**Original Paper** Plant Biotechnology, **16** (3), 213-218 (1999)

# *Agrobacterium*-mediated Genetic Transformation of Pigeonpea (*Cajanus cajan* L.) and Development of Transgenic Plants via Direct Organogenesis

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Recieved 25 August 1998; accepted 25 February 1999

**Abstract**

A reproducible system for production of transgenic plants via *Agrobacterium* mediated transformation of pigeonpea (*Cajanus cajan* L.) was developed. Shoot apices and cotyledonary node explants were transformed by cocultivation with *Agrobacterium tumefaciens* strain LBA4404 . The strain harbours a binary vector carrying the reporter gene ( $\beta$-glucuronidase (*uidA*) and the marker gene neomycin phosphotransferase (*nptII*). Cocultivated explants were cultured on shoot regeneration medium with 2mg $l^{-1}$ BAP and kanamycin ( 50 $\mu$g $ml^{-1}$ for selection (MS1). Approximately 45-62% of the explants produced putatively transformed shoots on the selection medium. Multiple shoots were repeatedly selected on 0.5 mg $l^{-1}$ BAP and 25 $\mu$g $ml^{-1}$ kanamycin medium(MS2) The elongated shoots were subsequently rooted on a medium supplemented with 25 $\mu$g $ml^{-1}$ kanamycin sulfate (MS3). The transgenic plants were later established in pots. Although transformation was achieved with both cotyledonary node and shoot apices, cotyledonary nodes responded better with 62% of the explants producing GUS positive shoots after selection on MS2 medium. The presence of *uidA* and *nptII* genes in the transgenic plants was verified by PCR analysis. Integration of T-DNA into the genome of transgenic plants was further confirmed by Southern blot analysis.

## 1. Introduction

The importance of grain legume is multipurpose and their seeds are mostly used to supply vegetative protein for human as well as animal consumption. Red gram or pigeonpea ranks high amongst the grain legumes of India, consumed by large population of the country. Nutritionally, they are two to three times richer in protein than cereal grains. However, the major seed proteins of leguminous crop species are deficient in the essential sulfur containing amino acid methionine. Therefore, the introduction of DNA sequences encoding methionine rich seed protein via gene transfer technologies is an attractive to classical methods for the improvement of protein quality of grain legume [Altenbach and Simpson,1990]. In addition, lepidopteron is one of the most serious and widespread pest of economically important crop plants including pigeonpea. Insect control proteins from a prokaryotic source, *Bacillus thuringiensis* (Bt) are specific for lepidopteran insects and exhibit no activity against humans, other vertebrates and beneficial insects. Attempts to obtain pest resistant genotypes of pigeonpea species by conventional breeding methods have not been successful because of limited genetic variation and incompatibility associated with wild species [Nene *et al.*,1990]. Therefore, genetically engineered resistance has been actively investigated in recent years as an alternative for insect resistance [Nayak *et al.*, 1997]. Genetic improvement through molecular techniques has been considered for a wide range of grain legumes. This has led to the establishment of transformation protocols in grain legumes like soybean [Hinchee *et al.*, 1988; Di *et al.*, 1996 and Trick *et al.*, 1997], chickpea [Kar *et al.*, 1996], peas [Grant *et al.*,1995 and DeKathen and Jacobsen, 1990], cowpea [Muthukumar *et al.*, 1996], peanut [McKently *et al.*, 1995 and Cheng *et al.*, 1997], dry bean [McClean *et al.*, 1991] and lentils [Warkentin and McHughen, 1992] using a naturally occurring gene transfer system involving *Agrobacterium tumefaciens*.

