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AGROBACTERIUM-MEDIATED PRODUCTION OF TRANSGENIC PIGEONPEA (*CAJANUS CAJAN* L. MILLSP.) EXPRESSING THE SYNTHETIC *BT CRY1AB* GENE

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Summary

Conventional breeding methods have not been very successful in producing pest-resistant genotypes of pigeonpea, due to the limited genetic variation in cultivated germplasm. We have developed an efficient method to produce transgenic plants of pigeonpea by incorporating the *cry1Ab* gene of *Bacillus thuringiensis* through *Agrobacterium tumefaciens*-mediated genetic transformation. The novel tissue culture protocol is based on the direct regeneration of adventitious shoot buds in the axillary bud region of *in vitro* germinating seedlings by suppressing the axillary and primary shoot buds on a medium containing a high concentration of N^6-benzyladenine (22.0 μM). The tissue with potential to produce adventitious shoot buds can be explanted and used for co-cultivation with *A. tumefaciens* carrying the synthetic *cry1Ab* on a binary vector and driven by a CaMV 35S promoter. Following this protocol, over 75 independently transformed transgenic events of pigeonpea were produced and advanced to T2 generation. Amongst the recovered primary putative transformation events, 60% showed positive gene integration based on initial polymerase chain reaction (PCR) screening. PCR analysis of the progenies from independent transformants followed gene inheritance in a Mendelian ratio and 65% of the transformants showed the presence of single-copy inserts of the introduced genes. Reverse transcription–polymerase chain reaction analysis showed that the transcripts of the introduced genes were normally transcribed and resulted in the expression of Cry1Ab protein in the tested T2 generation plants. Interestingly, the content of Cry1Ab protein as a percent of total soluble protein varied in different tissues of the whole plant, showing the highest expression in flowers (0.1%) and least in the leaves (0.025%) as estimated by enzyme-linked immunosorbent assay. The transgenic plants produced in this study offer immense potential for the improvement of this important legume of the semi-arid tropics for resistance to insect pests.

Key words: Agrobacterium tumefaciencs; *Bt cry1Ab*; *Cajanus cajan*; insect resistance; pigeonpea; shoot regeneration; transgenic plants.

Introduction

Pigeonpea [*Cajanus cajan* (L) Millsp., red gram] is a protein-rich grain legume of the semi-arid tropics. A lepidopteran insect, the legume pod borer or *Helicoverpa armigera* L., is the most serious and widespread pest of pigeonpea. Its larvae attack the flowers and pods of pigeonpea, resulting in substantial damage and yield losses of over US$300 million annually worldwide (Shanower et al., 1999). This is despite the use of over US$211 million worth of chemical pesticides to control this pest. Intensification of agriculture has exacerbated the pest problems, and farmers are responding by using more toxic pesticides more frequently. For pest problems as complex and intractable as *Helicoverpa*, no single control strategy is successful in keeping its population below economic threshold levels. Although chemical control is one of the effective methods of controlling this pest, it has led to the emergence of secondary pest problems, besides contamination of food and food products with insecticide residues. Moreover, their indiscriminate use has also resulted in the development of resistance to various insecticides, thus making it more difficult to address this problem through chemical control. Therefore, development of crop cultivars with insect resistance can form the backbone for integrated management of this pest (Sharma et al., 2004).

Attempts to produce pest-resistant genotypes of pigeonpea by conventional breeding methods have not been successful due to limited genetic variation in the cultivated germplasm and incompatibility with wild species (Nene and Sheila, 1990). In recent years, genetically engineered resistance has been actively investigated to enhance the breeder's arsenal. It is imperative to have an efficient regeneration and transformation system in order to introduce novel traits in pigeonpea. Genes conferring resistance to insect pests have been introduced successfully in a wide array of crop plants (Dunwell, 2000).

In pigeonpea, attempts to regenerate plants from various explants have been attempted and direct shoot induction has been obtained from various explants. These include leaves (Eapen and George, 1993; Eapen et al., 1998; Geetha et al., 1998; Singh et al., 2002; Dayal et al., 2003), cotyledonary node (Shiva Prakash et al., 1994; Franklin et al., 1998; Geetha et al., 1998; Mohan and Krishnamurthy, 1998;

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Singh et al., 2002), epicotyl (George and Eapen, 1994), and shoot apices (Geetha et al., 1999, Singh et al., 2004). However, the transformation efficiency in explants derived from cotyledonary nodes has been low (Geetha et al., 1999). More recently, shoot apices from 16-d-old seedlings were used to obtain shoot regeneration and transformation with marker genes to obtain polymerase chain reaction (PCR)-positive primary transformants of pigeonpea (Singh et al., 2004). In our laboratory, seedling-derived leaf explants have also been employed successfully to obtain high frequency of genetic transformation in pigeonpea through biolistics (Dayal et al., 2003). The use of genes conferring resistance to insect pests and the progress in developing transgenic crops carrying the genes coding for Cry toxins from the bacterium *Bacillus thuringiensis* (*Bt*) for insect resistance have been reviewed extensively (Sharma et al., 2004).

In this report, we have developed a direct shoot regeneration protocol by using the axillary bud region of the *in vitro* germinated seedlings as an explant. We report a novel method for the regeneration of adventitious shoot buds and efficient transformation of pigeonpea by using *Agrobacterium tumefaciens*-mediated transfer of a synthetic *cry1Ab* gene of *B. thuringiensis*. Several events of transgenic pigeonpea were produced by this method that showed stable integration, inheritance, and expression of the introduced genes in subsequent generations.

Materials and Methods

Plant materials and culture conditions. Seeds of pigeonpea varieties ICPL 87 were used for all the experiments in the present studies. Unless mentioned otherwise, all media contained MS salts and organic constituents (Murashige and Skoog, 1962), 3% sucrose, 0.8% (w/v) Difco-Bacto agar, and the pH was adjusted to 5.8 before autoclaving. For explant culture and shoot bud development, 100 × 25 mm sterile Petri dishes were used to initiate cultures, and sealed with Parafilm® (American Can Company), whereas 10 × 100 mm culture tubes were used for seed germination, shoot elongation, and rooting, and closed with plugs made with non-absorbent cotton. All the growth regulators including N^6-benzyladenine (BA), gibberellic acid (GA_3), and indole-3-acetic acid (IAA) were added after filter sterilization. The cultures were incubated at 26 ± 2°C under continuous light of 100 $\mu E\, s^{-1}\, m^{-2}$ irradiance provided by cool white fluorescent lamps.

