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Original Paper

In vitro shoot grafting on rootstock: An effective tool for *Agrobacterium*-mediated transformation of pigeonpea (*Cajanus cajan* (L.) Millsp.)

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Abstract Lack of reproducible in vitro transformation method in pigeonpea limits the application of biotechnological breeding approaches for its genetic improvement. The present study describes a transformation method using novel in vitro shoot grafting technique for two cultivars ICPL87 and ICPL87119. Modified Murashige and Skoog (MS) medium with 1 mg l^{-1} 6-benzylaminopurine and 0.2 mg l^{-1} α-naphthaleneacetic acid induced an average of 25 shoots from decapitated embryonic axis explants after six weeks of culture. These shoots were further elongated in a modified MS medium containing 0.5 mg l^{-1} 6-benzylaminopurine along with 0.5 mg l^{-1} gibberellic acid for another four weeks. Grafting of pigeonpea shoots to seedling rootstock allowed 95% recovery of shoots. The whole regeneration process, starting from explant preparation to complete plant development, took 12–13 weeks. Further, the explants were infected with *Agrobacterium tumefaciens* harboring a binary vector pBI121. Transient and constitutive β-glucuronidase expressions were obtained in putative transgenic shoots selected at 100 mg l^{-1} kanamycin. The selected shoots were grafted on non-transgenic root stock to establish putative transformants. T_0 and T_1 transformants were confirmed through polymerase chain reaction for presence of *neomycin phosphotransferase* gene. An overall 9% of transformation efficiency was recorded in both cultivars.

Key words: β-glucuronidase, pigeonpea regeneration, pigeonpea transformation, shoot grafting.

Pigeonpea (*Cajanus cajan* (L.) Millsp.) is an important legume crop of rain-fed agriculture in the semi-arid tropics. Pigeonpea is grown on *ca.* 4.75 million hectares making it the sixth most important legume food crop globally (FAO 2012). It is grown extensively in about fifty countries (Kamble et al. 1998). In India, it is the second important food legume contributing to 90% of global production. It is a rich source of proteins (20–22%) and leaves are used as fodder and dry crushed seeds as animal feed. Dry seeds are used widely in India as dry split pea for dahl preparation for human consumption. In the Caribbean region, the pea is consumed as green vegetable (Nene and Sheila 1990).

In spite of its large demand, global production and yield of pigeonpea has not been increased markedly in the past few decades. This is due to major factors such as, inappropriate production practices, inadequate biological nitrogen fixation, and damage of the crop by several insects and pathogens as well as susceptibility of cultivars to abiotic stresses (Upadhyaya et al. 2013). Conventional breeding approaches to overcome such stresses have limited success because of narrow genetic variation in cultivated germplasm and incompatibility with the wild species (Nene and Sheila 1990). The recent developments in plant genetic engineering have provided immense potential in overcoming some of these constraints, thereby offering opportunities for its genetic improvement which could be successfully integrated with conventional crop improvement strategies.

For the successful development of transgenic plant an effective regeneration system is essential. Pigeonpea is one of the most recalcitrant crops with poor tissue culture responses. Regeneration efficiency was reported to be affected by explant origin, culture maintenance conditions, age of explants and most importantly successful root induction in micro shoots (Sharma et al. 1990). Protocols for obtaining stable regenerants in pigeonpea have been reported through organogenesis from undifferentiated callus, cotyledonary nodes, apical meristem, differentiated non-meristematic tissues like

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leaf and cotyledons (Dayal et al. 2003; Geetha et al. 1998; George and Eapen 1994; Singh et al. 2002; Villiers et al. 2008). Somatic embryogenesis were reported in diverse genotypes using various explants such as mature seeds, shoot apices, intact seedlings, leaves, petioles, hypocotyls, epicotyls, cotyledonary nodes, cotyledons, internodes, roots, endosperm, and cell suspensions (Anbazhagan and Ganapathi 1999; George and Eapen 1994; Mohan and Krishnamurthy 2002; Nalini et al. 1996; Singh et al. 2003; Sreenivasu et al. 1998). The recovery of plants through somatic embryogenesis has been found to be very low.

Development of transgenic pigeonpea containing various foreign genes was attempted over the last decade to improve resistance to insects, fungal diseases and nutrient quality using cotyledonary node and leaf explants. Various genes like *Bacillus thuringiensis cry1Ab*, *cry1E-C*, *cry1AcF* (Ramu et al. 2012; Surekha et al. 2005; Verma and Chand 2005) and cowpea *protease inhibitor* (Lawrence and Koundal 2001) were used for conferring insect resistance. Rice *chitinase* and *Nicotiana sylvestris dihydrodipicolinate synthase*, were incorporated for fungal resistance and lysine content enhancement, respectively (Kumar et al. 2004b; Thu et al. 2003). Edible vaccine genes like *hemagglutinin* of rinder pest virus and *hemagglutinin neuraminidase* of peste des petits ruminants' virus were integrated into pigeonpea to immunize goat and sheep for rinder pest virus and peste des petits ruminants' virus, respectively (Prasad et al. 2004; Satyavathi et al. 2003).