The availability of a genetic transformation system would facilitate the agronomic traits affecting

production efficiency as well as the nutritional quality of red gram [Mehta and Mohan Ram, 1980; Kumar *et al.*, 1983; Prakash *et al.*, 1994; Mohan and Krishnamurthy, 1998 and Eapen and George, 1993]. For successful genetic modification and production of transgenic plants, effective transformation and regeneration systems is imperative. In spite of its economic importance so far there are no reports available on transformation and regeneration of transgenic plants in red gram. There are, however, few reports of regeneration in one or two varieties [Prakash *et al.*, 1994; Mohan and Krishnamurthy, 1998 and Eapen and George, 1993]. Recently we have reported high efficiency regeneration from cotyledonary explants of pigeon pea [Geetha *et al.*,1998]. This paper describes the development of transgenic plants via *Agrobacterium tumefaciens* mediated transformation in red gram with the marker gene, *nptII*, and the reporter gene, *uid A*, using this protocol. Transgenics were confirmed by molecular analysis.

## 2. Materials and Methods

*2.1 Plant material and seed germination*

Seeds of *Cajanus cajan* L. 'Hyderabad C' used in this study were obtained from the Amrudhi Seed Corporation, Bangalore. Seeds were surface sterilized with 0.1% (w/v) aqueous mercuric chloride solution for 7 min and then rinsed five times with sterile distilled water. These seeds were germinated under aseptic conditions on cotton wool soaked with sterile distilled water in Majenta jars for 5 days with 16h photoperiod at 25 ± 2 ℃ with a light intensity of 60 $\mu$E $m^{-2}s^{-1}$. Shoot apices and cotyledonary nodes of 5 - 8 mm in length excised from 5 - day - old seedlings were used as explants for cocultivation with *Agrobacterium tumefaciens*.

*2.2 Culture medium and conditions*

Both shoot apices and cotyledonary node explants were placed on MS [Murashige and Skoog, 1962] medium with 3% (w/v) sucrose supplemented with BAP (1.0 - 5.0 mg $l^{-1}$) for direct shoot bud regeneration. BAP at 2.0 mg $l^{-1}$ was proved to be effective for maximum frequency of shoot bud regeneration (MS1) and was used routinely in subsequent transformation experiments. All media were adjusted to pH 5.8 prior to the addition of 0.8% (w/v) agar and autoclaved at 121 ℃ and 1.02 kg $cm^{-2}$ pressure for 15 min. The cultures were maintained at 25 ± 2 ℃ in the culture room with 16 h photoperiods with 60 $\mu$E $m^{-2}s^{-1}$ light intensity provided by cool white fluorescent tubes.

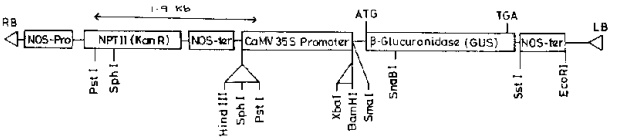


**Fig. 1** Schematic representation of pBI121 plasmid *NPTII*, coding region of the neomycin phosphotransferase gene; CaMV35S, Cauliflower Mosaic Virus 35S gene; NOS - Pro, nopaline synthase gene promoter; NOS - ter, nopaline synthase gene terminator; GUS, $\beta$ - glucuronidase.

*2.3 Agrobacterium strain and plasmid vector*

The disarmed *Agrobacterium tumefaciens* strain LBA4404 [Hoekema *et al.*, 1983] harboring a binary plasmid pBI121 [Jefferson *et al.*, 1987] was used as vector system for transformation. Plasmid pBI121 containing the *uidA* (GUS) gene linked to the Cauliflower Mosaic Virus (CaMV) 35S promoter and nopaline synthase (NOS) terminator sequences served as reporter gene and *nptII* (neomycin phosphotransferase) gene under the control of a NOS promoter and terminator was used as selectable marker (**Fig. 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 50 $\mu$g $ml^{-1}$ kanamycin sulfate and 25 $\mu$g $ml^{-1}$ rifampicin.