Seed germination and seedling pre-culture. Mature seeds were surface-sterilized by rinsing in 70% alcohol for 1 min, followed by treatment with a solution of 0.1% (w/v) $HgCl_2$ containing 1–2 drops of Tween-20 for 8 min. The seeds were rinsed thoroughly with sterile-distilled water and soaked for 4–5 h. Seed coats from the pre-soaked seeds were removed under aseptic conditions and seeds cultured for 2–14 d in culture tubes containing the shoot induction medium (SIM) comprising of MS, 3% (w/v) sucrose and 22.0 μM BA for their germination and pre-culture. Unless mentioned otherwise, 14–15-d-old seedlings were used to obtain explants for transformation experiments. To test the effect of basal medium, the seeds were cultured on various media including MS, B5 (Gamborg et al., 1968), and L2 (Phillips and Collins, 1979) for seed germination and seedling pre-culture. The media were supplemented with 22.0 μM BA to select the optimal basal medium that would suppress the axillary buds and form multiple adventitious shoots in the axillary region. To study the effect of age of the explant donor seedlings, the explants were obtained from 7–15-d-old seedlings pre-cultured on SIM to study their responses on the induction of adventitious shoot buds and their further elongation.

Explant preparation and shoot regeneration. The pigeonpea seedlings pre-cultured on SIM for 14–15 d (Figs. 1 *A*, *B*, 2*C*) were used to obtain the explants for adventitious shoot formation and development. During this stage, the pre-existing axillary bud was completely suppressed and was encircled by a whorl of meristematic tissue forming initials of adventitious shoot buds. This tissue (Fig. 2*C*) was carefully excised to obtain the axillary meristem explant (AME) and cultured on SIM for 10–12 d for further development of shoot buds. The induced shoot buds were cultured for their development on MS containing 3% sucrose and various cytokinins [BA, thidiazuron (TDZ) and kinetin] in different combinations (see Table 2). Amongst these, BA at a concentration of 22.0 μM was selected for the development of shoot buds. For the elongation of fully developed shoots, they were further sub-cultured on shoot elongation medium (SEM) containing MS, 3% sucrose, and 0.58 μM GA_3.

Rooting of shoots and transplantation. The elongated shoots were transferred to MS containing 3% sucrose and different concentrations of IAA or IBA at 0.5, 2.5, 5.0, and 10.0 μM levels for rooting. In the absence of consistent rooting, the shoots were subjected to a pulse treatment with 25.0 μM filter-sterilized IAA for 2–3 min, followed by their culture on root induction medium (RIM) containing MS with 3% sucrose, which proved beneficial for root induction. The rooted shoots growing on MS basal medium for 2–3 wk were transferred to pots (3 in. diameter) containing autoclaved sand and thiram (fungicide) along with Hoagland's nutrient solution, and maintained in a greenhouse at 25–28°C with 95% humidity under natural light and photoperiod. After 2 wk, when the transplanted shoots resumed normal growth, they were transferred to larger pots (12 in. diameter) containing autoclaved sand:soil (1:1) for further growth and seed production. The details on transplantation and hardening of the plants were discussed earlier (Dayal et al., 2003).

Plasmid constructs and bacterial strain. The *Bt cry1Ab* gene was provided by Plantec corporation as plasmid pBT 1291 (Fujimoto et al., 1993). The 2180-bp (*Sal* I–*Xba* I) fragment containing the N-terminal half of *cry1Ab* was cloned into an intermediate plasmid pRTL2 (Carrington and Freed, 1990), consisting of the $35S^{DE}$ promoter and fused with a leader sequence of the tobacco etch virus (TEV), at *Sma* I–*Xba* I sites after blunting the *Sal* I site. To obtain the binary plasmid pHS723:Cry1Ab (Fig. 3), the *Pst* I fragment (2.2 kb) from pRTL2:Cry1Ab containing the *cry1Ab* gene along with double-enhanced CaMV 35S promoter, TEV leader sequence, and 35S polyA termination sequences from pRTL2:Cry1Ab was blunt-end ligated into the *Sma* I site of the binary plasmid pHS723 (kindly provided by Dr Gopalan Selvaraj, Plant Biotechnology Institute, NRCC, Saskatoon, Canada). The plasmid pHS723 carried the fused *uid*A and *npt* II genes driven by a double-enhanced CaMV 35S promoter and polyA sequence producing a fusion protein. The plasmid also contained a resistance gene for bacterial selection. The resulting binary plasmid pHS723:Cry1Ab was mobilized into the disarmed *A. tumefaciens* strain C58 and used for co-cultivation.

Genetic transformation and selection of transformants. A single colony of *A. tumefaciens* strain C58 carrying the binary plasmid pHS723:Cry1Ab was inoculated and grown overnight at 28°C in 25 ml of YEB (Sambrook et al., 1989) supplemented with appropriate antibiotics. Bacterial suspension (5 ml) was pelleted by centrifugation for 10 min at 5000 rpm. The cells were washed with 10 ml of half-strength MS and re-suspended in 30 ml of half-strength MS-containing 2% sucrose. This suspension was stored at 4°C for 1–2 h and used for co-cultivation. The bacterial suspension was transferred to a sterile Petri plate so as to make a thin film (2–3 mm) at the base of the Petri plate. Adventitious shoot-forming AME were prepared and the cut end of the axillary bud region was immersed into the bacterial suspension for few seconds and immediately placed on SIM with the cut ends embedded in the medium. The explants were co-cultivated with *Agrobacterium* for 72 h before transfer to SIM supplemented with filter-sterilized cefotaxime (250 $\mu g\, ml^{-1}$) to prevent bacterial growth. Plating density was maintained at 10 explants per Petri plate (90 mm × 15 mm).

The AMEs after treatment with *Agrobacterium* were maintained on SIM containing 250 $\mu g\, ml^{-1}$ cefotaxime for 2 wk when multiple shoot buds appeared. At this stage, the explants bearing shoot buds were transferred to SIM containing 250 $\mu g\, ml^{-1}$ cefotaxime and 125 $\mu g\, ml^{-1}$ kanamycin to initiate antibiotic selection and enrichment of transformed cells. Organogenic tissues differentiated into shoot buds for another 2 wk. Subsequently, each explant containing multiple shoot buds was cut into 3–4 pieces and transferred to SEM containing 125 $\mu g\, ml^{-1}$ kanamycin for three sub-cultures at 2 wk intervals. After this stage, the elongated shoots were pulse treated with filter-sterilized 25.0 μM IAA and cultured on MS without antibiotic for rooting. All the shoots cultured on RIM produced multiple roots within 2 wk. The rooted shoots were transferred to pots (3 in. diameter) containing autoclaved sand. The pots were covered with a polyethylene bag for 1 wk to provide high humidity (90–100%) and maintained in the greenhouse. The bags were cut at the corners after 7 d and removed after 10 d and maintained under high humidity (85%) at 23–28°C in a containment greenhouse. The plants were allowed to flower and set seed. Upon flowering and pod formation, the mature seeds were collected and analyzed for the presence and expression of introduced genes.