In spite of rigorous exercises, the transformation efficiency in pigeonpea has been very discrete, ranging from 0.2–80% (Ghosh et al. 2014; Krishna et al. 2010). Induction of root in regenerated shoots is the major problem for all types of culture based transformation strategies. The resulting loss of regeneration efficiency can vary from 30–80% due to in vitro root induction failure and represents a significant bottleneck in the overall recovery of plants from culture after transformation (Ghosh et al. 2014; Krishna et al. 2010). The present work describes an improved and novel regeneration method in pigeonpea using root grafting strategy. Further *Agrobacterium*-mediated transformation has been performed successfully with higher efficiency.

Materials and methods

Materials

Seeds of two cultivars, ICPL87119 and ICPL87 were collected from the Indian Institute of Pulses Research, Kanpur, India. *Agrobacterium tumefaciens* strain EHA105, harbouring the binary vector pBI121 was used for plant transformation. The T-DNA contains β-glucuronidase (*gus*) under the control of the *cauliflower mosaic virus 35S* (*CaMV35S*) promoter and *nopaline synthase* (*nos*) terminator and *neomycin phosphotransferase* (*nptII*) gene as selection marker under the control of *nos* promoter and terminator.

Explant preparation and multiple shoots induction

Surface sterilization of the seeds was performed in 0.1% $HgCl_2$ along with 0.1% Tween 20 for 20 min and soaked in sterile double distilled water for 16–18 h. After removing seed coat, the seeds were kept in modified Murashige and Skoog (MS) (Murashige and Skoog 1962) basal medium 4 to 5 days. Ten to twelve seeds were cultured in each 90 mm petri dish. Tips of plumule and radical were excised and decapitated embryonic axis with cotyledon was chosen as the explant for multiple shoots induction.

A basal medium made up of MS major salts, 4X MS minor salts, B5 (Gamborg et al. 1968) vitamins, 5 mM proline, 10 mM MES buffer along with 3% (W/V) sucrose and 0.8% bacto-agar (Difco) supplemented with different concentration and combination of plant growth regulators (PGRs) were employed at different stages of regeneration. The pH of all culture media was adjusted to 5.8 prior to autoclaving at 1.05 kg cm^{-3} for 15 min. The cultures were incubated at 22±2°C under a 16 h photoperiod, with a light intensity of 45 μmol m^{-2} s^{-1}. Regular re-culturing was done at the interval of 7 days.

Explants were cultured in the basal medium supplemented with 1 mg l^{-1} 6-benzylaminopurine (BAP) and 0.2 mg l^{-1} α-naphthaleneacetic acid (NAA) for multiple shoot induction for six weeks. Explants with emerging shoots were further cultured in medium containing 0.5 mg l^{-1} BAP along with 0.5 mg l^{-1} kinetin for another 5 weeks.

Root organogenesis, grafting and hardening

Properly elongated shoots (length ≤3 cm) with distinct nodes and internodes were transferred to 0.5X and 1X basal media supplemented with varying concentrations of IAA (1–2.5 mg l^{-1}) and IBA (0.5–1.5 mg l^{-1}). Ten shoots were cultured per treatment for 4 weeks. The experiment was repeated thrice.

Surface sterilized and germinated seeds were cultured in Hoagland (Hoagland and Arnon 1950) agar medium in above-mentioned culture condition for preparation of rootstock. After three weeks of growth, shoots were cut at first node and 2 mm vertical splits were made on rootstocks. Each pigeonpea shoot scion was taken from culture and cut at base to form a deep 'V.' Scion was inserted into the base of a vertically split stem until it fit securely supported by a sterile Teflon ring and incubated in same condition for 7 days until the healing of the tissue of the grafted region was complete. Ten in vitro grafting were performed in each experiment and the experiment was repeated thrice.

After 4 weeks of incubation in root induction media and one week of grafting, the plants with developed root system were transferred to liquid 1/2 strength of Hoagland solution on filter paper bridge, and kept for 5–7 days. Then they were transferred to plastic pots containing autoclaved synthetic soil (soilrite) and sprayed with 1/4 strength of Hoagland Solution and covered with other transparent plastic pots. In the process of

acclimatization the covering pots were temporarily removed for 2 to 3 h every day. After 10 days the plantlets were transferred to 25–30 cm diameter pots containing soil, sand and organic manure (6 : 3 : 1). The number of plants established using each media combination as well as grafting technique was recorded.