*2.4 Cocultivation and transformation*

A single bacterial colony was inoculated into 25 m*l* of liquid LB medium with 50 $\mu$g $ml^{-1}$ kanamycin sulfate and 25 $\mu$g $ml^{-1}$ rifampicin and incubated at 28 ℃ on a shaker at 120 rpm for overnight (16 - 18 h), and used in the late log phase $A_{600}$ at 0.6. Precultured explants were treated with an *Agrobacterium* suspension. Explants were immersed in the *Agrobacterium* suspension under gentle agitation (40 rpm) for 10 min at room temperature, drained on filter paper and placed on the regeneration medium (MS1) for cocultivation. Explants were precultured for 2 days on a regeneration medium before cocultivation with *Agrobacterium* in petri plates. Fifteen to 20 explants were cultured per plate. 150 - 200 explants were used for each treatment, with three replications. All explants were cocultivated for a period of 2 days in darkness at 25 ± 2℃. In control experiments, explants were placed on the regeneration medium for 2 days without cocultivation with *Agrobacterium*.

*2.5 Selection and plant regeneration*

To identify the killer concentration of kanamycin for effective selection of putatively transgenic plants, regeneration medium (MS+2.0 mg $l^{-1}$ BAP)

with different concentrations of kanamycin sulfate ( 20-100 $\mu$g $ml^{-1}$) was tested. At 40 $\mu$g $ml^{-1}$ and above, the explants turned brown and did not show further growth (data not shown). Hence, 50 $\mu$g $ml^{-1}$ was used as selection pressure. Explants were cultured on selection medium with antibiotics (50 $\mu$g $ml^{-1}$ kanamycin sulfate and 300 $\mu$g $ml^{-1}$ cefotaxime) 2 days after cocultivation. Shoots regenerated after 4 weeks on this medium. These were further subcultured on MS2 medium with reduced BAP (MS macro- and micro salts and vitamins, 0.5 mg $l^{-1}$ BAP, 30 g/*l* sucrose, 8 g/*l* agar, 25 $\mu$g $ml^{-1}$ kanamycin sulfate and 300 $\mu$g $ml^{-1}$ cefotaxime) to allow shoot elongation. Putative transformants were subcultured for additional 4 weeks on the same medium for further shoot elongation (longer than 3 cm). These were transferred to a rooting medium in test tubes which contained MS macro- and micro salts and vitamins, 30 g/*l* sucrose, 8 g/*l* agar, 0.2 mg $l^{-1}$ IBA and 25 $\mu$g $ml^{-1}$ kanamycin sulfate (MS3). Putative transgenic plants were transferred to plastic cups containing soil and maintained in the controlled environment at 25 ± 2 ℃ and 16 h photoperiod.

*2.6 Assay of GUS activity*

Leaves from regenerated and kanamycin-resistant plants were analysed for GUS (*uidA*) gene expression *in situ* with X-Gluc according to Jefferson *et al.* [1987]. Plant materials were stained with 2 mM X-Gluc, 100 mM Tris-HCl (pH 7.0), 50 mM NaCl, 2 mM potassium ferricyanide and 0.1% (v/v) Triton X-100 at 37 ℃ overnight. After staining, plant tissues were soaked in 70% ethanol to clear chlorophyll content and were subsequently fixed in 70% ethanol. Tissue was observed under light microscope and photographed with a Nikon stereomicroscope.