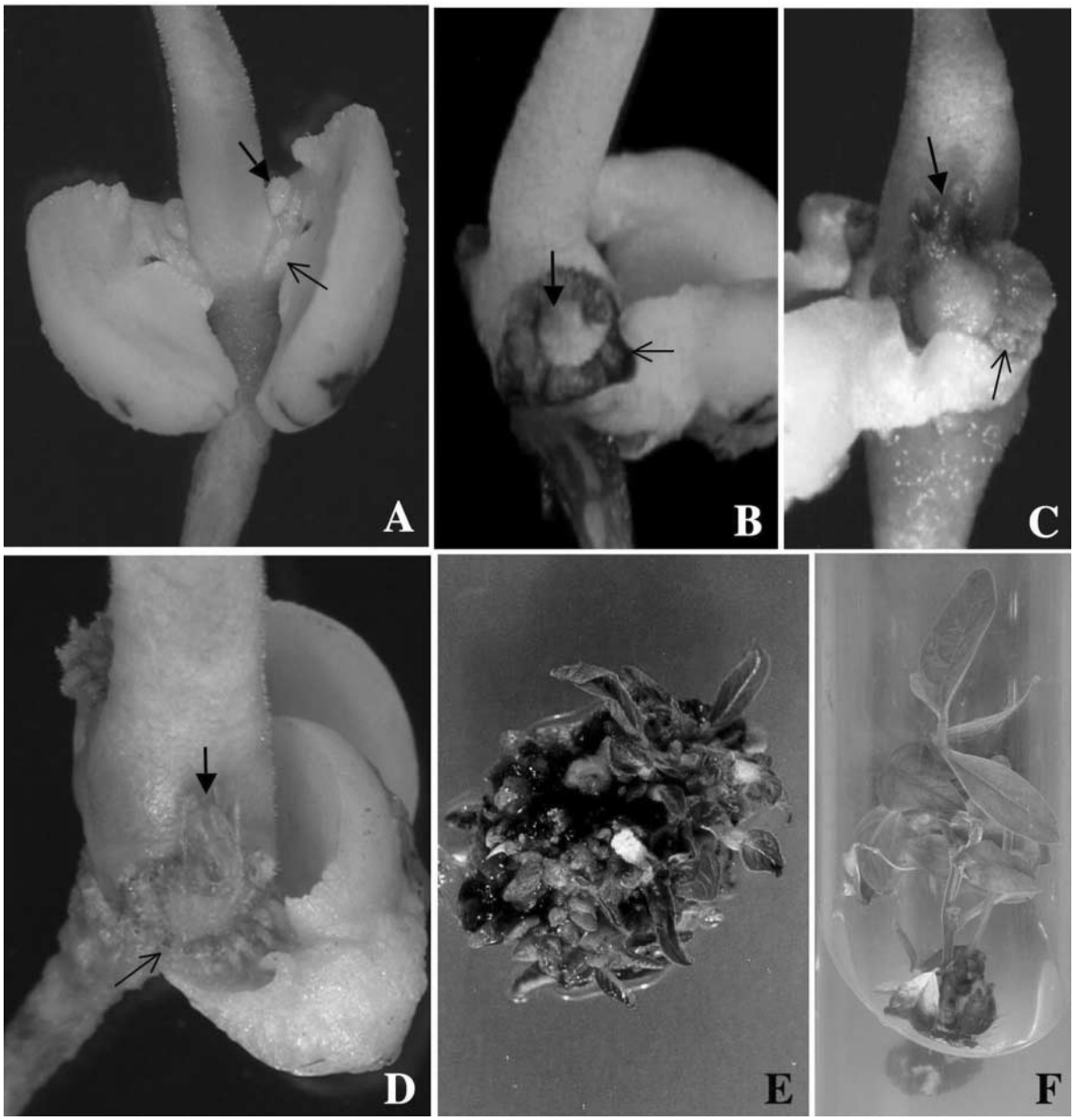

FIG. 1. Regeneration of multiple shoots from the axillary bud region of *in vitro* germinated seedlings of pigeonpea pre-culture on SIM containing MS and 22.0 μ*M* BA. *A*, Germinated seedlings after 8 d on SIM. Note the suppression of the axillary bud (*closed arrow*) and initiation of bulging at the cotyledonary nodal region (*open arrow*) surrounding the suppressed axillary bud. *B*, Differentiation of adventitious shoot buds (*open arrow*) in the cotyledonary nodal region after 12 d pre-culture on SIM. *C*, Suppressed axillary bud region (*closed arrow*) resembling a dome surrounded by adventitious shoot initials (*open arrow*) after 15 d on SGM. *D*, Development of fully differentiated shoots (*open arrow*) after 20 d on SIM. Note that the suppressed axillary bud (*closed arrow*) starts elongating at this stage. *E*, Development of shoot buds on axillary bud region excised from Fig. 1*B* after 5 d on SIM containing 5 μ*M* BA. *F*, A rooted shoot after 1 wk on RIM containing MS. The shoots were pulse treated with 25 μ*M* IAA for 2–3 min prior to culture.

Molecular analyses of putative transgenic plants. For histochemical analysis, β-glucuronidase (*uid*A) enzyme activity was detected histochemically in unfixed leaves and free-hand sections of petiole and stem sections of regenerated plants growing *in vitro* on medium containing 125 μg ml^{-1} kanamycin or in the containment glasshouse, by using X-Gluc (5-bromo,4-chloro,3-indolyl-D-glucuronide) as the substrate (Jefferson, 1987). After the histochemical reaction at 37°C for 6–12 h, tissue was cleared in 70% ethanol and examined for presence of the blue color.

For PCR analysis, the total genomic DNA was isolated from fresh leaves of *in vitro* or glasshouse-grown putative transformants (T0, T1, and T2 generations). Leaf tissue (500 mg) was ground in liquid nitrogen with mortar and pestle by following the modified CTAB method (Porebski et al., 1997). Putative transformants in T0, T1, and T2 generations were screened by PCR for the presence of *npt* II, *uid*A, and *cry1Ab* genes. The 700 bp region of *npt* II and 1200 bp coding region of *uid*A were amplified by using the respective 22-mer and 21-mer oligonucleotide primers as previously reported (Hamil et al., 1991). A 908-bp coding region of the *cry1Ab* gene was amplified by using 25-mer oligonuceotide primers (*Bt* primer I: 5′-TCACCCAGT TCCTCCTCAGCGAGTT-3′ and *Bt* primer II: 5′-GGCGTTGCCCATCGTGCCGTAGAGC-3′). The amplification

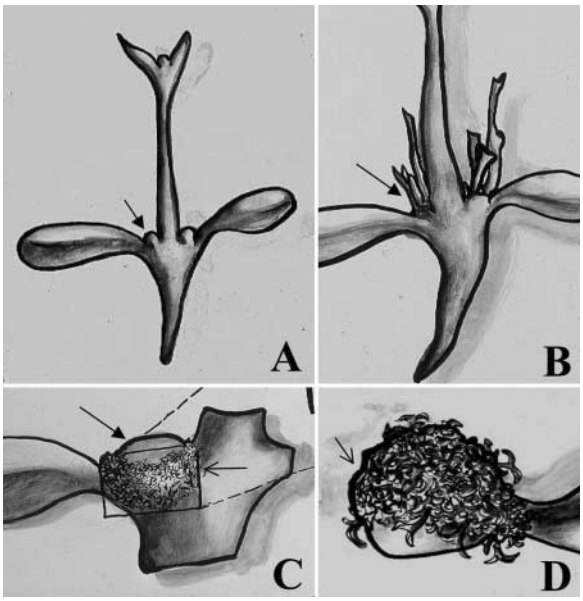

FIG. 2. Diagrammatic representation of multiple shoot formation from the cotyledonary node region of the pigeonpea seedling. *A*, Germinated seedling of pigeonpea showing the axillary bud (*arrow*) at the cotyledonary region. *B*, Axillary shoot formation (*arrow*) from the axillary bud region cultured on MS in absence of SIM. *C*, Suppression of axillary bud region (*arrow*) and initiation of multiple shoots (*open arrow*) after culture on SIM. *D*, Proliferation of multiple shoots at the lateral sides of the suppressed axillary bud region after prolonged culture on shoot germination medium.

