
	70	75
	100	90
	130	81
pH	5.0	48
	5.2	75
	5.5	84
	5.8	61
Co-cultivation temperature (°C)	22	57
	24	75
	26	68
	28	47

Note: Each treatment repeated thrice. 20 explants per replication.

procedures were based on a direct shoot organogenesis protocol previously described by Eissa *et al*., (2002).

The sensitivity of plant cells to the selective agent depends upon the genotype, the explant type, size and developmental stage of tissue(s). Hence it is compulsory to determine the lowest concentration of the selective agent that suppresses growth and proliferation of untransformed cells. The survival, regeneration frequency and the average number of shoots per explant decreased with increasing hygromycin concentration.

The maximum transformation frequency was observed at a concentration of 10^7 cells/ml with a constant increase up to 10^7 cells/ml and decrease their off, as reported in tobacco, *Arabidopsis thaliana* (Lin *et al.,* 1994) and other grain legumes (Bean *et al.*, 1997).

Co cultivation duration directly affect the transformation efficiency where as longer incubation with *Agrobacterium*, led to bacterial leaching. Longer period of co-cultivation resulted in negative effects due to the overgrowth of the bacterium in *V. radiata* (Sonia *et al.,* 2007). Higher transformation efficiency could be achieved by 2-3 days of incubation period. Transformation efficiency can further be enhanced by adding acetosyringone at various concentrations to the co-cultivation medium (Table 1). Acetosyringone was applied to improve the *Agrobacterium* infection which is a phenolic compound produced by the wounding of plant tissues that induces the transfer of T-DNA from *Agrobacterium* to the plant genome (Lee *et al*., 1995). This might be due to vir gene activation which facilitated the transgene integration via wound of the explant under the influence of acetosyringone. In this present investigation, addition of 100 ìM acetosyringone to freshly excised cotyledonary nodes tissues could increase the transformation efficiency (Table 1). The increase in the transformation efficiency of freshly cut tissues in the presence of acetosyringone suggests that the synthesis of *vir* inducers during incubation, and the accumulation of *vir* inducing compounds in the wounded and pre incubated plant tissues are an important factors contributing to the increased competence of the incubated common bean cotyledonary node tissues to *Agrobacterium* mediated transformation. Stimulation of plant cell division and activation of the DNA replication machinery during the incubation period may play an important role in the integration of plasmid DNA leading to stable transformation.

Droste *et al.* (2000) used 100 µM of acetosyringone to increase the virulence of *Agrobacterium tumefaciens* in *Glycine max*. Similarly, acetosyringone has been used to achieve higher transformation efficiency in several *Vigna* species, such as *V. radiata* (Sonia *et al.,* 2007) and *V. mungo* (Sainger *et al.,* 2015). Higher transformation frequency has been reported at low pH of bacterial inoculation and co-culture media (Solleti *et al.,* 2008). Temperature plays an important role in co cultivation process affecting T-DNA transfer. Co-cultivation at low temperature has been reported in several instances (Popelka *et al.,* 2006). It has also been found that the temperature dependence profile of T-DNA transfer does not 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).

Table 2: Determination of the sensitivity of *Agrobacterium* to various level of cefotaxime concentration.

Treatments	Conc of cefotaxim (mg/l)	Bacteria growth reappearance	Bacteria growth reappearance (%)	Bacteria growth
T_1	0	20	100	++++
T_2	100	16.8	84.	+++
T_3	200	14.2	71	+++
T_4	300	8.3	41.5	++
T_5	400	4.6	23	+
T_6	500	0	0	-
CV%		2.31	3.8	
CD(p=0.05)		1.29	1.34	

Note : Each treatment repeated thrice. 20 explants per treatment.

- No growth, + little growth, ++ moderate, +++ Prominent growth, ++++ over growth of *Agrobacterium.*

Table 3: Transformation efficiency of T_0 plants based on hygromycin B selection and PCR analysis.

Total no of explant assayed	No. of shoots obtained after co cultivation	No. shoots selected based on Hygromycin selection	Transformation efficiency (%) based on Hygromycin Selection	No. plants selected based on PCR analysis	Transformation Efficiency (%) based on PCR
175	136	17	12.5	06	4.41

Table 4: Transformation efficiency of T_1 plants based on PCR analysis.

