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Agrobacterium tumefaciens Mediated Genetic Transformation of Pigeonpea [*Cajanus cajan* (L.) Millsp.]

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Abstract

Optimal protocol for efficient genetic transformation has been defined to aid future strategies of genetic engineering in pigeonpea with agronomically important genes. Transgenic pigeonpea plants were successfully produced through *Agrobacterium tumefaciens*-mediated genetic transformation method using cotyledonary node explants by employing defined culture media. The explants were co-cultivated with *A. tumefaciens* strain C-58 harboring the binary plasmid, pCAMBIA-1301 [conferring β-glucuronidase (GUS) activity and resistance to hygromycin] and cultured on selection medium (regeneration medium supplemented with hygromycin) to select putatively transformed shoots. The shoots were then rooted on root induction medium and transferred to pots containing sand and soil mixture in the ratio of 1:1. About 22 putative T0 transgenic plants have been produced. Stable expression and integration of the transgenes in the putative transgenics were confirmed by GUS assay, PCR and Southern blot hybridization with a transformation efficiency of over 45%. Stable integration and expression of the marker gene has been confirmed in the T0 and T1 transgenics through PCR, and Southern hybridization.

Key words: *Agrobacterium tumefaciens*, genetic transformation, pigeonpea

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Introduction

Pigeonpea [*Cajanus cajan* (L.) Millsp.] is the second most important food legume of India, valued as food and fodder and possesses the capacity for biological nitrogen fixation in symbiosis with *Rhizobium* sp. Pigeonpea is a good source of dietary protein in the tropics and subtropics. The constraints for enhancing the productivity include the damages caused by various fungi, bacteria, virus and insect pests. Genetic improvement of pigeonpea has been restricted due to the non-availability of better genetic resources, presence of strong sexual barriers and incompatibilities among wild relatives. Genetic engineering technology can therefore be used as an additional tool for introduction of agronomically useful traits into established cultivars.

Crop improvement through recombinant DNA technology is possible only after developing efficient plant regeneration and transformation systems. For successful development of transgenic plants, identification of suitable target tissue and efficient gene transfer protocols are essential (Taylor and Vasil 1991). Whereas, regeneration of plantlets from callus cultures is a time consuming and labor intensive task, the direct regeneration of multiple-shoots can simply be obtained from explants taken from *in vitro* germinated seedlings and uniform explant sources can be obtained at any time of the year with quick high efficiency rooting and plant regeneration (Devi et al. 2000). Though considerable work has been done in regard to the genetic transformation of legumes, the common approach for genetic transformation was through *Agrobacterium tumefaciens* to produce transgenics such as soybean (Hinchee et al. 1988), chickpea (Kar et al. 1996), Peas (Pounti-Kearlas et al. 1992; Davies et al. 1993; Grant et al. 1995) and

groundnut (Eapen and George, 1994, Sharma and Anjaiah 2000). To date, there are three transformation reports in pigeonpea (Geetha et al. 1999; Lawrence and Koundal 2001; Dayal et al. 2003), however, with varying transformation efficiency. Therefore, in the present study, standardization of regeneration and transformation protocol was given prior importance to produce transgenic pigeonpea plants at a larger scale.

With a long-term plan to develop transgenic pigeonpea with resistance to fungal disease, a protocol for efficient *in vitro* plantlet regeneration from cotyledonary nodes has been developed. Coty ledonary nodes were used as target tissue for *Agrobacterium* mediated genetic transformation to develop transgenic plants. As a first step towards the development of an efficient transformation system, we transferred a marker plasmid to cotyledonary nodes of pigeonpea. The report describes a rapid, reliable method for the co-cultivation and genetic transformation of pigeonpea. These defined conditions will aid future strategies of genetic engineering in pigeonpea with agronomically important genes.