reactions were carried out using a Techne™ PHC3 thermal cycler under the following conditions: 94°C for 4 min (one cycle), 93°C for 60 s (denaturation), 58.5°C (*npt* II), 59°C (*uid*A), and 63.1°C *(cry1Ab)* for 45 s (annealing), with an extention cycle at 72°C for 90 s (extension) for 32 cycles, followed the final extension at 72°C for 4 min (one cycle). The PCR was performed by using ~150 ng of purified genomic DNA and the recombinant *Taq* DNA polymerase (Invitrogen) according to the manufacturer's recommendations. The amplified products were assayed by electrophoresis on 1.2% agarose gels. The fidelity of the amplicons were verified by transferring the amplicons from gels onto the Hybond + nylon membranes (Amersham Pharmacia Biotech) by Southern blotting (Sambrook et al., 1989), followed by hybridizing with *uid*A and *cry1Ab* gene fragments prepared from the plasmid DNA labeled with AlkPhos Direct® kit (Amersham Pharmacia Biotech). The blots were also hybridized with the *Pst* I fragment containing 800 bp coding sequence of *npt* II from the plasmid pRT99 (Topfer et al., 1988) and 2337-bp *Xho* I–*Xba* I fragment of *cry1Ab* gene from pHS 723:Cry1Ab.

For Southern blot analysis, the genomic DNA (15–20 μg) from each of the putative transformants in T1 generation and untransformed plants was separately digested with *Bam* HI that possessed one restriction site within the T-DNA region to ascertain the copy number and the integration pattern of the *cry1Ab* gene. The digested DNA was separated on 0.8% agarose gel and transferred to the nylon membrane (Hybond N +) by capillary transfer

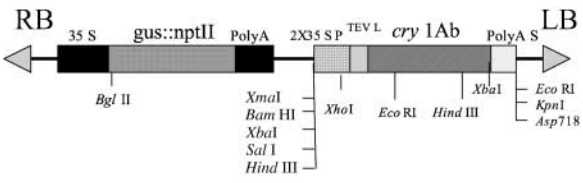

FIG. 3. Restriction map of the binary vector construct pHS723:Cry1Ab used for *Agrobacterium*-mediated transformation of axillary bud region of pigeonpea.

(Sambrook et al., 1989). PCR amplified fragments of the coding sequence (908 bp *cry1Ab*) was used as probe after labeled using Alkphos Direct® system. The labeling, hybridization, and detection were performed according to the manufacturer's instructions.

For reverse transcription-polymerase chain reaction (RT-PCR) analysis, total RNA was isolated from the transgenic plants using TRIzol® reagent (Invitrogen) from 100 mg leaf tissue according to the manufacturer's instructions. The integrity of RNA was verified by visualizing the RNA bands on 1.2% denaturing agarose gel (Sambrook et al., 1989). RT-PCR analysis was carried out for the selected plants from the T2 generation by using Thermoscript RT-PCR system® (Invitrogen) according to the manufacturer's instructions. PCR of the coding sequences of *npt* II, *uid*A, and *cry1Ab* genes in the cDNA were carried out by using respective primers as described above.

To test for the presence of Cry1Ab protein in different plant parts, total soluble protein was isolated from different tissues, including leaf, pod-wall, seed, and flowers from transgenic pigeonpea plants in the T2 generation by using the TRIzol® reagent. Enzyme-linked immunosorbent assay (ELISA) was carried out using a pathoscreen kit (Agdia®) for Cry1Ab endotoxin according to the manufacturer's instructions. The data on Bt Cry1Ab protein were calculated from three assays per plant for respective tissue and analyzed statistically. The Bt protein was expressed as percent of the total soluble protein in the respective tissues.

RESULTS

Shoot bud differentiation. The seeds were germinated on MS basal medium with various concentrations of BA to suppress the growth of the primary shoot. Culturing the seedlings on SIM containing 22.0 μ*M* BA suppressed the axillary bud growth; it instead resulted in multiple shoot formation from the suppressed axillary bud region. Bulging at the cotyledonary node region was observed after 8 d (Fig. 1*A*) and by 12 d the differentiation of adventitious shoot buds occurred at the bulged portion surrounding the axillary bud (Fig. 1*B*). These shoot buds became more defined by 15 d, while growth of the axillary bud was further suppressed (Fig. 1*C*). Fully differentiated shoots developed in tissue surrounding the suppressed axillary bud (Fig. 1*D*), resulting in multiple shoots. If allowed to grow further, multiple shoots were formed in the axillary regions of the seedlings (Fig. 1*D*). However, tissue carrying the shoot buds at a stage indicated in Fig. 1*C* can be explanted and cultured on SIM for genetic manipulation and recovery of transformed shoots (Fig. 1*E*). The developed shoots can be separated and cultured on SEM containing reduced concentration of BA (5.0 μ*M*) resulting in further development of the shoot buds. The elongated shoots can be easily rooted after culture on RIM (Fig. 1*F*).

The unique pattern of differentiation of multiple adventitious shoot buds from the cotyledonary node region of pigeonpea seedlings is shown diagrammatically in Fig. 2*A*–*D*. If the pre-soaked and de-coated seeds are germinated on hormone-free MS medium (Fig. 2*A*), normal germination occurs, resulting in the development of two axillary buds (Fig. 2*B*). However, when these seeds are germinated on SIM containing 22.0 μ*M* BA, it results in complete suppression of the axillary and apical buds, and differentiation of adventitious shoot buds occurs in the tissue surrounding the axillary bud (Fig. 2*C*). Hence, for all studies on shoot regeneration, MS containing 22.0 μ*M* BA was used. For genetic transformation, the tissue containing the adventitious shoot bud initials (Fig. 2*C*) was explanted and co-cultivated with *Agrobacterium* that resulted in the formation of multiple adventitious shoot buds (Figs. 1*E*, 2*D*).