Total no of T_1 plant assayed	No. of plants obtained after germination	No. plants Obtained in PCR Selection	Transformation efficiency (%) after PCR analysis
44	44	28	63.6

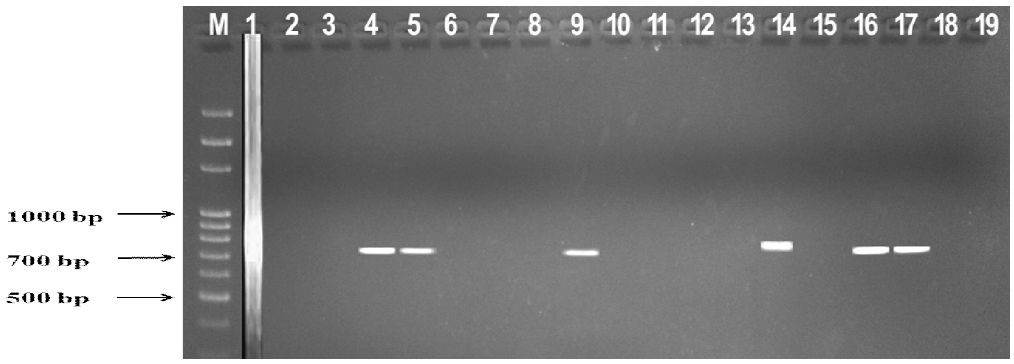

Fig 3: PCR analysis of T0 transformed plants using *hptII* as primer.
M. 100bp DNA ruler.
1. Positive control (Plasmid DNA), 2. Negative control, 3-18. Putative transformants 19. Untransformed plant.

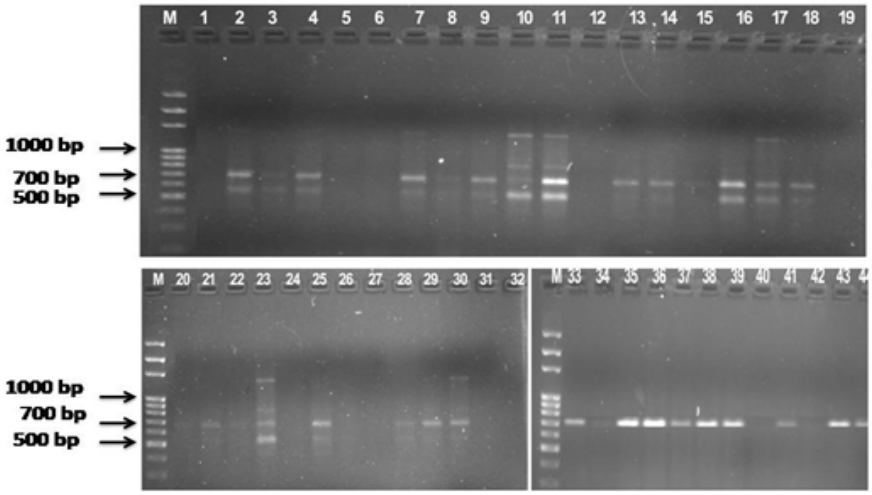

Fig 4: PCR analysis of T_1 transformed plants using *hptII* primer.

In the present study, the *hptII* gene coding was tested by selection of hygromycin stable transgenic shoots. The transgenic character of hygromycin resistant shoots was confirmed by detection through PCR in resistant tissues. Thus, these studies provide strong confirmation for a hygromycin resistant gene transferred to plant cells. Similar results were achieved by Jaiwal *et al*. (2001) who regenerated transformed shoots directly from cotyledonary node explants of mungbean cultured on medium containing 75 mg/l kanamycin after co-cultivation with LBA4404 (pTOK233).

CONCLUSION

Transgenics technology supplements the breeding programme for genetic improvement of crop plants where conventional breeding cannot be achieved due to sexual barrier and limited availability of genes within the germplasm.

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Conflict of interests

The authors declare that there is no conflict of interest in the present investigation.

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