Anatomical studies of graft union

To examine the anatomical structure of the graft unions, samples were collected after 3 weeks of grafting. The Teflon ring was removed and transverse sections (T. S.) were made thorough the different parts of graft union. The sections were kept in 30% ethanol for 5 min, followed by transfer in 1% safranin solution dissolved in 50% ethanol for 30 min. Then they were kept in 70%, followed by 80% and 90% ethanol, each for 5 min. The sections were then kept in 0.5% light green solution dissolved in 95% ethanol, washed in absolute ethanol for 5 min, mounted on glycerol and observed under compound microscope.

Agrobacterium-mediated transformation

A. tumefaciens culture was prepared by growing a single colony in yeast extract broth medium for 16–18 h. After adjusting the OD_{600} at 0.8–1.0, culture was collected by centrifugation at 5,000 rpm for 5 min. Culture was re-suspended in 20 ml of 150 μM acetosyringone (Himedia, India) supplemented regeneration medium (basal medium with 1 mg l^{-1} BAP and 0.2 mg l^{-1} NAA). The 40–50 explants were immersed in 20 ml of mentioned medium and incubated for 45 min. Excess bacterial suspension was removed from the explants followed by co-cultivation on 150 μM acetosyringone supplemented regeneration medium for 3 days. Then explants were washed and maintained on the 100 mg l^{-1} kanamycin supplemented regeneration medium for 6 weeks. Re-culturing was done at 1 week interval. Explants were transferred to the kanamycin supplemented elongation medium (basal medium with 0.5 mg l^{-1} BAP and 0.5 mg l^{-1} GA_3) and similar re-culturing was followed for another 4 weeks. Explants with elongated shoots were grafted on root stock, acclimatized and transferred to soil following the procedure mentioned earlier.

Analyses of putative transgenic plants

Histochemical studies were performed to determine the GUS activity in whole and transverse sections of leaves from regenerated plants after 3, 6 and 9 weeks of antibiotic selection using X-Gluc (5-bromo,4-chloro,3-indolyl-D-glucuronide) as the substrate following the protocol of Truernit et al. (2008).

Total genomic DNA was isolated from fresh leaves of green house grown T_0, T_1 and untransformed plants using the method described by Chakraborti et al. (2006a). Polymerase chain reaction (PCR) analysis for detection of the *nptII* gene was carried out using the gene specific primers (forward-5′ GAG GCT ATT CGG CTA TGA CTG 3′ and reverse-5′ ATC GGG AGC GGC GAT ACC GTA 3′).

Statistical analysis

Mean ± standard errors were calculated for all experimental treatments. Tabulated results were analyzed using one-way ANOVA and statistical differences between means were estimated (p, 0.05) using Duncan's multiple range test with the Statistica Software v. 10.0 (StatSoft, Tulsa, 2010).

Results

Establishment of shoot multiplication protocol

Different media combinations were tested for the production of multiple shoots using decapitated embryonic axis explants (Supplemental Table 1). One milligram per liter BAP and 0.2 mg l^{-1} NAA was found to be the best for multiple shooting which produced an average of 21 shoots per explant after 6 weeks of incubation.

Among various concentrations and combinations of PGRs applied, 0.5 mg l^{-1} BAP along with 0.5 mg l^{-1} GA_3 were found to be the most effective for elongation of regenerating shoots (Supplemental Table 2). Optimally elongated shoots were bright green in color with opened leaves, distinct nodes, and internodes and with average length of 3–4 cm (Supplemental Figure 1). The shoots obtained after 5 weeks of incubation in elongation media were ready for rooting/grafting and subsequent transfer to soil.

Root organogenesis, grafting and plant establishment

Shoots obtained after 5 weeks of incubation in optimised elongation media, were used for root induction. Ten root induction media along with shoot-grafting method were assessed for plant recovery. Among all the combinations, 1/2 modified MS supplemented with 1 mg l^{-1} IBA showed best response and 38% plants have been established in soil, whereas, the grafting showed a success rate of 95% (Figure 1). The varietal differences between root-stock and scion did not hamper the healing process of graft union and subsequent plant establishment.

The grafted shoots on root stocks (Figure 2a) were hardened by placing them on a filter paper bridge in 1/2 strength Hoagland liquid medium (Figure 2b). All the successfully grafted shoots survived in soil (Figure 2c). This grafting technique allowed rapid establishment of plantlets in soil with a well developed root system (Figure 2d–f) within 90–95 days (12–13 weeks) from culture initiation.