TABLE 1

EFFECT OF AGE OF THE EXPLANT DONOR SEEDLINGS ON THE FREQUENCY OF ADVENTITIOUS SHOOT REGENERATION IN PIGEONPEA ON MS MEDIUM CONTAINING 22.0 μ*M* BA

Seedling age (days)	No. of explants cultured	Frequency of explants with shoots (%)
10	50	17.5
11	50	25.0
12	50	45.0
13	50	55.0
14	50	95.0
15	50	92.0

It was found that adventitious shoot induction from axillary tissue was influenced by the seedling age. The best explant was tissues from 14–15-d-old seedlings that gave rise to the highest number of adventitious shoot buds when cultured on SIM with 5.0 μ*M* BA (Table 1).

Amongst the various basal media tested for the germination of seeds, MS containing 22.0 μ*M* BA was found to be superior to B5 and L2 media with respect to the number of seedlings showing the induction of adventitious shoot buds, shoot elongation, and rooting on RIM (data not shown). To optimize the cytokinin for adventitious shoot induction, BA, kinetin, and TDZ were tested in different combinations (Table 2). The combination of two cytokinins, BA and kinetin, did not help in increasing the shoot induction capacity, where the induced shoots were found to be difficult to elongate in subsequent passages. TDZ resulted in profuse callus formation and less or no induction of adventitious shoot buds (Table 2). Maintaining the concentration of BA at 22.0 μ*M* in the SEM resulted in restricting the growth of the preformed shoots. However, decreasing the BA concentration is beneficial to elongation of the preformed multiple shoots. Using GA_3 at low concentrations, 0.58 μ*M* also enhanced shoot elongation (data not shown) but high concentration or no GA_3 resulted in weak stems with longer internodal distance.

The elongated shoots were further sub-cultured for rooting on basal MS or MS containing IAA or IBA. The rooting response was found to be best on hormone-free MS when compared to other media (data not shown), but rooting was not consistent and only a few shoots (20%) formed roots and they were not healthy. However, over 70% of the shoots, when pulse-treated with 25 μ*M* IAA for 2–3 min prior to culture on MS, produced roots and appeared healthy. The remaining 30% shoots could be rooted after another pulse treatment with IAA. In general, the root formation occurred within 10–15 d after pulse treatment with IAA.

Genetic transformation and selection of putative transgenic plants. Explants from the axillary bud region were used for genetic transformation experiments. The explants co-cultivated on SIM for 72 h were subjected to a selection pressure after 2 wk of infection. The explants subjected to higher concentration of kanamycin (125 mg ml^{-1}) resulted in bleaching, thus playing a selective role on the suppression of shoot bud induction from the untransformed cells. Histochemical GUS staining of the explants to screen putative transgenic plants resulted in blue stain in transformed shoots. However, GUS staining is not a reliable screening system in pigeonpea as some of the untransformed pigeonpea plants also showed the inherent weak GUS-like expression.

Characterization of transgenic plants. About 75 primary putative transgenic plants were transplanted in the greenhouse and were allowed to set seeds (Table 3). The putative transgenic plants were subjected to molecular analysis for the presence of *npt* II, *uid*A and *cry1Ab* genes using PCR. Plants from all transgenic plants showed the expected size of the amplified fragment for the respective genes (Fig. 4*A*–*C*). No amplification was observed in untransformed plants, used as negative controls. The PCR amplification products for the two genes were independently blotted for Southern hybridization to verify the authenticity of the

TABLE 2

EFFECT OF GROWTH REGULATORS ON INDUCTION OF ADVENTITIOUS SHOOT BUDS FROM EXPLANTS OF PIGEONPEA. MS BASAL MEDIUM WAS USED FOR ALL COMBINATIONS

Plant growth regulators (μ*M*)					
BA	Kinetin	TDZ	No. of explants with shoots[a]	No. of explants with elongated shoots[a]	Explant with shoots (%)
0	0	0	10 ± 0.4	10 ± 0.1	28.6
8.8	–	–	15 ± 1.5	12 ± 0.3	42.9
22.0	–	–	30 ± 2.3	20 ± 4.5	85.7
44.3	–	–	28 ± 1.2	20 ± 3.0	80.0
66.4	–	–	28 ± 0.8	15 ± 4.0	80.0
4.44	0.46	–	20 ± 2.0	15 ± 0.6	57.1
–	0.46	–	15 ± 7.0	10 ± 0.5	42.9
–	–	9.08	15 ± 2.0	0	42.9
–	–	18.36	15 ± 5.0	0	42.9
22.0	–	–	30 ± 2.5	15 ± 3.0	85.7
22.0	–	–	30 ± 2.5	25 ± 4.0	85.7
22.0	–	–	30 ± 2.5	30 ± 2.0	85.7

[a] Results are averages of three replications where each replicate included 35 explants. Values are expressed as mean ± SE. The experiment was repeated three times.

TABLE 3

SUMMARY OF T0 TRANSGENIC PLANTS OF PIGEONPEA VAR. ICPL 87 CARRYING *cry1Ab* GENE

Transformation experiments[a]	No. of plants regenerated	No. of transgenic plants containing *cry1Ab* gene by PCR analysis	Transformation frequency (%)
1	21	14	66.7
2	19	12	63.2
3	22	12	54.6
4	21	13	61.9
5	20	14	70.0
6	22	14	63.6
Total	125	75	60.0

[a] Each experiment consisted of 60 explants. However, the frequency of regeneration of transformants was calculated based on the analysis of the regenerated whole plants.

amplicons and probed with the purified plasmid fragment from the plasmids pRT99GUS and pHS723:Bt. The amplicons showed positive in Southern hybridization. The seeds from T0 generation were advanced to T1 generation and the plants were analyzed for their segregation pattern. PCR analysis of the T1 progeny plants showed that the introduced gene (*cry1Ab*) was inherited in the self-pollinated T1 generation and segregated according to 3:1 Mendelian inheritance pattern (Table 4). Southern blot analysis was carried out for the genomic DNA from the T1 generation transgenic plants in order to determine the integration pattern and the copy number. PCR amplicon of *cry1Ab* fragment was used as probe for the Southern hybridization analysis. Hybridization signals were observed in eight of the 12 plants analyzed for the integration of *Bt* gene in the genome of these plants. These samples were taken from six transgenic plants, four of which showed single-copy integrations. There was no signal detected in untransformed plants

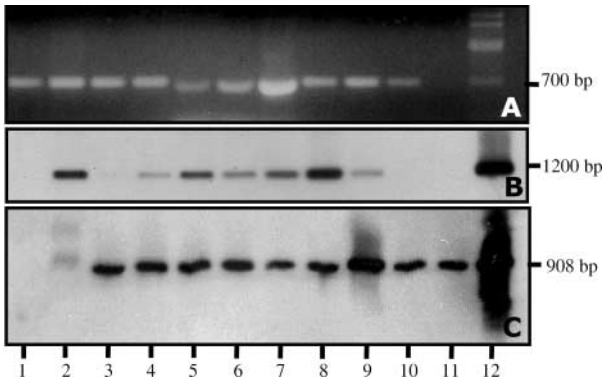

FIG. 4. Molecular analysis of putative transgenics of pigeonpea transformed with pHS723:Cry1Ab plasmid. *A*, PCR amplification of the genomic DNA showing the amplified product of 700 bp fragment of the *npt* II gene from T0 plants. *Lanes 1–6* and *8–10*, putative transgenic plants; *lane 7*, binary plasmid pHS 723:Cry1Ab; *lane 11*, untransformed plant; *lane 12*, λ DNA restricted with *Bst*E II. *B*, PCR amplification of the genomic DNA showing the amplified product of 1200 bp fragment of *uid*A gene from T0 plants. *Lane 1*, untransformed plant; *lanes 2–11*, T0 transgenic plants; *lane 12*, pHS723:Cry1Ab. *C*, PCR amplification of the genomic DNA showing the product of 908 bp fragment of *cry1Ab* gene from T0 generation plants. *Lane 1*, untransformed plant; *lanes 2–11*, putative transgenic plants; *lane 11*, pHS723:Cry1Ab (the PCR products in *B* and *C* were resolved on 1% agarose gels and transferred to nylon membranes prior to probing the *uid*A or *cry1Ab* from pHS723:Cry1Ab labeled with AlkPhos Direct® kit).