*2.7 Molecular analysis of putative transformants*

2.7.1 PCR anaysis

Total DNA was extracted from putative transgenic plants or untransformed plants following the procedure described by Edwards *et al.* [1991]. One $\mu$g of RNase treated DNA was used for polymerase chain reaction (PCR) amplification as template with oligonucleotide primers for the *uidA* and *nptII* genes, resulting in fragments of 0.53 kb and 0.8 kb, respectively. Two primers, 5′-TTC GCG TCG GCA TCC GCT CAG TGG CA-3′ and 5′-GCG GAC GGG TAT CCG GTT CGT TGG CA-3′ for uidA and two primers ,5′-GAG GCT ATT CGG CTA TGA CTG-3′ and 5′-ATC GGG AGG GGC GAT ACC GTA-3′ for *nptII* were used. Each PCR reaction was performed in 25 $\mu l$ (final volume) of reaction mixture consisting of 10X reaction buffer, 5 $\mu l$ DNA, 200mM dNTPs, 25 mM $MgCl_2$, 100 ng of each primer DNA and 1 unit of Taq DNA polymerase. The PCR was performed 35 cycles with 4 min preheating at 94℃, then 1 min melting at 94℃ , 1 min annealing at 58℃ and 1 min synthesis at 72℃ and another 10 min final extension at 72℃ for *uidA* gene amplifications, and 35 cycles with 4 min preheating at 94℃, then 1 min melting at 94℃, 1 min annealing at 55℃ and 1 min synthesis at 72℃ and another 10 min final extension at 72℃ for *nptII* gene amplifications. Amplified PCR products were analysed by electrophoresis in 1% agarose gel, visualized by staining with eithidium bromide and photographed under ultraviolet light.

2.7.2 Southern blotting analysis

Genomic DNA was isolated from leaves of putatively transformed and untransformed plants using the CTAB (cetyltrimethylammonium bromide) method described by Doyle and Doyle [1990]. Ten $\mu$g of DNA was digested with restriction endonuclease *Hin*dIII, separated by electrophoresis through a 0.8% agarose gel and transferred onto Hybond Nylon membrane. Prehybridization and hybridization were performed according to Sambrook *et al.* [1989] at 65℃. A 0.7 kb PCR fragment of the *nptII* gene was used as a probe .The probe was labelled with $\alpha$-$^{32}$P probe (Amersham) using a random primed labeling kit. Hybridized blots were washed with constant agitation in 2X SSC+0.1% SDS and 1X SSC+0.1% SDS for 15 min each at room temperature and then washed 30 min each in 0.5X SSC+0.1% SDS and 0.1X SSC+0.1% SDS at 65℃ . The blots were exposed to Kodak X – Omat film with two intensifying screens for 2 days for autoradiography at – 70℃ .

## 3. Results and Discussion

*3.1 Transformation and selection*

Shoot regeneration was observed from cotyledonary node explants and shoot apices after 3-4 weeks on the regeneration medium(MS1) with kanamycin. During the initial 2 days on non-selective shoot regeneration medium, all cocultivated and control explant materials retained a healthy green colour. Cocultivated explants retained a healthy green colour and developed shoots on MS1 medium after 3 weeks. However some explants have become necrotic. Shoots which survived this selection step were subcultured to fresh medium of the same composition where they developed multiple shoots (**Fig. 2- A**). Preculture of explants enhanced the transformation efficiency as shown in

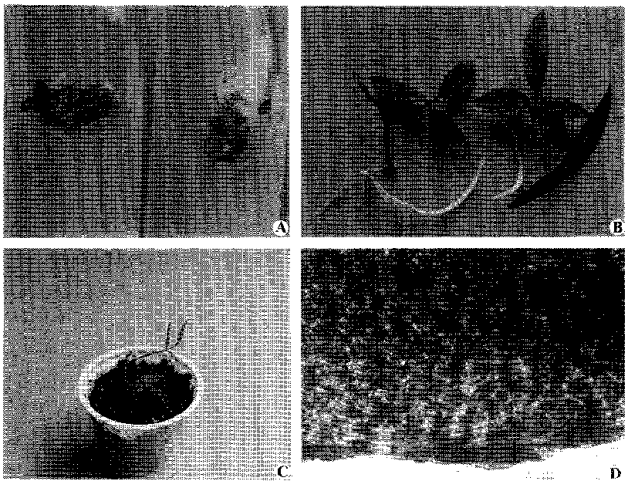

**Fig. 2** A. Regeneration of putatively transformed shoots on selection medium.
B. Putative transgenic shoots with well developed roots.
C. A putative transgenic plant established in pot.
D. Transgenic leaf showing characteristic blue colour of GUS expression.

another grain legume, (cowpea), by Muthukumar *et al.* [1996].

