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ORIGINAL ARTICLE

Optimization of *Agrobacterium*-mediated genetic transformation of shoot tip explants of green gram (*Vigna radiata* (L.) Wilczek)

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Abstract Shoot tip explants prepared from seedlings of ML-267 genotype of green gram were inoculated on MSB5 medium supplemented with BAP (0–20 µM) individually or in combination with minimal concentration of auxins (NAA/IAA/IBA) for adventitious shoots formation. BAP alone without auxins was observed to be efficient in multiple shoot induction and optimum shoot proliferation was achieved on MSB5 medium containing 10 µM BAP with 100% shoot induction frequency. 3-day-old explants gave best shoot multiplication response and the mean shoot number decreased significantly in 4-day and 5-day-old explants. The induced shoots rooted profusely on ½ MSB5 + 2.46 µM IBA and about 90% of the plantlets survived after acclimatization and set seed normally. Shoot tip explants infected with *A.tumefaciens* (LBA4404) harboring pCAMBIA 2301 + *AnnBj*1 recombinant vector. Various factors which influence the competence of transformation were optimized based on the frequency of transient GUS expression in shoot tip explants. Optimum levels of transient GUS expression were recorded at pre-culture of explants for 2 days, infection for 10 min with Agro-culture of 0.8 OD and co-cultivation for 3 days on co-cultivation medium containing 100 µM acetosyringone in dark at 23 °C. Putative transformed shoots were produced on selection medium (shoot inductionmedium with100 mg/l kanamycin and 250 mg/l cefotaxim). PCR analysis confirmed the presence of *AnnBj1, npt*II, and *uidA* genes in T_0 plants. Stable GUS activity was detected in flowers of T_0 plants and leaves of T_1 plants. PCR analysis of T_1 progeny revealed *AnnBj1* gene segregated following a Mendelian segregation pattern.

Keywords Green gram (*Vigna radiata* (L.) Wilczek) · Shoot proliferation response · *Agrobacterium*-mediated genetic transformation · Shoot tip explants

Abbreviations

BAP	6-Benzyleaminopurine
Cefo	Cefotaxim
GUS	β-Glucuronidase
IAA	Indole-3-acetic acid
IBA	Indole-3-buteric acid
Kan	Kanamycin
MSB5	MS basal salts with gamborg vitamines
NAA	1-Naphthaleneacetic acid
OD	Optical density
SBIM	Shoot bud induction medium

Introduction

Green gram is an important leguminous pulse crop and major source of vegetarian protein for humans and animals. It improves the soil fertility by association with nitrogen fixing bacteria and accumulation of organic matter. The

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productivity of this crop is very low and the production has been stagnant over the years because of its susceptibility to various biotic and abiotic stresses and cultivation as an orphan crop (Sahoo and Jaiwal 2008). Efforts to develop stress tolerant varieties by traditional breeding achieved limited success owing to its narrow genetic base (Jaiwal and Gulati 1995) because of itsessentially self pollinated nature and the use of very few parental lines repeatedly in breeding programmes. Regeneration of whole plant through tissue culture provided an opportunity to introduce alien genes into plant cells and derive transgenic plants (Chandra and Pental 2003). Generally, legumes are highly recalcitrant and genotype specific to in vitro regeneration (Somers et al. 2003) and progress in transgenic development in legumes, specifically in green gram, has been very slow owing to their recalcitrant nature in culture (Dita et al. 2006; Eapen 2008; Varshney et al. 2009). Several researchers reported plant regeneration protocols for green gram through somatic embryogenesis (Girija et al. 2000; Kaviraj et al. 2006; Sivakumar et al. 2010), and organogenesis methods (Gulati and Jaiwal 1992, 1994; Rao et al. 2005; Himabindu et al. 2014). But, in most of these studies, the regeneration potential remained very low except few reports using cotyledonary node explants (Amutha et al. 2006; Vijayan et al. 2006; Yadav et al. 2010a, b; Sagare and Mohanty 2015). However, regenerating axillary meristmatic cells in cotyledonary node explants were quite inaccessible to *Agrobacterium*, which resulted in inefficient transformation (Sonia et al. 2007). Till date, very modest success has been achieved and there are only four published reports on genetic transformation of green gram describing the successful recovery of transgenic plants and inheritance of transgene to further generations (Mahalakshmi et al. 2006; Sonia et al. 2007; Vijayan and Kirti 2012; Yadav et al. 2012). A routine transformation method with high transformation efficiency is required for the development of transgenic green gram. Various parameters, which influence the efficiency of transformation need to be systematically standardized and a high throughput transformation system needs to be developed. This study reports, an efficient plant regeneration and *Agrobacterium*-mediated transformation protocols with optimization of critical parameters using shoot tip explants of green gram.

Materials and methods

Plant material and culture conditions

Seed material of ML-267 genotype of green gram used in the present study was obtained from AP State Seed Development Corporation (APSSDC) and Regional Agricultural Research Station, ANGRAU, Lam Farm, Guntur. Uniform and good quality seeds of ML-267 were washed with distilled water two–three times and surface sterilized by rinsing with 70 % (v/v) ethanol for 1 min followed by 0.1 % (w/v) aqueous $HgCl_2$ solutionfor 8–10 min. Then the seeds were thoroughly washed with sterilized distilled water for 4–5 times to remove any residual disinfectant and kept for germination on MSB5 medium.

Shoot tip explants were excised from 3 day-old aseptically germinated seedlings of ML-267 genotype. The seed coat was removed and cut the seedling approximately 2 mm below the nodal region followed by a horizontal division along the middle of the seed leading to production of shoot tip explants with embryoaxis comprising proximal half of the two cotyledons attached at the cotyledonary node. These explants were inoculated in vertically upright position in shoot bud induction media MSB5 supplemented with different concentrations of BAP (0, 5, 10, 12.5, 15 and 20 µM) and 10 µM BAP in combination of NAA/IAA/IBA (0.05, 0.5 µM). After 15 days of initial culture, the shoot tip explants with multiple shoot buds were excised from embryo axis and cultured for 45 days with three subcultures after every 15 days on shoot proliferation media containing half the initial concentration of growth hormones.

To study the effect of age of explants on shoot multiplication, shoot tip explants prepared from different age seedlings (3, 4 and 5 days old) were cultured on MSB5 medium with 10 µM BAP. To assess the beneficial effect of seedlings preconditioning on BAP during seed germination, 3 day old shoot tip explants were prepared from seedlings germinated on BAP (8.89 µM) and cultured on MSB5 + 10 µM BAP. A total of 35 explants were inoculated in each treatment in five replicates and the experiment repeated three times. Percent shoot induction frequency, mean shoot number and mean shoot length were recorded. In all the media, 0.8 % (w/v) phyto agar as the gelling agent and 3 % (w/v) sucrose as a carbon source were used unless otherwise mentioned. The pH of the media was set to 5.8 ± 0.2 before sterilization in autoclave at 121 °C at 15 lbs pressure for 15 min. All these cultures were maintained at 12/12 h light/dark photoperiod under white fluorescent lamps (50 $\mu Em^{-2}\ s^{-1}$) at 25 ± 2 °C.