Materials and Methods

Plant materials

Seeds of pigeonpea [*Cajanus cajan* (L.) Millsp.] cv. LRG-30 obtained from LAM Agricultural Farm, Guntur, India, were surface-sterilized in 70% ethanol for 5 min, washed in sterile distilled water and then soaked in 0.1% (w/v) mercuric chloride containing 2 drops of Tween-20 for 10 min with intermittent shaking. Surface-sterilized seeds were washed thrice with sterile distilled water and soaked in distilled water for 6 h. The seed-coats of the pre-soaked seeds were removed aseptically and germinated on Murashige and Skoog's (MS) basal medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose and 0.8% (w/v) Phytagar, and maintained with 16 h photoperiod at 25 ± 2℃ with a light intensity of 60 μE m^{-2} S^{-1}.

Explants and culture conditions

Cotyledonary nodes of 7-8 mm in length excised from 12-day-old aseptically germinated seedlings were used as explants for co-cultivation with *Agrobacterium tumefaciens.* The explants were cultured on shoot initiation medium (SIM) comprising the MS medium supplemented with 6-benzyladenine (BA) (2.0 mg L^{-1}) for induction of shoot-buds. They were transferred after 2 weeks to shoot elongation medium (SEM) comprising the MS medium supplemented with 0.5 mg L^{-1} gibberellic acid-A (GA_3). The elongated shoots (longer than 3 cm) were then transferred to magenta bottles containing the root induction medium (RIM) [comprising the MS medium supplemented with 1.0 mg L^{-1} indole butyric acid (IBA)]. The culture conditions employed were the same as described above. Rooted plants were transferred to pots containing a 1:1 mixture of sand and soil and acclimatized for week (by covering with a plastic bag initially and gradually exposing the plant to the open environment) prior to the transfer to glasshouse.

Agrobacterium strain and plasmid vector

The disarmed *Agrobacterium tumefaciens* strain C58 harboring a binary plasmid pCAMBIA-1301 was used as vector system for transformation. The plasmid contained the reporter gene, *uidA* gene (GUS) (Jefferson, 1987) from *Escherichia coli* with an intron, driven by the Cauliflower mosaic virus (CaMV) 35 S promoter and *nos* poly-A terminator sequences and the hygromycin phosphotransferase (*hpt*) gene (used as a selectable marker) under the control of CaMV 35 S promoter and CaMV 35 S poly-A terminater (Figure 1). Bacteria were maintained on LB (Sambrook et al. 1989) agar plates (1% w/v tryptone, 0.5% w/v yeast extract and 1% w/v sodium chloride, pH 7.0) containing 50mg L^{-1} kanamycin sulfate.

Co-cultivation and transformation

A single bacterial colony was inoculated into 25 mL of

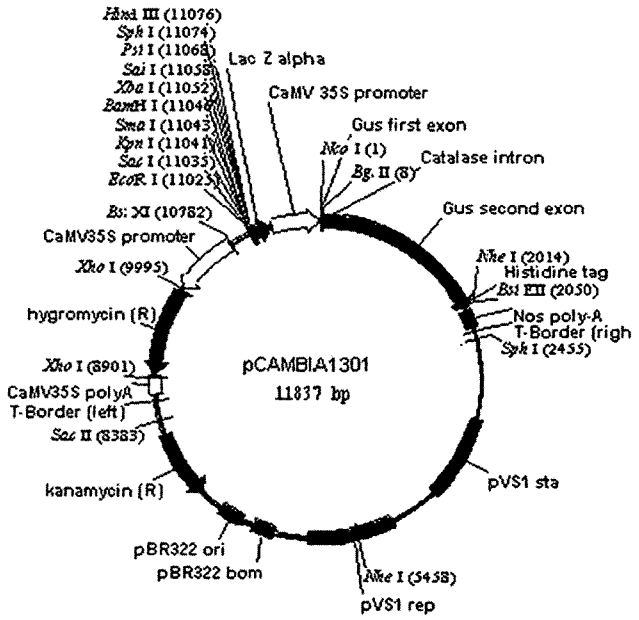

Figure 1. Restriction map of pCAMBIA-1301.