Anatomical studies of graft union

Graft wound healing was complete after 3 weeks of grafting (Figure 3a, b). T. S. above the graft union exhibited normal dicot anatomy with characteristic vasculature and secondary growth (Figure 3c). T. S. through the region of the graft union exhibited vascular

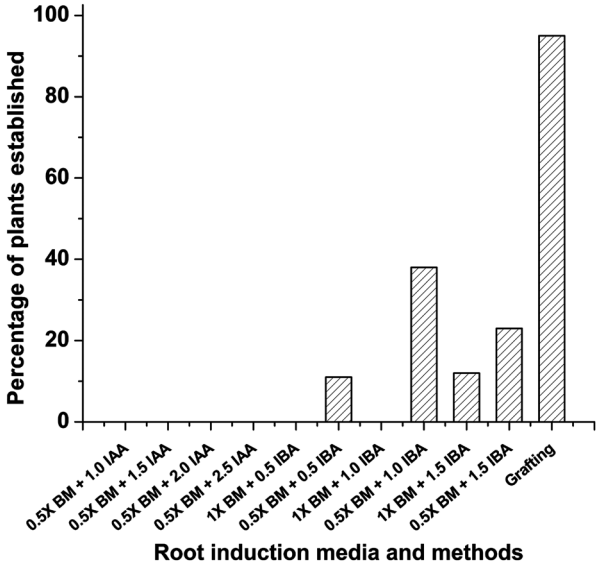

Figure 1. Bar graph representing percentage of plants established using different root induction media and the grafting method.

bundles of the stock and scion lying on different planes along with new xylem development (Figure 3d). Conspicuous protuberance of callus from the wounded scion was observed in graft union (Figure 3e). Callus tissue was undifferentiated and resurgence of cambium or vascular bundle was not observed. Below the graft union, self-healing of stock tissue was evident in the form of strips on either side of cortical and stellar region (Figure 3f).

Development of putative transgenic pigeonpea plants

Co-cultivation for 3–4 days was found to be optimal for transformation of decapitated explants. Prolonging co-cultivation period resulted in the excessive growth of *Agrobacterium* which inhibited the regeneration frequency. Subsequently they were transferred to selection media and allowed to grow there for 5 to 6 weeks. Bleached portions of the explants were eliminated and recultured at an interval of 7 days. Untransformed explants did not grow and turned brown. Explants with well-formed, multiple shoots were sub-cultured in elongation medium for 4 to 5 weeks in the same selection pressure. After a minimum of ten selection cycles, each of 1 week, matured elongated shoots of 3–5 cm with green leaves and distinct nodes and internodes were selected for grafting. They were grafted on 3 weeks old rootstock, hardened and transferred to soil. Ninety five percent of the grafted shoots were successfully established in soil. A total of 105 and 77 putative transgenic plants were obtained, using cultivars ICPL87119 and ICPL87, respectively, with average transformation efficiency (percentage of T_0 plants obtained out of total explants co-cultivated with *A. tumefaciens*) of 9% (Table 1).

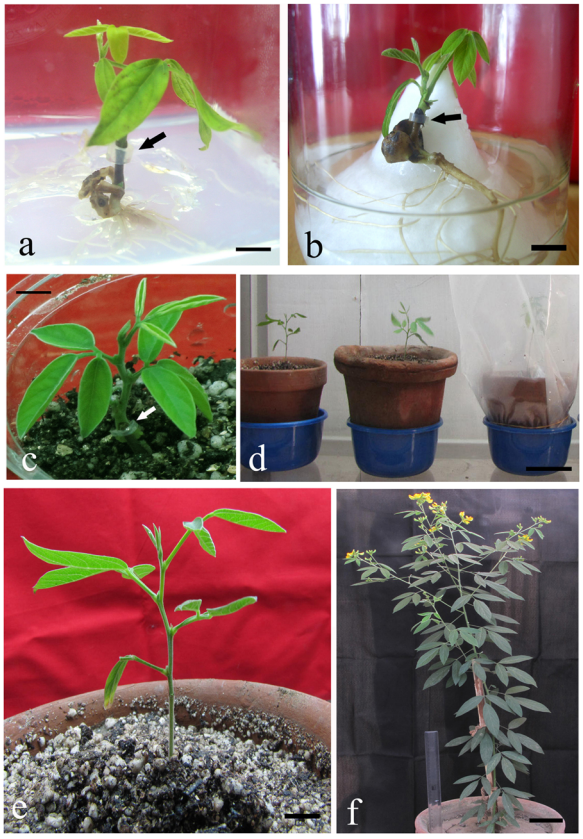

Figure 2. Different stages of hardening and transplantation. (a) Grafted shoot on rootstock; (b) grafted plant in Hoagland salt solution; (c) plantlet in soilrite during hardening; (d) plants growing in culture room after 3 weeks of soil transfer; (e) plant growing at poly-house after 4 weeks of soil transfer; (f) established plant with flowers at poly house after 12 weeks of soil transfer. Bars in figure a–c and e represent 1 cm; bars in figure d and f represent 10 cm. Arrows in figure a–c indicate the Teflon supporting ring.