In vitro rooting and acclimatization

The elongated shoots (~2–3 cm) were excised and kept for rooting on ½ MSB5 medium containing 2.46, 4.92 µM IBA, 2.85, 5.70 µM IAA and 2.68, 5.37 µM NAA with 1.5 % sucrose and cultured for 3 weeks. An observation on frequency of rooting was recorded. Well rooted plantlets were taken out from the culture bottles carefully and their roots washed thoroughly under running tap water to remove any traces of agar and transferred into small nursery covers containing autoclaved soil: sand (1:1) and covered with polypropylene transparent bags to reduce transpiration

losses. These plantlets were slowly acclimatized for 10–15 days by gradually exposing them to the open environment by making holes to the polypropylene bags. Finally primary hardened plantlets were shifted to 15 l pots having garden soil: farm yard manure (12:3) without disturbing their roots and grown till their maturity in transgenic glass house under controlled conditions. The percentage survival of plants during acclimatization was recorded.

Ontogeny studies

To confirm the shoot bud differentiation from the apical meristems, shoot tip explants from 2, 4 and 6 day-old cultures were collected and fixed separately in a solution containing methanol and acetic acid in 3:1 ratio for 48 h. The explants were dehydrated by passing through a graded ethyl alcohol series and embedded in paraffin block at 58–60 °C. Serial sections of 8 µ thickness were cut on an automated rotary microtome and floated on water and affixed to slides by de-waxing at higher temperatures. The sections were then stained with hematoxylin and eosin (H&E) and viewed under trinocular research microscope (Olympus).

Bacterial strain and gene construct used

The disarmed *A. tumefaciens* (LBA4404) harboring a binary vector pCAMBIA 2301, which contains *npt*II & β-glucuronidase (*uidA*) as plant selection marker and reporter genes, and *annexin* gene cassette (*CaMV35S promoter-AnnBj1-nos terminator*) of 1.7 kb size cloned in the MCS region in-between *npt*II and *uidA* genes was used in transformation studies (Fig. 1).

Antibiotic selection

Before transformation, the optimum dose of antibiotic for the selection of transformed explants was identified by culturing control shoot tip explants on shoot bud induction medium (MSB5 + 10 µM BAP) containing different doses of kanamycin (0, 50, 100, 150, and 200 mg/l) for 4 weeks with one subculture after 2 weeks onto the same fresh medium. About 15 explants were taken for each concentration in three replicates and the experiment has been repeated thrice.

Optimization of transformation protocol for shoot tip explants

The 3-day-old shoot tip explants prepared from ML-267 genotype seedlings were used and pre-cultured for 0–2 days on shoot bud induction medium (MSB5 + 10 µM BAP) prior to infection. These explants were injured by pricking two–three times with sterile fine needle at the apical meristematic region and submerged in bacterial suspension of 0.6, 0.8 and 1.0 O.D. at 600 nm for 10–30 min with continuous shaking at 100 rpm. The infected explants were dried on autoclaved filter paper and co-cultured for 2–4 days on co-cultivation medium at 23 °C in dark. The effect of different concentrations of acetosyringone (0–200 µM) in co-cultivation medium during co-cultivation was also assessed. After co-cultivation, the explants were rinsed with sterilized distilled water containing cefotaxime (250 mg/l) four to five times, dried on sterilized filter paper and then transferred on to selection medium (SBIM + 100 mg/l kan + 250 mg/l cefo) for multiple shoot induction. Few explants were randomly picked and incubated over night in GUS solution at 37 °C for histochemical GUS assays. Various parameters viz. explants pre-culture period, culture OD, infection time, co-cultivation days and acetosyringone concentration, which could influence transformation efficiency were optimized based on transient GUS expression. Around 36 explants were employed for each variable in an experiment, and each experiment repeated three times.

Selection and regeneration of transformants of shoot tip explants

To produce green gram transgenic plants, 3 day old shoot tip explants were prepared and pre-cultured for 2 days on shoot bud induction medium. These explants were then injured with sterile syringe needle at the apical meristematic region. Overnight grown *Agro* culture was centrifuged and the pellet

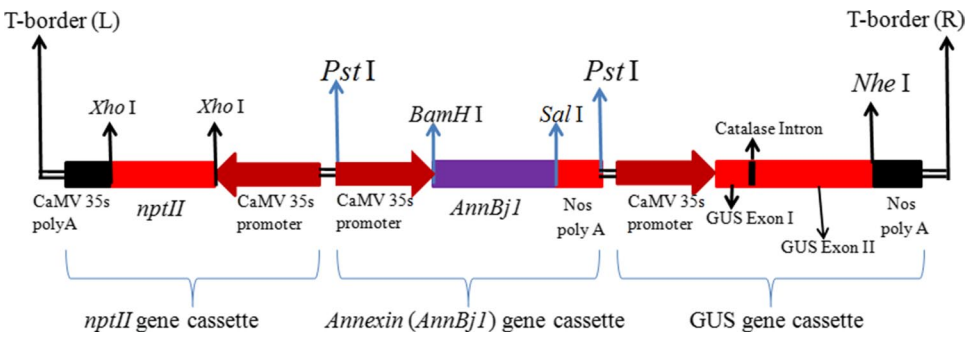

Fig. 1 Linear diagram of T-DNA region of pCAMBIA 2301 containing *AnnBj1* gene along with *npt*II and GUS genes

was re-suspended in liquid co-cultivation medium to a final concentration of 0.8 O.D. at A600. The injured explants were then submerged in infection medium (bacterial suspension) for about 10 min with gentle mixing at 100 rpm. These infected explants were blot dried on sterilized filter paper to remove excess bacterial culture and co-cultured for 3 d on co-cultivation medium supplemented with 100 µM acetosyringone at 23 °C under continuous dark. The co-cultivated explants were washed with cefo-water (sterile distilled water + 250 mg/l cefotaxime), blot dried on autoclaved filter paper and cultured on selection medium (MSB5 + 10 µM BAP + 100 mg/l kan + 250 mg/l cefotaxume) for 4 weeks with one subculture for multiple shoot induction. Untransformed explants were also cultured under similar conditions on selection-free (without kanamycin) medium as well as on selection (with kanamycin) medium as shoot induction and transformation controls respectively. After 4 weeks, explants with proliferating shoots on selection medium were sub-cultured onto fresh selection medium (containing 100 mg/l kan and 250 mg/l cefotaxime) for next cycle of 30 d with one subculture after 15 d time interval. The elongated shoots (with 2–3 internodes) were then transferred on to rooting medium ½ MSB5 + 2.46 µM IBA + 250 mg/l cefotaxime. The profusely rooted putative transformed plantlets were acclimatized as above mentioned and grown till maturity to collect seeds.