liquid LB medium containing 50 mg L^{-1} kanamycin sulfate and incubated at 28℃ on a shaker at 100 rpm for 16-18 h and used in the late log phase A_{600} at 0.6. The bacterial culture was centrifuged at 5,000 rpm and half MS liquid medium added to the bacterial pellet to makeup a volume of 25 ml. Freshly cut explants were dipped into this suspension, blotted on sterile filter paper and transferred to SIM. Twenty explants were co-cultivated and cultured per-petriplate and a total of 200 explants were used with three replicates. The co-cultivated explants were then transferred after 48 h to SIM-Cef medium comprising the SIM, supplemented with 200 mg L^{-1} cefotaxime to eliminate the bacteria.

Selection and plant regeneration

To identify the toxic concentration of hygromycin for effective selection of putatively transgenic plants, the control explants were cultured on SIM with different concentrations of hygromycin (0.5-10 mg L^{-1}). At 4 mg L^{-1} and above, the explants turned brown and did not show further growth (data not shown). Hence, 5 mg L^{-1} was used as selection pressure for the culture of co-cultivated explants. The explants cultured on SIM-Cef for 1 week were transferred to the selection medium, SIM-Sel-1 (comprising the SIM supplemented with 2 mg L-1 hygromycin and 200 mg L^{-1} cefotaxime) and later transferred to SIM-Sel-2 medium (after 2 wk), comprising SIM supplemented with 5 mg L^{-1} hygromycin. The cultures were maintained in stringent selection on SIM-Sel-2 for 3 weeks by which time the regenerated putatively transgenic shoots would have grown considerably. The shoots were then transferred to RIM for rooting and subsequently transferred to pots and moved to the glasshouse after acclimatization (as described previously). Control explants (not co-cultivated) were cultured simultaneously to regenerate untransformed control plants. The T0 plants were grown to maturity and seeds harvested to raise the T1 generation. The T0 and T1 transgenics were subjected to molecular genetic analysis

Histochemical GUS assay of the co-cultivated explants

Histochemical GUS assay was carried out on the co-cultivated explants with regenerated putatively transformed shoots, 4 weeks after co-cultivation by a modified method of Jefferson et al. (1987). The modified histochemical assay buffer consisted of 100 mM $NaPO_4$ buffer, 100 mM Na_2 EDTA, 50 mM K4Fe $(CN)_6$. 3 H_2O and 0.1% Triton X-100 (pH-7.0). 5-Bromo-4-chloro-3-indolyl-β-D-glucuronic acid (Clontech Laboratories, Palo Alto, CA, USA) was dissolved in 50% (v/v) ethanol, stored at -20℃ and added to the buffer to a final concentration of 0.5 mg mL^{-1} prior to the assay.

To rule out the possibility of *Agrobacterium* contamination, the putatively transformed material (2 weeks after co-cultivation) were cultured on antibiotic-free medium for 1 week before the analysis for GUS activity only after ascertaining that the *Agrobacterium* did not appear on the culture medium. The putatively transformed material and the controls were then subjected to histochemical GUS assay by scoring one petri plate (containing 20 explants) from each replicate (out of a total of three replicates). For the GUS assay, the material was immersed in the GUS substrate mixture immediately followed by vacuum treatment for 10 m, and then incubated at 37℃. The histochemical localization of GUS activity was examined under a Zeiss SV8 stereomicroscope. Chlorophyll was extracted from the material by successive incubation in 70% (v/v) ethanol for 2 h and 100% ethanol overnight to facilitate better examination. The data from the experiments were evaluated as number of GUS positive explants (explants having atleast one blue spot) in co-cultivated explants per replicate.

Molecular analysis of putative transformants

Molecular studies were carried out to confirm the integration of the foreign genes in the putatively transgenic plants. Genomic DNA was isolated from the control and the putatively transgenic plants by a modified method of Rogers and Bendich (1988).