*3.2 Transgenic plant regeneration*

Green shoot bud initiation was observed from both cotyledonary node explants and shoot apices after 3 weeks on MS1 medium. Along with the green shoot buds, some buds were found to be yellowish in colour. Green and healthy shoots measuring to 2-3cm in length were excised and planted separately in MS2 medium for further selection. The efficient uptake of kanamycin by shoots during their rooting stage might provide more effective selection for positive transformants. During rooting stage, shoots which apparently escaped from previous selection eventually died within 2 weeks. Putatively transformed shoots which survived on selection medium produced roots on MS3 medium within 3 weeks. After 1 month, more than 50% of the shoots rooted (**Fig. 2 - B**). According to Draper *et al.* [1988] roots are generally much more sensitive to antibiotics, and thus the ability to root on selection medium containing high levels of selective agent is therefore a strong indication of its transformed nature. Escapes failed to root in the presence of the selective agent in the rooting medium. Rooted shoots were transferred to pots containing soil and kept under controlled environment (**Fig. 2 - C**). Cotyledonary node explants had a higher transformation efficiency (62%) whereas the transformation efficiency of shoot apices was 45%. Shoot differentiation and the number of shoots was generally better from cotyledonary node explants compared to shoot apices.

*3.3 Confirmation of transformants*

In the histochemical assay, leaf segments taken from putative transformants were positive for GUS activity (**Fig. 2 - D**), while tissues from untransformed plants were found to be negative. These results combined with the fact that putative transgenic plants had survived on selective medium, indicated that functional copies of the *uidA* and *nptII* genes were present in the transformants. To rule out the possibility of *Agrobacterium* contamination, putatively transformed shoots were cultured on antibiotic-free medium before the analysis of GUS activity. *Agrobacterium* did not appear on this medium after 3-4 weeks of culture, indicating that no contamination might interfere with the analysis. GUS expression was reported histochemically in chickpea [Kar *et al.*, 1996], in soybean [Hinchee *et al.*, 1988] and in lentil [Warkentin and McHughen, 1992] and by fluorometric method in *Pisum sativum* transformants [Grant *et al.*, 1995]. The influence of *Agrobacterium* growth conditions and application of *Agrobacterium* by vacuum infilteration were studied in *Phaseolus vulgaris*, a similar grain legume, by Kapila *et al.*. [1988] using the *Agrobacterium tumefaciens* strain pTJK136, which contains a GUS gene interrupted by an intron. Although positive for GUS activity, regeneration was not observed.

The putatively transformed plantlets were subjected to PCR analysis to detect the presence of *uidA* and *nptII* genes. In the transformants with GUS activity, an expected fragment of 500 nucleotide *uidA* (**Fig. 3 - A**) was amplified. An additional confirmation was also done using *nptII* primers and PCR analysis revealed the amplification of a 700 base pair fragment (**Fig. 3 - B**). No amplification was found in non transgenic plants. Southern hybridization was also carried out in order to further confirm the T-DNA integration. A 0.7 kb PCR fragment of the *nptII* gene was used as probe. DNA from transgenic and untransformed plants was digested with *Hin*dIII, which cuts only one site-located between the *uidA* and *nptII* genes-in the T-DNA insert of pBI121. As shown in **Fig. 4**, a $\alpha$-$^{32}$P labelled *nptII* DNA probe hybridized to *Hin*dIII-digested nuclear DNA of transgenic plants (lanes 3-5) and plasmid DNA double digested with *Pst*I revealed a strong hybridizing signals to the *nptII* probe at about 1.9 kb, used as positive control (lane 1). No hybridization could be detected for untransformed plants (lane 2). The same hybridization patterns observed for transgenic plants indicates that regenerants have originated from the same explant.