Histochemical GUS assay

Transient GUS expression in infected shoot tip explants was scored after 3 days co-cultivation, whereas stable GUS expression was checked in flowers of putative T_0 plants and in young leaves of T_1 plants. After co-cultivation, explants were incubated in GUS solution containing 0.1 M sodium phosphate buffer (pH 7.0), 0.5 mM potassium ferri and ferrocyanide, 10 mM Na_2 EDTA (pH 8.0), 0.5 % Triton X-100 and 1 mM X-GlcA at 37 °C over night in dark. Before analysis, chlorophyll was completely removed from the tissues by extraction in 100 % ethanol. Untransformed explants, which were cultured under similar culture conditions served as controls. The GUS expression frequency was measured as number of explants showing GUS expression at the proliferating sites per number of explants treated.

Molecular characterization of putative transgenics

PCR amplification was carried out with *AnnBj1, npt*II and GUS gene specific primers using gDNA isolated from young leaves of putative T_0 transgenic plants, non-transformed plants (negative control) and pCAMBIA 2301 plasmid (positive control) as templates. A PCR reaction volume of 20 µl solution was prepared in 0.2 ml PCR strip tubes with 10X *Taq* DNA polymerase buffer (with $MgCl_2$), 2 mM of dNTP mix, 10 pico moles each of forward and reverse primers, 2 units of *Taq* DNA polymerase, 50–100 ng of gDNA or 5 ng/µl plasmid DNA and milliQ water to make up the total volume. Amplification using specific primer sets for *AnnBj1* (forward: 5′-ATGGCGACTCTTAAGGTTTC-3′ and reverse: 5′-TTAAGCATCATCTTCACCGAG-3′), *npt*II (forward: 5′-AGACAATCGGCT GCTCTGAT-3′ and reverse: 5′-CACCATGATATTCGGCAAGC-3′) and GUS (forward: 5′-TGGACGATATCACCGTGGTG 3′ and reverse: 5′-GGCACAGCACATCAAAGAGA-3′) was carried out in a thermal cycler under the subsequent conditions: 94 °C for 5 min for initial denaturation followed by 35 cycles of 94 °C for 45 s for denaturation, 58 °C for 45 s for annealing for *AnnBj1* gene and 60 °C for 45 s for *npt*II and GUS genes, 72 °C for 1 min for extension and 72 °C for 10 min for final extension. The PCR amplification products were resolved by electrophoresis using 0.8 % agarose gel, visualized and documented.

Generation advancement and evaluation of T_1 plants

The T_0 primary transgenic plants testing positive by PCR using *AnnBj1, npt*II and GUS primers were grown till maturity in transgenic glass house and their seeds were harvested. Subsequently, seed material of one randomly selected independent T_0 transgenic plant (P-19) were germinated to get T_1 plants for further evaluation. The total gDNA was isolated from T_1 plants and the inheritance pattern of *annexin* gene was analyzed by PCR using *AnnBj1* primers. Chi square test was performed on the T_1 plants of P-19 event for the presence of *annexin* gene in the expected Mendelian ratio (3:1) for a dominant gene.

Statistical analysis

The complete data collected were subjected to statistical analysis using single factor completely randomized block design in order to study the effect of different treatments on shoot proliferation and genetic transformation of green gram. Tukey's HSD test was carried out for comparison of means at significance (P) level 0.05. Analysis of variance and mean separation by Tukey's HSD test was computed by using SAS version 9.2.

Results and discussion

Effect of hormone concentration, age and genotype on shoot proliferation from shoot tip explants

Shoot tip explants with embryo axis (Fig. 2a) underwent moderate swelling, turned green and embryo axis elongated within 2 days of culture initiation (Fig. 2b). Mostly single

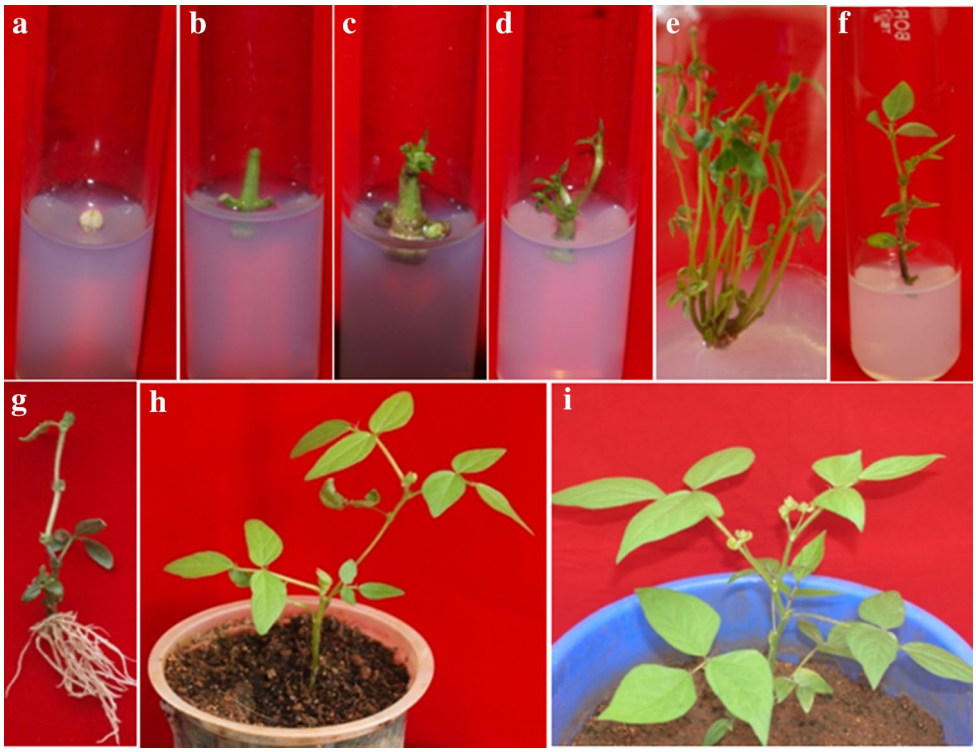

Fig. 2 Different stages of shoot multiplication from shoot tip explants of green gram. **a** Initial shoot tip explant. **b** Shoot tip explant with embryo axis after 2 days culture on shoot bud induction medium. **c** Differentiation of multiple shoot buds on MSB5 medium supplemented with 10 µM BAP. **d** Multiple shoots on shoot proliferation medium. **e** Elongated multiple shoots after 8 weeks of culture (**f**) an elongated shoot placed on ½ MSB5 + 2.46 µM IBA for rooting. **g** Profuse rooting after 3 weeks of culture (**h**) primary hardened plant. **i** Well established mature plant with flowers