PCR analysis

Polymerase chain reaction (PCR) analysis was carried out on the T0 putative transgenics for amplification of the coding regions of *uidA* and *hpt* genes. PCR was also carried out on the T1 transgenics for the amplification of *uidA* gene. One μg of RNase treated DNA was used as template for PCR amplification. Each PCR reaction was performed in 25 μL (final volume) of reaction mixture consisting of 2.5 μL 10X PCR amplification buffer, 2 μL of template DNA, 0.5 μL 10 mM dNTPs, 0.75 μL 50 mM $MgCl_2$, 100 ng (0.5 μL) of each primer, 10.5 μL sterile distilled water, 7.5 μL enhancer (Invitrogen) and 1 unit (0.25 μL) of Platinum Taq DNA polymerase (Invitrogen). The following primers were used to amplify 1.2 kb fragment of the *uidA* gene: forward primer: 5'-GGT GGG AAA GCG CGT TAC AAG-3' ; reverse primer: 5'-GTT TAC GCG TTG CTT CCG CCA-3'. The samples were heated to 94℃ for 4 min and then subjected to 34 cycles of 1 min melting at 93℃, 45 sec annealing at 59℃ and 90 sec synthesis at 72℃ followed by another 5 min final extension at 72℃. The following primers were used to amplify the 819 bp fragment

of the *hpt* gene: forward primer: 5'-CGT TAT GTT TAT CGG CAC TTTG-3'; reverse primer: 5'-GGG GCG TCG GTT TCC ACT ATCG-3'. The samples were heated to 94℃ for 4 min and then subjected to 34 cycles of 1 min at 93℃, 1 min at 58.5℃ and 90 sec at 72℃ followed by another 5 min final extension at 72℃. The amplified products were assayed by electrophoresis on 1.2% agarose gels, stained with ethidium bromide, visualized and photographed under ultraviolet light.

Southern blot hybridization analysis

Well-established T0 transformants were subjected to Southern blot hybridization analysis. Ten μg of genomic DNA from the putatively transformed and untransformed control plants was digested with *Xba* I, which recognizes a unique site within the pCAMBIA 1301 plasmid DNA. The digested DNA was separated by electrophoresis through a 0.8% agarose gel and transferred onto Nylon N+ membrane (Amersham) according to the manufacturer's instructions. The blot was probed with a non-radioactively labeled (Alkphos Direct Labeling and Detection system of Amersham Biosciences, Uppsala, Sweden) 819 bp PCR-amplified *hpt* gene fragment. For positive control, the plasmid pCAMBIA-1301 was restricted with *Xho-1* to release the *hpt* gene. The blot was exposed to X-Omat film (Kodak) for 15 m for autoradiography.

Results

Tissue culture

The regeneration system from cotyledonary node explant developed was rapid, reliable, reproducible and efficient. Moreover, it is capable of producing plantlets independently through organogenesis and development of multiple shoots without any callus phase. The survival rate of the *in vitro* regenerated plantlets was over 70%.

GUS assay of transformants

The *Agrobacterium tumefaciens* co-cultivated explants were cultured on selection medium (containing hygromycin) to recover and regenerate transgenic plants. However, transformation of the plasmid pCAMBIA-1301 to pigeonpea using cotyledonary node explants was also confirmed by the GUS assay. The transformation efficiency (through GUS assay) was 60% (Table 1). The histochemical evaluation revealed intense blue sectors in the explants after co-cultivation with *Agrobacterium*. No blue cells were observed after histochemical staining of untransformed shoots (control).

The shoots regenerated from co-cultivated cotyledonary explants on hygromycin selection media were rooted on RIM. The putative T0 transformants were transplanted to pots and moved to the glasshouse (Table 2). A total of 22 putative T0 transformants were established in the glasshouse and all the plants flowered and set seed normally. The T0 plants were grown to maturity and seeds harvested to raise the T1 generation. The T0 and T1 transgenics were subjected to molecular genetic analysis.