Stable GUS expression in pigeonpea transformed by A. tumefaciens

Histochemical GUS staining of the explants at various developmental stages to screen putative transgenic plants showed blue colouration of the transformed shoots whereas no GUS activity was observed in untransformed control (Figure 4a–d). GUS analysis was done in explants/leaves after 3, 6 and 9 weeks of growth under selection. The blue colouration was considerably increased with the increasing age of the explant. Transverse section of the leaf tissues after GUS staining showed constitutive pattern of expression (Figure 4e, f).

Molecular characterization of the putative transformants

The selected putative transgenic T_0 plants were subjected to PCR analysis with *nptII* gene specific primers, which yielded 700 bp DNA fragments corresponding to the part of coding region of the gene (Figure 5a). One hundred and eighty two T_0 plants were established and allowed to self-pollinate in green house condition (Table 1). Two T_0

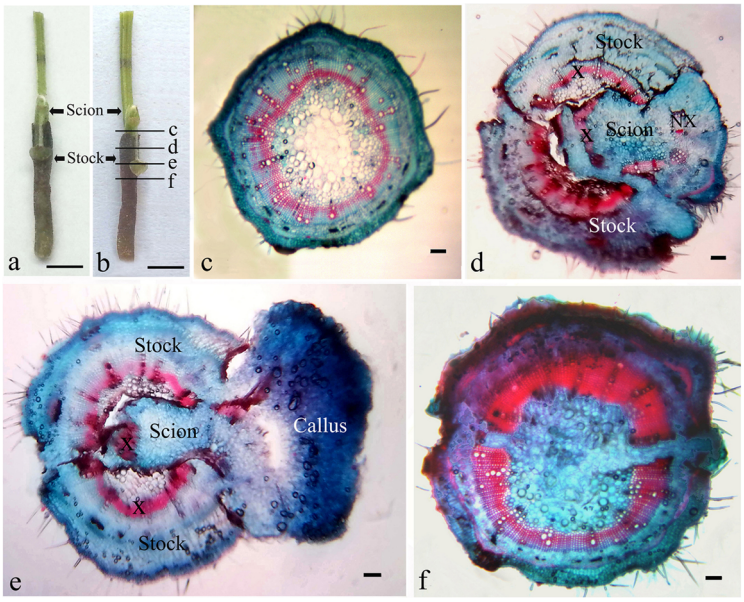

Figure 3. Morphological and anatomical features of graft union in pigeonpea. (a) and (b) front view and side view of graft union, respectively; c–f, are the positions of different transverse sections; (c) transverse section of the scion above the graft union; (d) healing of wounded region between stock and scion after 3 weeks of grafting; (e) profuse callusing from scion tissue at the wounded region; (f) self-healing of the stock below the graft union manifested by the interruption of the vascular bundle. 'X' and 'NX' represent xylem and new xylem, respectively; Bars in figure a and b represent 1 cm; bars in figures c–e and f represent 100 μm.

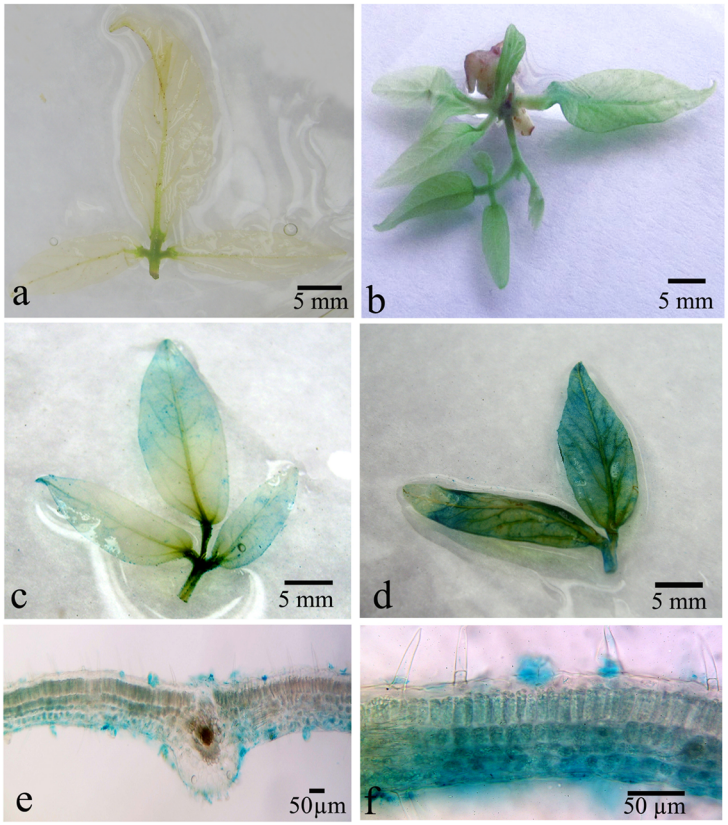

Figure 4. GUS expression of transformed shoots at different stages of growth. (a) 6 weeks old culture regenerated untransformed leaf as negative control; (b) 3 weeks old regenerated transformed shoots; (c) and (d) leaflets from 6 and 9 weeks old transformed shoots, respectively; (e) and (f) transverse section of 9 weeks old transformed leaflets at lower and higher magnifications, respectively.