Table 1 Proliferation response of shoot tip explants cultured on MSB5 medium containing different concentrations of BAP alone and with low concentrations of auxins

Conc. of BAP (µM)	Conc. of NAA (µM)	Conc. of IAA (µM)	Conc. of IBA (µM)	Mean shoot number	Mean shoot length (cm)
0	–	–	–	1.62 ± 0.22 g	6.65 ± 0.43 a
5	–	–	–	4.28 ± 0.15 f	5.33 ± 0.22 b
10	–	–	–	9.45 ± 0.68 a	4.43 ± 0.35 bc
12.5	–	–	–	7.33 ± 0.36 bc	3.97 ± 0.20 cd
15	–	–	–	6.07 ± 0.52 de	3.83 ± 0.32 cd
20	–	–	–	5.05 ± 0.45 ef	3.23 ± 0.41 de
10	0.05	–	–	7.88 ± 0.39 b	3.67 ± 0.24 cde
10	0.5	–	–	6.48 ± 0.42 cd	3.50 ± 0.36 cde
10	–	0.05	–	6.60 ± 0.35 cd	3.23 ± 0.20 de
10	–	0.5	–	5.72 ± 0.40 de	2.70 ± 0.32 e
10	–	–	0.05	5.82 ± 0.38 de	3.80 ± 0.44 cd
10	–	–	0.5	4.40 ± 0.18 f	3.53 ± 0.61 cde

All treatments gave 100% shoot induction frequency. Values represent mean ± SE of three replicates. Means in a column followed by same alphabet are not significantly different according to Tukey's HSD test (P = 0.05)

shoot buds from apical meristems and roots from hypocotyl cut ends were formed from explants cultured on MSB5 medium without hormones. Addition of various concentrations of BAP to MSB5 basal medium induced callus at the bottom of the explants as well as multiple shoot differentiation from shoot apices within 15 day of culture (Fig. 2c). Multiple shoot number increased along with shoot length on subsequent transfer of explants to shoot proliferation media containing reduced (half the initial) concentration of the growth regulators (Fig. 2d, e). This approach is in consistence with the previous reports in green gram (Vijayan et al. 2006) and groundnut (Beena et al. 2005; Srinivasan et al. 2010) to get more shoot number and shoot length. Yadav et al. (2010b) followed similar approach for shoot proliferation and elongation of multiple shoots in green gram. Further, Sagare and Mohanty (2015) obtained elongation of shoots on 0.1 mg/l BAP which is 1/10th concentration of BAP that was initially used for shoot regeneration. Almost three fold increase in mean shoot number was observed with addition of BAP (5.0 µM) to MSB5 basal medium (Table 1).

BAP is the most extensively used growth hormone for multiple shoot regeneration in various legumes including green gram (Sonia et al. 2007; Yadav et al. 2010a, b). Application of BAP at 10 µM concentration has resulted in highest number of multiple shoots (9.45) per explant in 100 % of the cultures (Table 1) while a significant decrease in shoot number was observed when explants were cultured with concentrations above 10 µM concentration of BAP. However, Rao et al. (2005) reported an increase in shoot proliferation with an increase in BAP concentration from shoot tip explants of green gram, but at the cost of regeneration frequency. The mean shoot length gradually decreased with an increase in BAP concentration (Table 1). There was no considerable increase in multiple shoot number when explants cultured on media containing 10 µM BAP supplemented with 0.05 and 0.5 µM concentrations of different auxins (NAA/IAA/IBA) (Table 1). BAP alone without auxins was observed to be efficient in inducting shoot regeneration from shoot tip explants in green gram (Gulati and Jaiwal 1992) and in other legumes like bean (Aragao et al. 1998), black gram (Saini and Jaiwal 2005) and cow pea (Brar and Anderson 1997).

Presence or absence of embryo axis with proximal halves of cotyledons along with shoot tip explants affected multiple shoot formation to great extent. Preliminary experiments showed that presence of embryo axis with proximal halves of the cotyledons in initial 15 days of culture was essential for inducing multiple shoots from the shoot tip explants. 3 day old shoot tip explants without embryo axis showed poor proliferation response and even though a few shoot buds were produced, they failed to elongate. The morphogenic potential of the explants also varied with the age of the explants. 3 day old explants produced highest number of multiple shoots and the average number of proliferating shoots per explant decreased as the explants age increased (Fig. 3a). Young explants were shown to produce optimum shoot regeneration in green gram (Gulati and Jaiwal 1994; Sonia et al. 2007) and other related leguminous pulses such as pea (Jackson and Hobbs 1990), pigeon pea (Prakash et al. 1994; Srinivasan et al. 2004) and *Phaseolus* species (Franklin et al. 1991; Santalla et al. 1998). The poor regeneration frequency with the increase in age of the explants is possibly due to prolonged utilization of reserve food in the cotyledons by the growing seedling at the time of germination. The explants prepared from young donor seedlings were most frequently used for *Agrobacterium*-mediated transformation, where the cells not only grow actively but also more accessible to the *Agrobacterium* (Vijayan and Kirti 2012). There is no significant increase in shoot number when seedlings preconditioned at 8.8 µM BAP during seed germination for 3 days (Table 2). Pre-conditioning of germinating green gram seedlings in BAP stimulates development of axillary buds in cotyledonary node explants from axillary meristem resulting in multiple shoot proliferation (Avenido et al. 2001).

Rooting was induced within 10 days and most efficient rooting (75 %) was obtained on ½ MSB5 + 2.46 µM IBA (Fig. 2g). Among the three auxins tested for in vitro rooting, IBA gave better response than IAA and NAA (Fig. 4). Similar IBA mediated root formation was already reported in green gram by Avenido and Hautea (1990), Jaiwal et al. (2001), Yadav et al. (2010a), Prasad et al. (2014). About 90 % of the plantlets survived after acclimatization (Fig. 2h), which later flowered and produced pods with viable seeds upon reaching maturity (Fig. 2i).