TABLE 4

TRANSGENE INHERITANCE IN THE PROGENY OF FIVE PRIMARY TRANSGENIC EVENTS BASED ON PCR AMPLIFICATION OF THE *cry1Ab* GENE IN TRANSGENIC PIGEONPEA PLANTS

T0 event	No. of T1 plants tested	No. of progenies containing *cry1Ab* gene[a]	Chi-square segregation ratio[b]
PPBt-04	20	13	1.07
PPBt-07	20	15	0.10
PPBt-11	20	14	0.27
PPBt-23	20	12	2.40
PPBt-27	20	17	1.07

[a] *cry1Ab* genes in the progeny of transgenic pigeonpea plants were analyzed by PCR using the specific primer as mentioned in *Materials and Methods*.

[b] Tabulated chi-square value at 5% probability for 1 degree of freedom is 3.841, that is less than the calculated chi-square value at the same degree of freedom indicating a Mendelian ratio of 3:1 segregation.

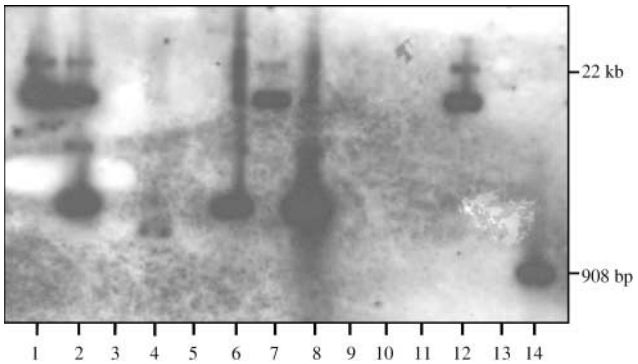

FIG. 5. Southern blot hybridization of *cry1Ab* gene in T1 generation. The plants used include those from six independent events. Genomic DNA isolated from individual putative transformants was digested with *Bam* HI, which cuts once within the T-DNA region. The blot was probed with a non-radioactive Alkphos Direct®-labeled 908 bp PCR amplified *cry1Ab* gene fragment. *Lanes 1*, *7*, *11*, and *12*, progenies from the same transgenic event showing three positive and one negative gene integrations; *lane 13*, untransformed plant; *lane 14*, 908 bp PCR amplified *cry1Ab* fragment.

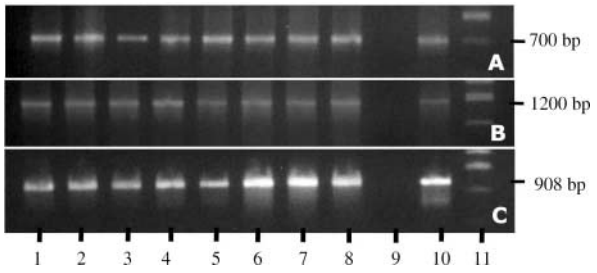

FIG. 6. RT-PCR analysis of transgenic plants for the transgenes. RT-PCR amplification of *npt* II (*A*), *uid*A (*B*), and *cry1Ab* (*C*). mRNA from randomly selected positive transgenic events were used. *Lanes 1–8*, T2 generation of transgenic pigeonpea; *lane 9*, untransformed plant; *lane 10*, pHS723:Cry1Ab; *lane 11*, lambda-DNA restricted with *Bst* EII.

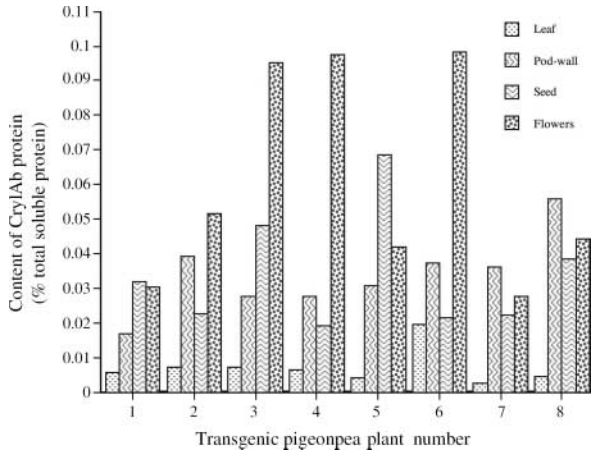

FIG. 7. Analysis of transgenic pigeonpea T2 generation for the Cry1Ab protein by ELISA. The protein from randomly selected positive transgenic events was used. The values are means of the three replicates. The protein content was expressed as percent of total soluble protein per unit fresh weight of the respective tissue.

(Fig. 5). RT-PCR results indicate expression of transgenes and there was no sign of gene silencing (Fig. 6*A–C*). Stable expression of Cry1Ab protein was observed even in the T2 generation using ELISA (Fig. 7). Since the *cry1Ab* gene was expressed under the constitutive CaMV 35S promoter, it is no surprise that the Cry1Ab protein was expressed in all parts of the transgenic plants. However, the level of Cry1Ab protein varied amongst the tissues, with flowers showing the highest Cry1Ab (~0.1% of the total soluble protein), while *cry1Ab* expression was lower in leaves (Fig. 7).

DISCUSSION

For the successful development of transgenic plants, an effective regeneration and transformation system is imperative. In pigeonpea, diverse tissues that have been used to obtain shoot regeneration include leaves (Eapen and George, 1993; Eapen et al., 1998; Geetha et al., 1998; Singh et al., 2002; Dayal et al., 2003), cotyledonary node (Shiva Prakash et al., 1994; Franklin et al., 1998; Geetha et al., 1998; Mohan and Krishnamurthy, 1998; Singh et al., 2002), epicotyl (George and Eapen, 1994), and shoot apices (Geetha et al., 1999). Different authors working on the same explant types have reported induction of shoot buds as well as embryos on the similar media. In some cases, it is difficult to distinguish between shoot meristem-like structures and embryo-like structure (Chandra et al., 2003). While some of these systems resulted in the regeneration of shoot buds with high frequencies (Dayal et al., 2003), others have reported mixed success rates with regards to regeneration of shoots and whole plants.