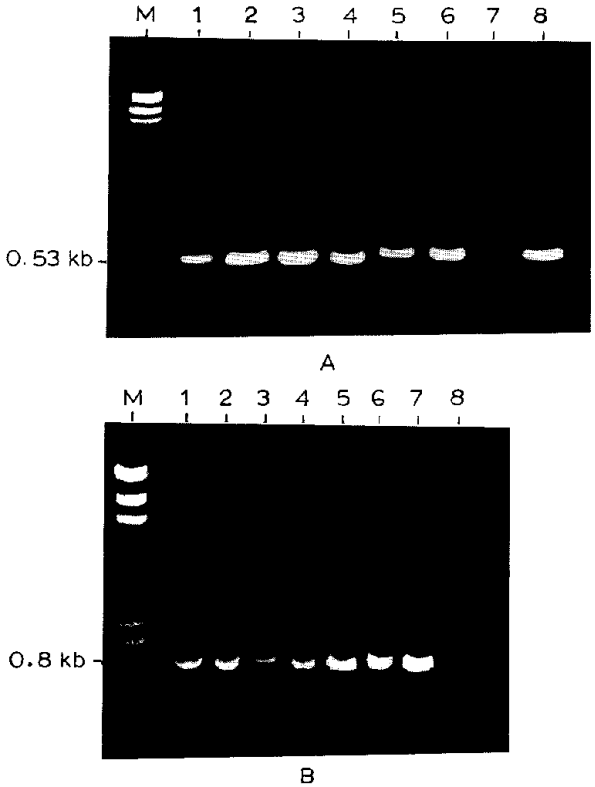


**Fig. 3** A. PCR analysis of transgenic plants showing the presence of an expected size of *uidA* (0.53 kb) gene.
Lanes 1-6. Transformed plants
Lane 7. Negative control (untransformed plant)
Lane 8. Positive control (pBI121 plasmid DNA)
B. PCR analysis of transgenic plants showing the presence of an expected size of *nptII* (0.8 kb) gene.
Lanes 1-6. Transformed plants
Lane 7. Positive control (pBI121 plasmid DNA)
Lane 8. Negative control (untransformed plant)

## 4. Conclusions

In the present investigation, a protocol for *Agrobacterium* mediated genetic transformation in red gram is demonstrated. This transformation system will be valuable tool for breeding and genetically engineer useful traits such as insect and disease resistance and genes for quality improvement. Molecular analysis confirms the transgenic nature of the plants. Optimization of protocols for the development of transgenic plants with appropriate vectors and promoters should enable the production of transgenics in red gram with improved nutritional quality like 2s albumin and insect resistance.

## Acknowledgement

The principal investigator (GLS) is thankful to Council of Scientific and Industrial Research (CSIR) for financial assistance.

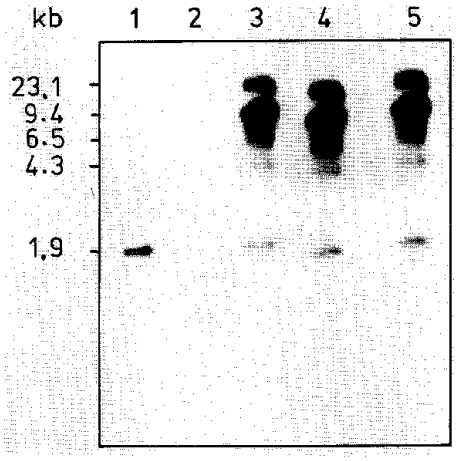


**Fig. 4** Southern blot analysis of the *nptII* gene
Lane 1. Positive control (plasmid DNA was cut with *Pst*I and probed with the *nptII* gene).
Lane 2. Negative control (untransformed plant).
Lanes 3-5. Transformed plants (genomic DNA from transgenic plants was cut with *Hind*III and probed with the *nptII* gene).

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