Table 1. Transformation efficiency of two cultivars expressed in percentage of established T_0 plants/total number of seeds co-cultivated with *A. tumefaciens*.

Cultivar	Explants co-cultivated	Explants produced green shoots on kanamycin	Established PCR positive T_0 plants	Transformation efficiency (%)
ICPL87119	1200	800	105	8.75
ICPL87	820	650	77	9.39

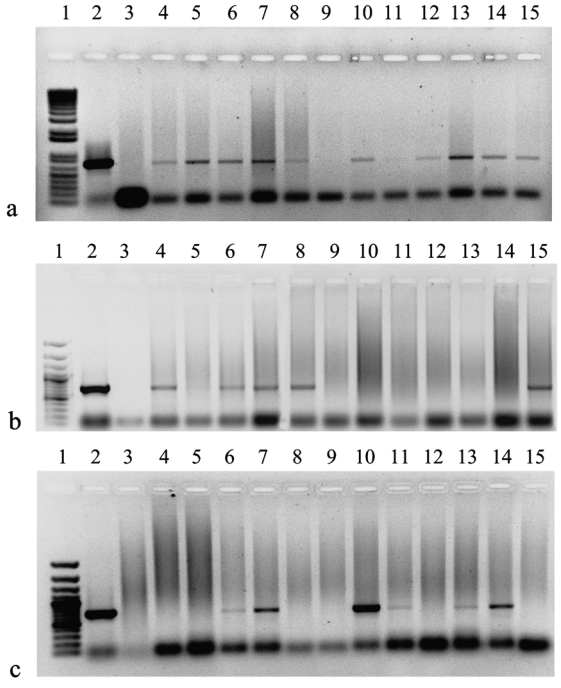

Figure 5. PCR analysis of established putative transformed plants for the presence of *nptII* gene. (a) Amplification of 700 bp *nptII* specific fragment of in T_0 plants; lanes 4 to 15, T_0 lines 1361, 1362, 1363, 1364, 1365, 1367, 1368, 1369, 13710, 13611, 13612 and 13613, respectively; (b) and (c), lanes 4–15, PCR analyses of 12 T_1 progenies obtained from each T_0 lines1362, 13611, respectively showing amplification of 700 bp *nptII* specific fragment. Lanes1–3, DNA ladder, positive and negative controls, respectively.

lines were selected for further analysis in T_1 generation to monitor the inheritance of *nptII* gene. Five and six T_1 progenies obtained from two T_0 lines 1362 and 13611, respectively, were fount to be positive for *nptII* gene (Figure 5b, c). Untransformed pigeonpea genomic DNA served as negative control and pBI121 plasmid as positive control for all the PCR analysis.

Discussion

The choice of freshly harvested well formed healthy seeds was considered to be important for the preparation of explants. The embryo axis was decapitated so that excised explant might be better amenable to *A. tumefaciens* infection. Explant preparation involved suppressing growth of apical bud and primary shoot bud while inducing multiple adventitious shoot buds in axillary regions of the seedlings. Axillary meristem based explant has been used earlier by several workers (Franklin et al. 2000; Sharma et al. 2006; Shiva Prakash et al. 1994). Decapitation of embryo axis enhanced the lateral dominance and thus higher number of multiple shoot production was experienced. Excision of the radical tip resulted in a significant increase in formation of number of shoots as found in chickpea (Chakraborti et al. 2006b; Polisetty et al. 1997). Presence of cotyledons attached to embryos was noted to be essential for higher number of shoot production.

Legumes are found to regenerate multiple shoots without an auxin supplementation due to the presence of high level of endogenous auxin; however, efficient regeneration can be optimised by balancing auxin-cytokinin level by exogenous supply (Franklin et al. 2000). The use of BAP along with NAA was advantageous for production of multiple shoots as documented by Franklin et al. (2000) and Srinivasan et al. (2004). In present study, initially single shoot possessed unifoliate and bifoliate leaf morphology and finally attained the original trifoliate morphology after 5 to 6 weeks of growth. This excluded occurrence of genotypic variations among the culture regenerated plants (Franklin et al. 2000). BAP and GA_3 each 0.5 mg l^{-1} showed optimum elongation of the regenerated shoots. Earlier workers reported the successful elongation of regenerated shoots with application of BAP and IAA together (Eapen and George 1993). However, GA_3 alone or in combination with BAP and IAA also elongated micro-shoots (Dayal et al. 2003; Eapen et al. 1998; Villiers et al. 2008).