In the present study, we report an efficient protocol by using the axillary bud region of the seedlings, which can be induced to differentiate into adventitious shoots that can be used for efficient production of transgenic pigeonpea plants. The method involves suppressing growth of the axillary bud and the primary shoot bud while inducing multiple adventitious shoot buds in the axillary regions of the seedlings. Although similar type of regeneration has been observed earlier from cotyledon explants of pigeonpea (Shiva Prakash et al., 1994), the shoot meristems were thought to differentiate directly without the formation of the intervening primary shoot-like structures, where the axillary shoots and possibly the adventitious shoots were not separated and were mixed. In this study, we were able to isolate the tissue surrounding the axillary bud, which could be isolated and induced to differentiate into multiple adventitious shoot buds. The buds could develop into normal shoots and rooted, followed by successful transplantation. The protocol described here has also been tested for other genotypes of pigeonpea and is genotype-independent for regeneration and transformation (unpublished results).

In recent years, results of several studies on pigeonpea transformation have indicated the feasibility of producing transgenics using either *A. tumefaciens* or biolistics-mediated gene transfer (Geetha et al., 1999; Lawrence and Koundal, 2001; Dayal et al., 2003; Thu et al., 2003). However, the transformation frequencies have been variable, ranging from <1 to $>50\%$. By using the leaf explants, we earlier reported high transformation frequencies using biolistics (Dayal et al., 2003) and the shoot regeneration method is also amenable to *Agrobacterium*-mediated transformation (Dayal et al., unpublished results). Pigeonpea transformation using the method similar to that described in this study has also been reported previously (Thu et al., 2003) but the shoots were obtained through a callus from cotyledonary node region and only about 25% of the shoots were able to root. Because of difficulties in rooting and transplantation of the putative transformants, only 6.5% of transformants could be established in soil, from which less than 30% produced seeds. More recently, the shoot apices from seedlings germinated for 16 h were used to introduce marker genes *hpt* and *uid*A to obtain PCR-positive primary transformants (Singh et al., 2004). However, in this study, 60% of the independently transformed plants showed positive gene integration and expression and 65% of the transformants showed single copy inserts of the introduced gene. Over 95% of the putatively transformed shoots could be rooted and readily transplanted to soil with 100% success rate (data not shown).

Our study also shows stable transgene integration and inheritance in 75 independent transgenic plants up to the T3. Stable inheritance and expression of the introduced *cry1Ab* gene (Wu et al., 2002), and protease inhibitor-II (Duan et al., 1996) in transgenic rice have been demonstrated under field conditions. Stable expression of

foreign genes in transgenic plants has also been observed in alfalfa (Micallef et al., 1995) and in maize (Fearing et al., 1997). The transgenic pigeonpea plants described in this work showed the integration and inheritance of *cry1Ab* gene in subsequent selfed generations as verified by PCR, Southern analysis, and RT-PCR. These transgenes displayed the Mendelian inheritance pattern of 3:1 segregation ratio. The Cry1Ab protein was expressed in all plant parts, including leaf, pod-wall, seed, and flowers, as shown from ELISA results. However, the content of Cry1Ab varied amongst the plant tissues. While the highest level of Cry1Ab protein (~0.1% of the total soluble protein) was observed in flowers, ~0.07% was detected in seeds and very low in pod-walls. An almost negligible amount of the Cry1Ab protein was detected in leaves of these plants. This pattern of Cry1Ab expression may be useful for insect control because pod borers infest initially on the reproductive parts like flowers, followed by pods and seeds. Nevertheless, similar spatial variation in transgene expression has also been reported in rice (Wu et al., 2002). In cotton, plants transformed with *cry1Ac* under the control of 35S promoter containing the small subunit leader region and transit peptide from *Arabidopsis thaliana* resulted in the accumulation of Cry1Ac protein up to nearly 1% of the total soluble protein (Wong et al., 1992). On the other hand, transgenic tobacco expressing full-length or truncated *cry1Ac* under the control of 35S promoter showed low levels of Cry1Ac protein accumulation (Barton et al., 1987; Fischhoff et al., 1987; Vaeck et al., 1987). Fujimoto et al. (1993) reported that an accumulation of equal to 0.05% toxin of the total soluble leaf protein conferred transgenic rice resistance to leaf folder (*Cnaphalocrosis medinalis*) and yellow stem borer (*Chilo suppressalis*).

The transgenic plants expressing insecticidal crystal proteins (ICP) are a powerful tool in an integrated pest management program. The susceptibility of *H. armigera* to various *cry* genes including the *cry1Ab* have been reviewed earlier (Sharma et al., 2004). In this study, the level of Cry1Ab protein in different parts of the transgenic plants varies from 0.02 to 0.1% of the total soluble protein. Since the LD_{50} (50% insect ICP concentration) for lepidopteran pests is 25–40 ng g^{-1} soluble protein (Vaeck et al., 1987), it is possible that the level of Cry1Ab protein in transgenic pigeonpea is sufficient against the pod borer. Nevertheless, the efficiency of transgenic pigeonpea produced in this study against *H. armigera* is currently being studied.

In conclusion, the protocol for pigeonpea transformation reported here is highly efficient. A large amount of meristematic tissue capable of shoot regeneration can be obtained and is highly amenable for genetic transformation. The method is genotype independent and the whole plants can be obtained within 120 d. In addition, a large number of independently transformed plants carrying the *cry1Ab* gene can be generated and assayed for insect resistance. This protocol provides an additional tool for genetic improvement of pigeonpea, an important legume crop of the semi-arid tropics.

Acknowledgments

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References

Barton, K. A.; Whitely, H.; Yanz, N. S. *Bacillus thuringiensis* δ-endotoxin in transgenic *Nicotiana tabacum* provides resistance to lepidopteran insects. Plant Physiol. 85:1103–1109; 1987.

Carrington, J. C.; Freed, D. D. Cap-independent enhancement of translation by a plant potyvirus 5′ nontranslated region. J. Virol. 64:1590–1597; 1990.

Chandra, A.; Gupta, V.; Burma, P.; Pental, D. Patterns of morphogenesis from cotyledon explants of pigeonpea. In Vitro Cell. Dev. Biol. Plant 39:514–519; 2003.

Dayal, S.; Lavanya, M.; Devi, P.; Sharma, K. K. An efficient protocol for shoot regeneration and genetic transformation of pigeonpea (*Cajanus cajan* (L.) Millsp.) by using leaf explants. Plant Cell Rep. 21:1072–1079; 2003.

Duan, X.; Li, X.; Xue, Q.; Abo-El-Saad, M.; Xu, D.; Wu, R. Transgenic rice plants harbouring an introduced potato proteinase inhibitor II gene are insect resistant. Nat. Biotechnol. 14:494–498; 1996.

Dunwell, J. M. Transgenic approaches to crop improvement. J. Exp. Bot. 51:487–496; 2000.