Table 2 Effect of seedlings preconditioning during seed germination on multiple shootinduction from shoot tip explants of green gram

Seed germination on	Mean shoot number	Mean shoot length (cm)
MSB5	9.10 ± 0.78 a	3.87 ± 0.44 a
MSB5 + 8.88 µM BAP	8.83 ± 0.80 a	3.47 ± 0.55 a

All treatments gave 100 % shoot proliferation. Values represent mean ± SE of three replicates. Means in a column followed by same alphabet are not significantly different according to Tukey's HSD test (P = 0.05)

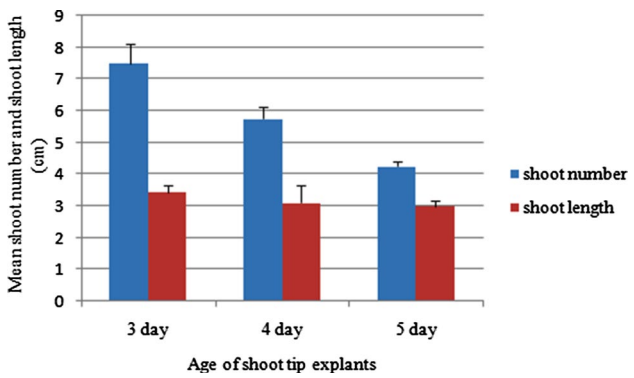

Fig. 3 Effect of explants age (3, 4 and 5 day-old) on shoot induction from shoot tip explants of ML-267 genotype

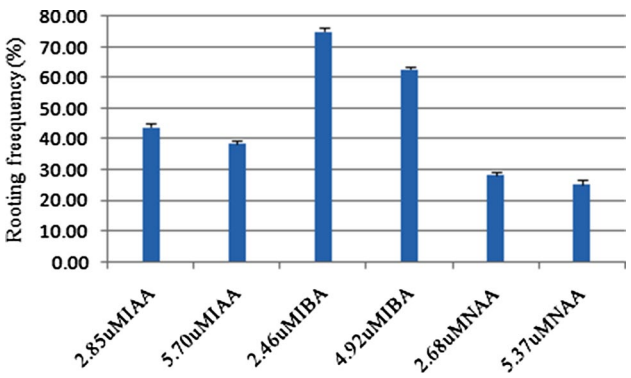

Fig. 4 Induction and frequency of rooting of the shoots in different concentrations of auxins supplemented to ½ strength MSB5 medium

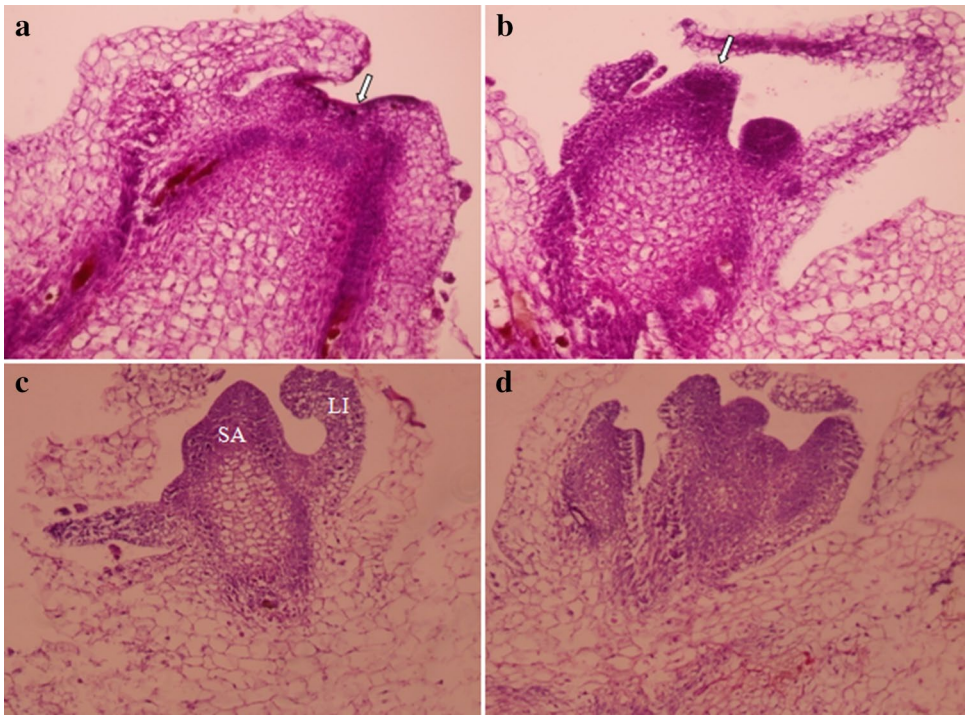

Fig. 5 Ontogeny of the shoot buds formation from the apical meristematic region of the shoot tip explants. **a** Meristematic zone in the peripheral cell layers (*arrow*) of 2-day-old culture. **b** Initiation of meristematic dome with shoot primordia and leaf initial from 4-day-old culture (**c, d**) well-defined shoot primordia with flanking leaf initials from 6-day-old culture

Histological analysis

Longitudinal sections of shoot apices cultured for 2 days on MSB5 medium containing 10 µM BAP showed formation of meristematic zone in the peripheral cell layers of shoot apex region (Fig. 5a). Cells present in this zone were small and very compact with dense cytoplasm. Initiation of nodular structures with dome-shaped shoot primordia and adjacent leaf initials was observed due to localized meristematic activity upon further culture of shoot apices for 4 days (Fig. 5b). The nodular structures resulting from the meristematic activity proliferated and gave rise to well-defined shoot apice (SA) with flanking leaf initials (LI) within 6 days of culture of shoot tip explants (Fig. 5c, d). Histological studies demonstrated the absence of vascular connection of shoot primordials to the main vascular system in 6 days old explants.

Optimization of antibiotic concentration

As the plasmid used in this study (pCAMBIA 2301) harboured *npt*II as the plant selection marker gene which confers resistance against the antibiotic kanamycin, the optimal concentration of kanamycin for the selection of transformed explants was identified by culturing the control shoot tip explants on shoot bud induction medium (MSB5 + 10 µM BAP) supplemented with various concentrations of kanamycin. Explants on plain MSB5 medium without kanamycin shown 100% survival and shoots of these explants were healthy with green leaves (Fig. 6). Whereas, 30% of the explants on 50 mg/l kanamycin medium produced pale green, bleached shoots. Kanamycin at 100 mg/l concentration was observed to be lethal as it completely bleached the proliferated shoots and hence, this concentration (100 mg/l) was used for the screening of transformed shoots (Fig. 6). For the stringent selection of transformed cells, choice of the suitable selectable marker gene and timing of its application are very important (Sonia et al. 2007). Kanamycin-based selection of transformants was well documented in leguminous species, viz. alfalfa (Jin et al. 2010), black gram (Saini et al. 2003; Saini and Jaiwal 2005, 2007), chickpea (Tripathi et al. 2013; Chakraborty et al. 2016), green gram (Jaiwal et al. 2001; Yadav et al. 2012), pea (Nadolska-Orczyk and Orczyk 2000) and peanut (Anuradha et al. 2006).