Molecular characterization of transgenic plants

Molecular analysis of the putative T0 transformants produced from co-cultivated cotyledonary node explants was carried out by PCR and Southern blot hybridization. The

Table 1. GUS assay of putatively transformed explants and transformation frequency.

Experiment No.	No. of co-cultivated cotyledonary node explants	No. of cotyledonary node explants with blue spots	TransformationFrequency (%)
1	20	11	55
2	20	13	65
3	20	12	60
Total	60	36	60

Table 2. Putatively transgenic (T0) plants produced on hygromycin selection.

Experiment No.	No. of co-cultivated cotyledonary node explants	No. of explants after 4 weeks on hygromycin selection	No. of rooted plants	No of transplanted plants
1	200	43	28	6
2	200	58	32	9
3	200	36	23	7
Total	600	137	83	22

PCR analysis carried out for amplification of coding regions of *uidA* and *hpt* genes revealed that more than 45% of the plants of T0 generation expressed the amplification of the expected size of the respective gene. Out of a total of 22 transformants (T0), 10 plants were found to be positive for the amplification of both the genes, *viz.* 1.2 kb fragment of the *uidA* gene (Figure 2A) and 819 bp fragment of the *hpt* gene. (Figure 2B).

Further, the transgene integration pattern in the nuclear genome of the putative transformed T0 plants was confirmed through Southern hybridization of the genomic DNA. Southern hybridization of ten PCR positive T0 transformants was carried out. The *hpt* gene hybridization signals were found in seven plants, of which, single copy of the gene was observed in three plants (P-8, P-18 and P-21), two copies in two plants (P-9 and P-22), and three copies in two plants

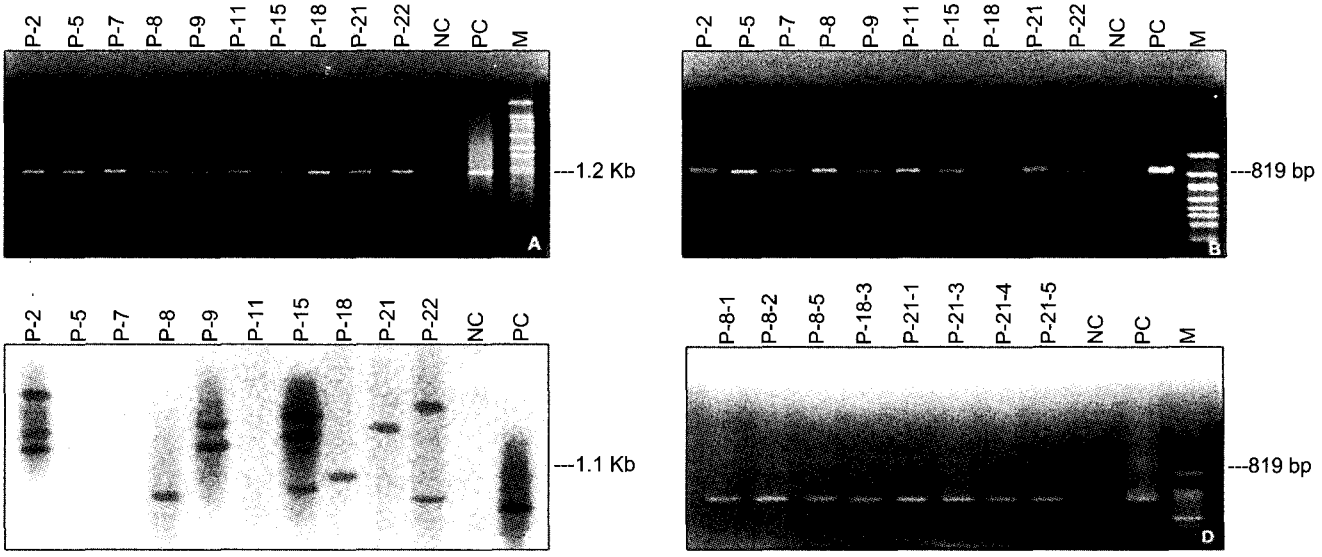

Figure 2. PCR and Southern blotting of genomic DNA of the T0 and T1 transformants obtained via *Agrobacterium*-mediated transformation of the cotyledonary node explant using the plasmid pCAMBIA-1301.