Eapen, S.; George, L. Plant regeneration from leaf discs of peanut and pigeonpea: influence of benzyladenine, indoleacetic acid and indoleacetic acid-amino acid conjugates. Plant Cell Tiss. Organ Cult. 35:223–227; 1993.

Eapen, S.; Tivarkar, S.; George, L. Thidiazuron-induced shoot regeneration in pigeonpea (*Cajanus cajan* L.). Plant Cell Tiss. Organ Cult. 53:217–220; 1998.

Fearing, P. L.; Brown, D.; Vlachos, D.; Meghji, M.; Privalle, L. Quantitative analysis of Cry1A(b) protein expression in Bt maize plants, tissues, and silage and stability of expression over successive generation. Mol. Breed. 3:169–176; 1997.

Fischhoff, D. A.; Bowdish, K. S.; Perlak, F. J.; Marrone, P. G.; McCormick, S. M.; Niedermeyer, J. G.; Dean, D. A.; Kusano-Kretzmer, K.; Mayer, E. J.; Rochester, D. E.; Rogers, S. G.; Fraley, R. T. Insect tolerant tomato plants. Bio/Technology 5:807–813; 1987.

Franklin, G.; Jeyachandran, R.; Melchias, G.; Ignacimuthu, S. Multiple shoot induction and regeneration of pigeonpea (*Cajanus cajan* (L.) Millsp.) cv. Vamban 1 from apical and axillary meristem. Curr. Sci. 74:936–937; 1998.

Fujimoto, H.; Itoh, K.; Yamomoto, Y.; Kyozuka, J.; Shimamoto, K. Insect resistant rice generated by introduction of a modified δ-endotoxin gene of *Bacillus thuringiensis*. Bio/Technology 11:1151–1155; 1993.

Gamborg, O. L.; Miller, R. A.; Ojima, K. Nutrient requirements for suspension cultures of soybean root cells. Exp. Cell. Res. 50:151–158; 1968.

Geetha, N.; Venkatachalam, P.; LakshmiSita, G. *Agrobacterium*-mediated genetic transformation of pigeonpea (*Cajanus cajan* L.) and development of transgenic plants via direct organogenesis. Plant Biotechnol. 16:213–218; 1999.

Geetha, N.; Venkatachalam, P.; Prakash, V.; LakshmiSita, G. High frequency induction of multiple shoots and plants regeneration from seedling explants of pigeonpea (*Cajanus cajan* L.). Curr. Sci. 17:1036–1041; 1998.

George, L.; Eapen, S. Organogenesis and embryogenesis from diverse explants in pigeonpea. Plant Cell Rep. 13:417–420; 1994.

Hamil, J. D.; Rounsley, S.; Spencer, A.; Todd, G.; Rhodes, M. J. C. The use of polymerase chain reaction in plant transformation studies. Plant Cell Rep. 10:221–224; 1991.

Jefferson, R. A. Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol. Biol. Rep. 5:387–405; 1987.

Lawrence, P. K.; Koundal, K. R. *Agrobacterium tumefaciens*-mediated transformation of pigeonpea (*Cajanus cajan* L. Millsp.) and molecular analysis of regenerated plants. Curr. Sci. 80:1428–1432; 2001.

Micallef, M. C.; Austin, S.; Bingham, E. T. Improvement of transgenic alfalfa by backcrossing. In Vitro Cell. Dev. Biol. Plant 31:187–192; 1995.

Mohan, M. L.; Krishnamurthy, K. V. Plant regeneration in pigeonpea (*Cajanus cajan* (L.) Millsp.) by organogenesis. Plant Cell Rep. 17:705–710; 1998.

Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant. 15:473–497; 1962.

Nene, Y. L.; Sheila, V. K. Pigeonpea: geography and importance. In: Nene, Y. L.; Hall, S. D.; Sheila, V. K., eds. The pigeonpea. Wallingford, UK: CAB International; 1990:1–14.

Phillips, G. C.; Collins, G. B. *In vitro* tissue culture of selected legumes and plant regeneration from callus cultures of red clover. Crop Sci. 19:59–64; 1979.

Porebski, S.; Grant, L.; Bailey, L.; Baum, B. R. Modification of CTAB DNA extraction protocol for plants containing high polysaccharides and polyphenol components. Plant Mol. Biol. Rep. 15:8–15; 1997.

Sambrook, J.; Fritsch, E. F.; Maniatis, T. Molecular cloning: a laboratory manual. New York: Cold Spring Harbor; 1989.

Shanower, T. G.; Romeis, J.; Minja, E. M. Insect pests of pigeonpea and their management. Annu. Rev. Entomol. 44:77–96; 1999.

Sharma, H. C.; Sharma, K. K.; Crouch, J. H. Genetic transformation of crops for insect resistance: potential and limitations. Crit. Rev. Plant Sci. 23:47–72; 2004.

Shiva Prakash, N.; Pental, D.; Bhalla-Sarin, N. Regeneration of pigeonpea (*Cajanus cajan*) from cotyledonary node via multiple shoots formation. Plant Cell Rep. 13:623–627; 1994.

Singh, N. D.; Sahoo, L.; Saini, R.; Sarin, N. B.; Jaiwal, P. K. *In vitro* regeneration and recovery of primary transformants from shoot apices of pigeonpea using *Agrobacterium tumefaciens*. Physiol. Mol. Biol. Plants 10:65–74; 2004.

Singh, N. D.; Sahoo, L.; Sonia; Jaiwal, P. K. *In vitro* shoot organogenesis and plant regeneration from cotyledonary node and leaf explants of pigeonpea (*Cajanus cajan* L. Millsp.). Physiol. Mol. Biol. Plants 8:133–140; 2002.

Thu, T. T.; Mai, T. T. X.; Dewaele, E.; Farsi, S.; Tadesse, Y.; Angenon, G.; Jacobs, M. *In vitro* regeneration and transformation of pigeonpea [*Cajanus cajan* (L.) Millsp]. Mol. Breed. 11:159–168; 2003.

Topfer, R.; Schell, J.; Steinbiss, H. H. Versatile cloning vectors for transient gene expression and direct gene transfer in plant cells. Nucleic Acids Res. 16:8725; 1988.

Vaeck, M.; Reynaerts, A.; Hoftey, H.; Jansens, S.; DeBeuckleer, M.; Dean, C.; Zabeau, M.; Van Montagu, M.; Leemans, J. Transgenic plants protected from insect attack. Nature 327:33–37; 1987.

Wong, E. Y.; Hironaka, C. M.; Fischhoff, D. A. *Arabidopsis thaliana* small subunit leader and transit peptide enhance expression of *Bacillus thuringiensis* proteins in transgenic plants. Plant Mol. Biol. 20:81–93; 1992.

Wu, G.; Cui, H.; Ye, G.; Xia, Y.; Sardana, R.; Cheng, X.; Li, Y.; Altosaar, I.; Shu, Q. Inheritance and expression of the *cry1Ab* gene in Bt (*Bacillus thuringiensis*) transgenic rice. Theor. Appl. Genet. 104:727–734; 2002.