Optimization of factors affecting transformation

To develop an efficient *Agrobacterium*-mediated genetic transformation system, especially, species like green gram, optimization of transformation procedures that result in the maximum number of independent transgenic events is critical. Various factors, which influence the transformation frequency were optimized based on the percent frequency of GUS expression in shoot tip explants. The effect of pre-conditioning of explants prior to transformation was evaluated to determine the suitable stage to attain optimum transformation efficiency. In the present work, pre-culture of explants for 2 days helped in a significant increase in transient GUS expression from 45% in cultures without pre-culture to highest of 70% (Fig. 7). Similarly, Sonia et al. (2007) also observed an increase in GUS expression frequency upon

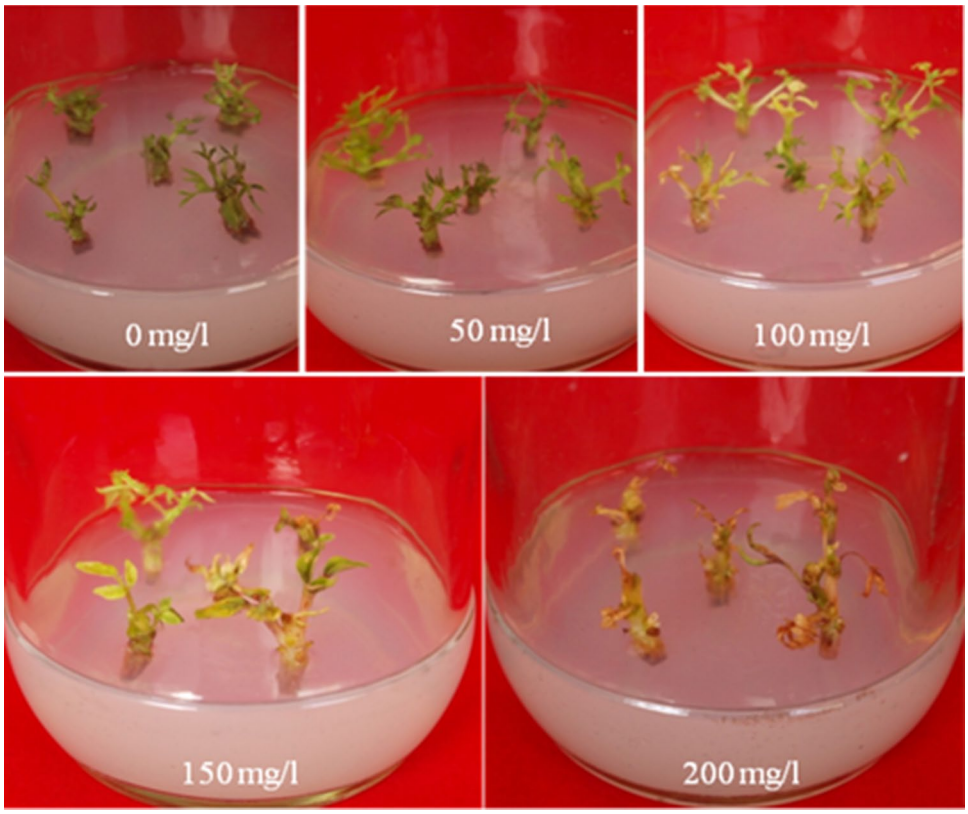

Fig. 6 Optimization of kanamycin concentration for selection of transformed explants by culturing un-transformed control explants on MSB5 + 10 µM BAP containing 0, 50, 100, 150 and 200 mg/l kanamycin media

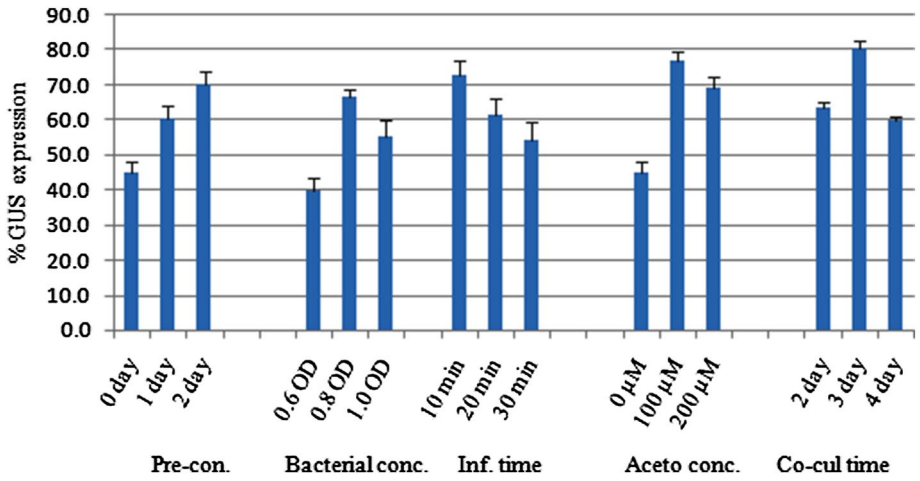

Fig. 7 Effect of different parameters of *Agrobacterium* mediated transformation on transient GUS expression in shoot tip explants of green gram

pre-culture of young cotyledonary node without cotyledons explants of green gram for 1 day. Yadav et al. (2012) obtained best results with pre-culture of the cotyledonary node explants of green gram. This increase could be due to integration of T-DNA by the presence of dynamic DNA replication machinery in the actively dividing cells (Baron and Zambryski 1995) induced by cytokinin during pre-culture (Sangwan et al. 1992). Increase in efficiency of transformation by pre-culture of the explants on regeneration medium before infection has been well reported in other grain legumes, like cowpea (Muthukumar et al. 1996) and pigeon pea (Lawrence and Koundal 2001; Krishna et al. 2011).

Optical density (OD) of infection medium after *Agrobacterium* re-suspension is directly related to the *Agrobacterium* cell density. Significant differences in GUS expression frequencies were observed when the shoot tip explants were infected with cultures of 0.6, 0.8 and 1.0 OD. Maximum GUS expression (67%) was observed with 0.8 OD culture and thereafter, the frequency decreased to 55% with a further increase of bacterial concentration to an OD of 1.0 (Fig. 7). Yadav et al. 2012 observed best transformation response at 0.8 OD in green gram. Further, Kim et al. (2009) reported severe wilting and yellowing of explants and lower transient expression when OD of cell suspensions was 1.2 and 0.3 or lower