A. PCR amplification of the genomic DNA of T0 transformants (P-2, P-5, P-7, P-8, P-9, P-11, P-15, P-18, P-21 and P-22) showing amplification of the 1.2 Kb fragment of *uid*A gene after *Agrobacterium*-mediated gene transfer using plasmid pCAMBIA-1301. NC (negative control) represents the genomic DNA from un-transformed plant; PC (positive control) represents the plasmid pCAMBIA-1301; M represents the DNA size marker (λ Bst EII Marker).

B. PCR amplification of the genomic DNA of T0 transformants (P-2, P-5, P-7, P-8, P-9, P-11, P-15, P-18, P-21 and P-22) showing amplification of the 819 bp fragment of *hpt* gene after *Agrobacterium*-mediated gene transfer using plasmid pCAMBIA-1301. NC (negative control) represents the genomic DNA from un-transformed plant; PC (positive control) represents the plasmid pCAMBIA-1301; M represents the DNA size marker (50 bp Marker).

C. Southern blot hybridization of *hpt* gene in the genomic DNA from putative transformants of T0 generation (P-2, P-5, P-7, P-8, P-9, P-11, P-15, P-18, P-21 and P-22). The plant genomic DNA was digested with *Xba*I to provide a single cut within the plasmid DNA. The blot was probed with non-radio Alkphos labeled 819 bp PCR-amplified *hpt* gene fragment. NC (negative control) represents the genomic DNA from un-transformed plant; PC (positive control) represents the plasmid, pCAMBIA-1301 restricted with *Xho-1* to release the *hpt* gene.

D. PCR amplification of the genomic DNA of T1 transformants (P-8-1, P-8-2, P-8-5, P-18-3, P-21-1, P-21-3, P-21-4, P-21-5) showing amplification of the 819 bp fragment of *hpt* gene. NC (negative control) represents the genomic DNA from un-transformed plant; PC (positive control) represents the plasmid pCAMBIA-1301; M represents the DNA size marker (100 bp Marker).

Table 3. Inheritance of *hpt* gene in T1 generation of transgenic pigeonpea.

Plant No.[a]	No. of T1 plants tested	PCR analysis of *hpt* gene		3:1 Segregation	Probability
		Positive	Negative	χ^2	
P-4	5	3	2	0.60	0.43
P-8	5	1	4	8.06	0.0045
P-9	5	4	1	0.066	0.79

[a]P-4, P-8, P-9 are independent T0 plants and seeds of 5 replicates were taken from the above independent transgenic plants for inheritance studies in T1 generation. All the calculated values are lesser than the tabulated value of χ^2 = 3.84 at 1 degree of freedom and 0.05 probability.

(P-2 and P-15). The 1.1 kb signal was observed incase of the positive control (the plasmid pCAMBIA 1301 was restricted with *Xho*I to release *hpt* gene). No hybridization signal was observed in case of the untransformed plant DNA. The transformation efficiency pertaining to PCR was 46%.