Direct rooting using IBA and IAA supplemented media were tried in pigeonpea but it required at least 4 weeks obtaining well developed roots with less than 40% success rate. This observation was similar to previous reports on direct rooting (Eapen et al. 1998; Tyagi et al. 2001; Villiers et al. 2008). It is also evident from earlier studies that, shoots failed to produce roots on rooting medium with kanamycin at high selection pressure during regeneration of putative transformants (Krishna et al. 2010). Prolonged incubation of shoots in culture for root induction resulted in the ageing which had negative effect on normal growth of plant and ultimate seed yield.

A shoot grafting technique was adapted from similar methods followed in cotton (Luo and Gould 1999) and chickpea (Chakraborti et al. 2006b) to overcome these problems. This technique allowed rapid establishment of plantlets in soil with well developed root system and 95% of grafted plants could be established in soil. The support of the tap root of root stock enabled quick recovery of

grafted shoots. The root-stock was non-transgenic in nature and was healthier from the roots emerging from transgenic shoots. Thus, the use of grafting was more effective in pigeonpea than direct rooting and the duration of grafting, hardening and transplantation was 15 days. Such results are in concurrence with Luo and Gould (1999) and Chakraborti et al. (2006b) who reported the shoot grafting on rootstock in cotton and chickpea respectively. Success of this grafting method was directly related to scion size (4–5 cm) and age of the seedling rootstock (3 to 4 weeks). The fine wedge-shaped scion was securely appressed to the stock and locked by a fine Teflon ring. The varietal differences between rootstock and scion did not affect the graft union formation. The differential staining enabled to distinguish the tissue differentiation between the stock and scion. Callus formation occurred at the healed portions of the scion, whereas, the longitudinal cut ends of the stock exhibited healing with discontinuous cortex, cambium and vascular bundle. The currently described protocol took 90–95 days, from seed sterilization to establishment of whole plant.

Here we report for the first time a stable and efficient *Agrobacterium*-mediated transformation of two cultivars of pigeonpea via in vitro root-stock grafting. Cotyledonary nodes proved to be the best explant for *Agrobacterium*-mediated transformation, as mentioned by previous workers (Kumar et al. 2004a, b; Prasad et al. 2004; Satyavathi et al. 2003; Thu et al. 2003; Verma and Chand 2005). The regeneration of multiple shoots was completely inhibited at 100 mg l^{-1} of kanamycin (data not shown) which was found to be the optimum concentration for selection of putative transformants using selection marker gene *nptII* as reported earlier (Satyavathi et al. 2003; Thu et al. 2003; Verma and Chand 2005). Selected transformants were easily grafted on non-transgenic root-stock, hardened and acclimatized in field condition and thereby reducing the time period of transplantation. In this way difficulties in root organogenesis under in vitro conditions, considered as a major concern in pigeonpea transformations, can be conveniently bypassed resulting in higher plant survival.

There are various reports on the stable integration of *nptII* and *gus* genes in transformed pigeonpea (Kumar et al. 2004a; Mohan and Krishnamurthy 2003; Surekha et al. 2007; Thu et al. 2003; Verma and Chand 2005). This is the first report where elaborative histochemical studies of GUS expression have been performed at different stages of growth, delineating the constitutive expression of this popular reporter gene under the control of *CaMV35S* promoter.

Conclusion

This is the first report on in vitro shoot grafting mediated transformation in pigeonpea. This is a unique protocol with minimum time requirement and maximum success rate as compared to other related reports. The high rate of successful establishment of transgenic plants using two different cultivars also broadens the practical scope of this protocol for developing improved pigeonpea plant through genetic engineering approach.

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Supplemental Table 1. Effect of different combinations and concentrations of BAP, NAA and kinetin on multiple shoot induction from decapitated embryonic axis explants

Plant growth regulators[a] (mg l^{-1})			Cultivars	
			Average number[b] of shoots per explants	
BAP	NAA	Kinetin	ICPL87119	ICPL87
1	0.02	0	16.8±0.2d	13.3±0.3e
1	0.2	0	25.1±0.4a	21.1±0.3a
1.5	0.2	0	20.2±0.4b	15.6±0.3c
2	0.2	0	14.2±0.4e	10.1±0.4fg
2.5	0.2	0	13.4±0.4e	9.7±0.3g
3	0.2	0	8.7±0.3f	4.1±0.3h
1	0.5	0	19.8±0.4b	13.2±0.3e
1	0	0.5	17.1±0.4d	14.2±0.4d
1	0	1	14.7±0.4e	10.8±0.4f
1	0	1.5	20.1±0.4b	15.1±0.3cd
1	0	2	20.9±0.3bc	16.0±0.4c
1	0	2.5	21.8±0.4bc	19.6±0.3b

[a]Five explants per 90 mm petri dish from each genotype in duplicate sets (i.e. 10 replications) were cultured per treatment. Each treatment was repeated thrice. Data were scored after 6 weeks of culture initiation.