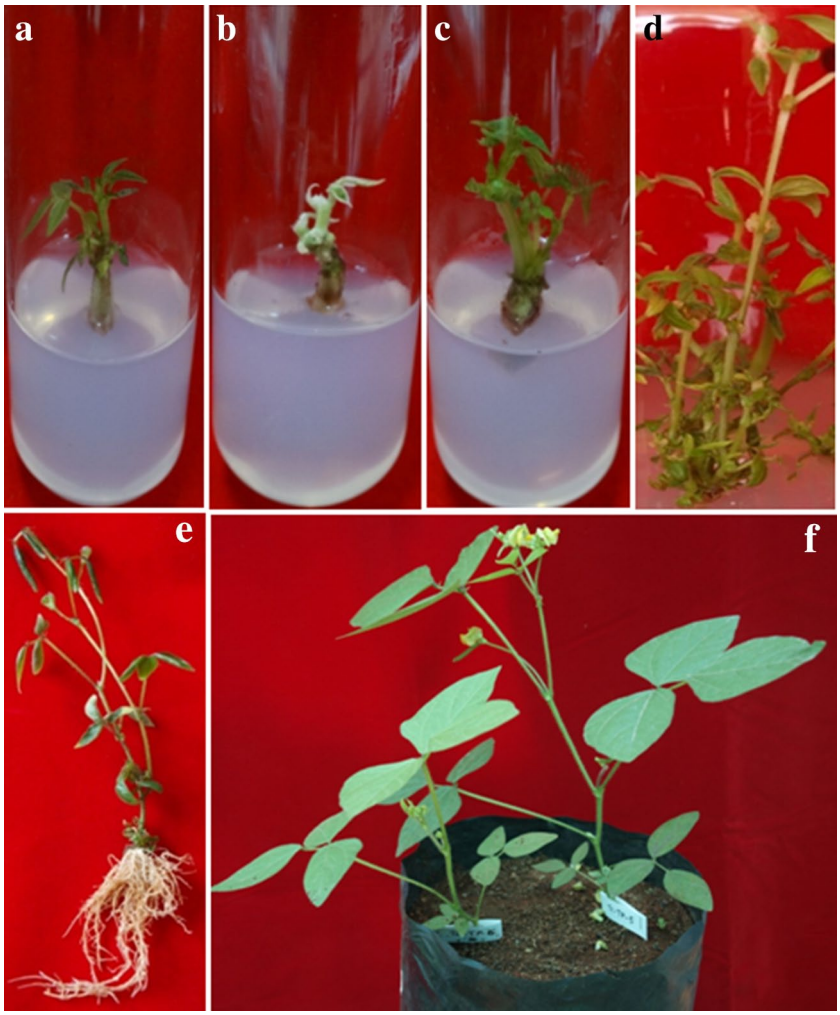

Fig. 8 Different stages of *Agrobacterium*-mediated transformation of shoot tip explants of green gram. **a** Non-transformed explant on shoot induction medium (**b**) non-transformed shoot tip explant on selection medium. **c** Multiple shoot proliferation from shoot tip explant survived on selection medium (**d**) elongated multiple shoots. **e** Putative transformed shoot rooted on MSB5 + 2.46 µM IBA + 250 mg/l cefotaxime. **f** Putative transgenic plants (T_0) setting flowers and pods

Table 3 Transformation efficiency using an optimized protocol for the transformation of shoot tip explants of green gram infected with *A.tumefaciens* (LBA4404) harbouring a binary vector pCAMBIA 2301 + *AnnBj1*

Explants inoculated with *Agrobacterium* (No.)	Plants established in soil (no.)	Plants positive for *AnnBj1*, GUS and *npt*II by PCR (no.)	Transformation frequency (%)
268	36 (50.7%)	4	1.492 (4/268)

respectively, whereas, highest transient GUS expression frequency was observed when OD was in between 0.6 and 0.9.

The transient GUS expression frequency was observed to decrease with an increase in infection time from 10 to 30 min and highest transformation frequency (73%) was obtained in explants infected for 10 min (Fig. 7). In contrast to this, Sonia et al. (2007) observed an increase in transient GUS expression with an increase in time up to 30 min. This difference could be due to variation in the type of explants used. High concentration of bacterial cells and prolonged infection timings not only resulted in overgrowth of the *Agrobacterium*, but also resulted in a decrease in regeneration potential due to hypersensitive response of explants. Addition of phenolic compounds to the co-cultivation medium would enhance the transfer of T-DNA region to plant cells by stimulating '*vir*' gene induction in *Agrobacterium* and acetosyringone is one such potent phenolic compound generally secreted by wounded plant tissue (Stachel et al. 1985, 1986; Nadolska-Orczyk and Orczyk 2000). In this study, inclusion of 100 µM acetosyringone to the infection medium before infection as well as to the co-cultivation medium resulted in considerable increase in transient GUS expression frequency from 45 to 77%. This result is in consistence with the earlier report in pigeon pea (Krishna et al. 2011). Similarly, Aldemita and Hodges (1996) observed that preconditioning of *Agrobacterium* with acetosyringone before co-cultivation was essential for rice transformation. Halder and Jha (2016) added 200 µM acetosyringone to the bacterial suspensions 2 h prior to inoculation of peanut explants to improve virulence. However, a further increase in acetosyringone concentration to 200 µM resulted in a decline in GUS expression to 69% (Fig. 7). The influence of co-cultivation period on transformation efficiency was also studied by culturing the infected explants on co-cultivation

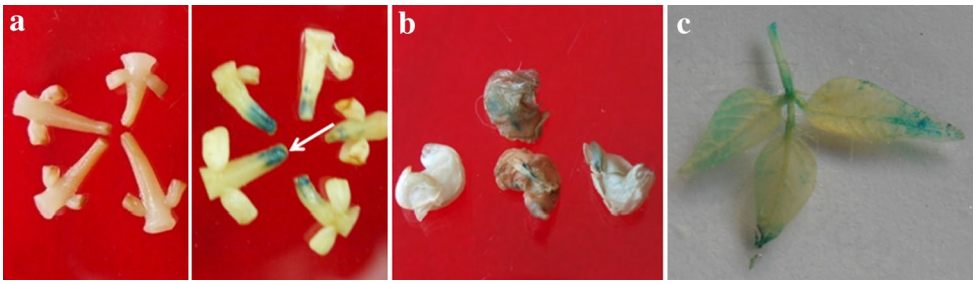

Fig. 9 Transient and stable GUS expression of shoot tip explants, flowers and leaf tissues. **a** GUS activity of shoot tip explants at the sites of shoot induction (*arrow*). **b** GUS expression in flowers of T_0 transgenic plants. **c** GUS expression in leaves of T_1 transgenic plants. Control explants and flower from non transgenic plant showing no GUS expression