Of the 7 independent transformed plants of T0 generation, 3 were advanced to T1 generation. In order to study the Mendelian inheritance pattern in the T1 progeny, 5 seeds from each of the three T0 plants were sown and PCR analysis was carried out on 15 plants for the amplification of the *hpt* gene. Out of the 15 T1 plants, 8 plants were positive for PCR (Table 3). The control plant DNA comprising the DNA from the untransformed plant did not yield any amplification

Discussion

In order to improve various traits of the plant through genetic engineering and transformation, it is necessary to have an efficient plant regeneration system to effect any genetic manipulation. In the present study, an efficient *in vitro* plant regeneration protocol from cotyledonary node explants of pigeonpea has been developed with a survival rate of more than 70%. The novel genetic transformation system for pigeonpea, developed in the present study, uses the cotyledonary node explants for integrative transformation and inherited expression of transgenes. After co-cultivation, the axillary meristematic cells can be multiplied for an indefinite period with or without selection and regenerated to transgenic plants. Although regeneration in pigeonpea was previously reported from cotyledonary node explants (Shiva Prakash et al. 1994) and from different seedling explants (Eapen et al. 1998; Naidu et al. 1995; Geetha et al. 1998; Mohan and Krishnamurthy, 1998) through organogenesis and somatic embryogenesis (Eapen and George 1993; Shiva Prakash et al. 1994; Sreenivasu et al. 1998), the protocols were not favorable for genetic transformation, because of low regeneration frequencies and long time taken for regeneration.

The GUS assay approach was found to be an easy and reliable way of establishing optimal conditions for transformation. Strongest GUS expression was concentrated in one area with unidirectional distribution of the blue precipitate. Therefore, we conclude that transformed nodal meristem cells that produced β-glucuronidase kept their potential to undergo anticlinal divisions. Geetha et al. (1999) also reported high transformation efficiency, using the cotyledonary node explants. Stringent hygromycin selection was applied presently to ensure the recovery of transformants with minimum escapes. While delayed application of the selective agent leads to excessive escapes, (Metz et al. 1995), too early selection pressure adversely affects shoot regeneration (Von Wordragen and Dons 1992). We found a delay period of 1 week is necessary for efficient recovery of transgenics. In spite of the difficulty in carrying out transplantation of the transformants, a total of 22 putative T0 transformants were established in the glasshouse, grown to maturity and the seeds collected to raise the T1 generation. Three T0 plants were advanced to the T1 generation. Fifteen T1 plants were produced from 5 seeds taken from each of the three T0 plants (P-8, P-18 and P-21). Molecular analysis of the T0 and T1 plants was carried out to study the gene segregation pattern.

Molecular analysis established the transgenic status of the T0 and T1 transformants. Our results show that by fine-tuning the conditions of transformation, even a recalcitrant crop like pigeonpea can be transformed with an optimum frequency. Optimal conditions standardized presently for efficient transformation of the cotyledonary nodes include the use of freshly cut explants, co-cultivation duration of 48 h, a delay period of 7 days followed by culture on selection medium and application of stringent selection for three weeks before rooting. Although the frequency of transformation is still low compared to the model species, the protocol is repeatable and can be used to mobilize genes of agronomic importance into elite cultivars of pigeonpea.

Previous reports on *Agrobacterium*-mediated transformation of pigeonpea have utilized shoot apices and cotyledonary nodes to achieve direct regeneration (Geetha et al. 1999) and embryonic axes to achieve indirect regeneration through callus (Lawrence and Koundal 2001). However, in the former report only three plants from T0 generation were reported, while in the latter, of the 898 explants, 213 explants (23.8%) produced callus on selection medium, of which only 11 explants (1.2%) produced 9 shoots and the effective frequency of transformed shoots (T0) was less than 1%. The present report is a significant improvement, with at least 45% of the transgenic plants showing positive gene integration. Further, out of the 15 T1 transformants, 8 plants were positive for PCR indicating the presence of the GUS gene and thereby the inheritance of the gene from T0 to T1 generation. The efficiency of this experiment involving *Agrobacterium*-mediated transformation of the cotyledonary node explant is, however, on par with the efficiency of 50% reported in our previous publication (Dayal et al. 2003) on the microprojectile-mediated transformation of pigeonpea using leaf explants.

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