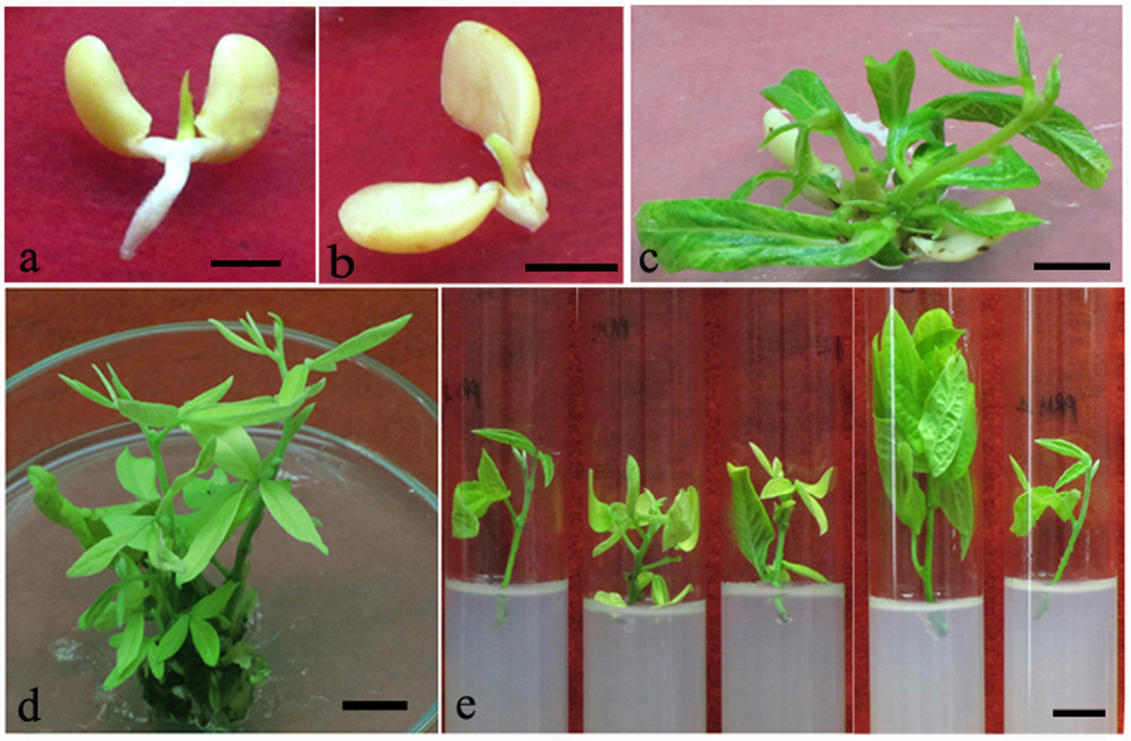
[b]Each value represents mean ±SE of all observations. The means followed by the same letters within a column do not differ statistically according to Duncan's multiple range tests at a 5% probability level.

Supplemental Table 2. Effect of different combinations and concentrations of IAA, BAP and GA_3 on shoot elongation

Plant growth regulators[a] (mg l^{-1})			Cultivars	
			Average number[b] of shoots with length ≤3 cm per regenerating explant	
IAA	BAP	GA_3	ICPL87119	ICPL87
0.1	0	0	2.2±0.3d	1.7±0.1efg
0.2	0	0	1.9±0.2d	1.9±0.1efg
0.5	0	0	1.5±0.1d	1.2±0.1f
0	0.5	0.05	2.4±0.1d	2.4±0.2e
0		0.1	5.1±0.2c	3.9±0.3d
0	0.5	0.1	9.0±0.3b	7.3±0.2c
0	0.5	0.2	9.1±0.4b	8.3±0.3b
0	0.5	0.5	19.8±0.4a	18.9±0.6a
0	0	0	9.8±0.5b	8.3±0.4b

[a]Five regenerating explants from each genotype in duplicate sets (i.e. 10 replications) were cultured per treatment. Each treatment was repeated thrice. Data were scored after five weeks of incubation.

[b]Each value represents the mean±SE of all observations. The means followed by the same letters within a column do not differ statistically according to Duncan's multiple range tests at a 5% probability level.



Supplemental Figure 1. Generation of multiple shoots from decapitated embryonic axis explants. (a) Stage of the germinated seed used for explant preparation; (b) the explant; (c) regeneration of multiple shoots from an explant after 35 days of incubation on 1 mg l^{-1} BAP and 0.2 mg l^{-1} NAA medium; (d) elongating multiple shoots generated from an explant after 4 weeks of incubation on 0.5 mg l^{-1} BAP and 0.5 mg l^{-1} GA_3 medium; (e) individual elongated shoots separated from shoot stock. Bar represents 1 cm.