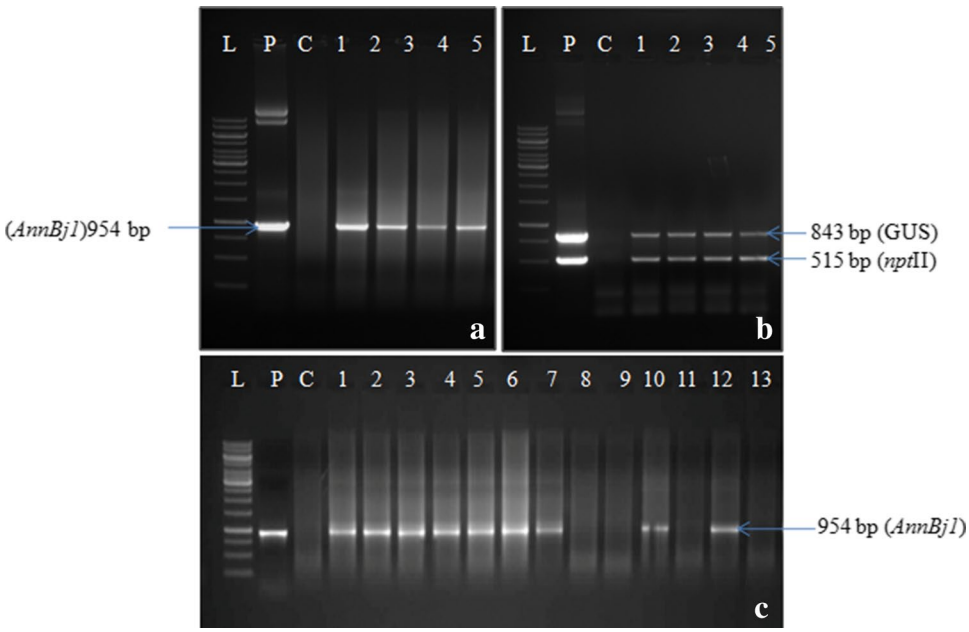

Fig. 10 Molecular characterization of T_0 and T_1 transgenic green gram plants. **a** PCR amplification of *AnnBj*1. **b** PCR amplification with GUS and *npt*II specific primers. **c** PCR analysis of T_1 transgenic green gram plants with *AnnBj*1gene specific primers. *L* represents 1 kb marker, *P* and *C* represents positive and negative controls (untransformed plant) respectively

Table 4 Inheritance of *annexin* gene in T_1 generation of transgenic green gram

T_0 plant	No. of T_1 plants tested for *annexin* by PCR			Segregation ratio	χ^2 value	p value
	Total	Positive	Negative			
P-19	13	9	4	3:1	0.195	0.658

medium containing 100 µM acetosyringone for 2, 3 and 4 days. Highest (80%) GUS expression was obtained with 3 days co-cultivation and a further increase in co-cultivation duration led to decreased GUS expression frequency (Fig. 7). A short co-culture period of 2 or 3 days with bacterial growth in log phase has been found to be optimum and the same was reported in *Vigna radiata* (Jaiwal et al. 2001; Mahalakshmi et al. 2006; Sonia et al. 2007; Yadav et al. 2012) and in other leguminous species such as, *Arachis hypogaea* (Geng et al. 2012), *Cajanus cajan* (Geetha et al. 1999; Mohan and Krishnamurthy 2003), *Cicer arietinum* (Tripathi et al. 2013; Chakraborty et al. 2016) and *Glycin max* (Li et al. 2004; Liu et al. 2012).

Development of transformants

Shoot apex morphology and its capacity to produce multiple shoots revealed that the shoot tip explants are ideal for achieving the development of transgenic plants, as shoot apical meristematic dome is directly accessible to gene delivery either through *Agrobacterium* or particle bombardment method. A total of 268 explants were infected in five different experiments for the production of transgenic green gram plants. Control explants cultured on regeneration medium without kanamycin produced healthy green shoots (Fig. 8a), whereas, shoots got bleached in explants cultured on regeneration medium containing kanamycin under similar conditions (Fig. 8b). *Agrobacterium*-mediated transformation of 3 days old shoot tip explants of ML-267 genotype of green gram with binary vector pCAMBIA2301 + *AnnBj1* using

the optimized conditions produced 106 shoots on kanamycin selection medium (MSB5 + 10 µM BAP + 100 mg/l Kan + 250 mg/l cefo) (Fig. 8c, d). Of these, 71 formed roots on ½ MSB5 + 2.46 µM IBA + 250 mg/l cefotaxim (1.5% sucrose) in 3 weeks of time (Fig. 8e). These plantlets were acclimatized, where 36 plants were survived and grown to maturity (Fig. 8f), (Table 3).

Histochemical GUS assay

The histochemical GUS assay was carried out following Jefferson et al. (1987). Shoot tip explants, which were pre-cultured for 2 days and wounded showed intense GUS activity at the site of shoot induction (Fig. 9a), whereas no GUS activity observed in control explants. GUS expression obtained in reproductive parts of flowers of T_0 plants (Fig. 9b) and leaves of T_1 plants (Fig. 9c) demonstrating the stable inheritance and expression of the transgenes in the progeny. Further, there was no GUS expression in the flowers and leaves of control plants.

Screening of transgenic plants by PCR

The PCR analysis of 36 T_0 plants with *AnnBj1*, GUS and *nptII* gene specific primers showed amplification of expected 954 bp (Fig. 10a), 843 and 515 bp (Fig. 10b) DNA fragments respectively corresponding to the coding regions of the respective genes confirming the integration of transgene in 4 plants (P-18, P-19, P-29 and P-36) with ~1.5% transformation frequency (Table 3). Similar to our results, Saini and Jaiwal (2005) achieved a significant increase in transformation frequency by employing shoot tip explants in place of cotyledonary node explants under similar conditions and bacterial strain in the background of *Vigna mungo*. In legumes higher efficiency was reported with shoot tip explants compared to other type of explants, which might be due to direct accessibility of shoot apical meristmatic dome to *Agrobacterium*, which possibly facilitated transformation of sub-epidermal germ line cells of the apical meristem. Further, it is very essential to characterize the inheritance pattern of the introduced gene in the T_1 population to understand the stability of the foreign gene integration and segregation pattern. Therefore, seedlings of one of T_0 putative transgenic plant (P-19) were analyzed with *AnnBj1* specific primers at T_1 level, which resulted in an amplicon of the expected size of 954 bp in nine out of 13 transgenic plants (Fig. 10c). Chi square (χ^2) analysis revealed that the line P-19 presented a monogenic segregation ratio of 3:1 in a normal Mendelian fashion (Table 4). These results suggested the integration and inheritance of transgene in transgenic plants developed in this study.

Conclusion

Regeneration is considered as a major obstacle in genetic transformation of green gram. Several researchers have been trying to break this obstacle. In this study we have reported maximum induction of shoots with 100% shoot proliferation frequency. Further, we have reported 1.5% transformation efficiency with this optimized regeneration protocol. To our knowledge, this is the first report in green gram on successful regeneration of highest number of multiple shoots with 100% frequency and genetic transformation employing shoot tip explant. The protocols developed in this study are simple and efficient and can be used to develop transgenic green gram for various traits of interest and also functional genomic studies.

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Authors contributions GKM contributed to the acquisition of data by conducting different experiments and manuscript preparation. VNJ has made substantial contribution in critically drafting and editing the manuscript. GM contributed by giving valuable suggestions in preparation of manuscript. PBK has helped in initial concept design and generously provided the *AnnBj1* gene construct. SKY conceived the study and contributed to designing experiments, data analysis and critical revision of manuscript.

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