In planta transformation of pigeon pea : a method to overcome recalcitrancy of the crop to regeneration *in vitro*

K. Sankara Rao[#], Rohini Sreevathsa[§], Pinakee D. Sharma[§], Keshamma E[§] and Udaya Kumar M[§]

[#]Centre for Ecological Sciences, Indian Institute of Science, Bangalore - 560 012

[§]Biocon Limited, Bangalore - 560 100

[§]Department of Crop Physiology, University of Agricultural Sciences, GKVK, Bangalore - 560 065

ABSTRACT

Development of transgenics in pigeon pea remains dogged by poor plant regeneration *in vitro* from transformed tissues and low frequency transformation protocols. This article presents a non-tissue culture-based method of generating transgenic pigeon pea (*Cajanus cajan* (L.) Millisp.) plants using *Agrobacterium*-Ti plasmid-mediated transformation system. The protocol involves raising of whole plant transformants (T₀ plants) directly from *Agrobacterium*-infected young seedlings. The plumular and intercotyledonary meristems of the seedling axes are targeted for transformation. The transformation conditions optimized were, pricking of the apical and intercotyledonary region of the seedling axes of two-day old germinating seedlings with a sewing needle, infection with *Agrobacterium* (LBA4404/pKIWI105 carrying *uid A* and *npt II* genes) in Winans' AB medium that was added with wounded tobacco leaf extract, co-cultivation in the same medium for 1h and transfer of seedlings to soilrite for further growth and hardening and subsequent transfer of seedlings to soil in pots in the greenhouse. Out of the 22-25 primary transformants that survived infection-hardening treatments from each of the three experiments, 15 plants on the average established on the soil under greenhouse conditions, showed slow growth initially, nevertheless grew as normal plants, and flowered and set seed eventually. Of the several seeds harvested from all the T₀ plants, six hundred were sown to obtain progeny (T₁) plants and 350 of these were randomly analysed to determine their transgenic nature. PCR was performed for both *gus* (*uid A*) and *npt II* genes. Forty eight of the 350 T₁ plants amplified both transgenes. Southern blot analysis substantiated the integration and transmission of these genes. The protocol ensured generation of pigeon pea transgenic plants with considerable ease in a short time and is applicable across different genotypes/cultivars of the crop and offers immense potential as a supplemental or an alternative protocol for generating transgenic plants of difficult-to-regenerate pigeon pea. Further, the protocol offers the option of doing away with a selection step in the procedure and so facilitates transformation, which is free of marker genes. [Physiol. Mol. Biol. Plants 2008; 14(4) : 321-328] E-mail : baradwaj@biochem.iisc.ernet.in; sankarakolluri@yahoo.com

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Red gram or pigeon pea (*Cajanus cajan* (L.) Millsp.) is an important protein-rich grain legume of the semi-arid tropics and is an important dietary protein source for humans, especially for the large vegetarian population of sub-tropics. It is also one of the oldest among cultivated plants. India now constitutes the center of diversity for this crop and among the pulses cultivated and produced in this region, pigeon pea ranks highest. Despite being so important, the production of this crop has not undergone any significant improvement in the last three decades. Classical breeding to obtain

genotypes/varieties of pigeon pea with desirable agronomical traits has met with limited success because of its narrow genetic base and incompatibility associated with wild species (Nene and Sheila 1990).

Large number of germplasm lines of pigeon pea have, nevertheless, been identified for resistance to *Fusarium* wilt, *Phytophthora* stem blight and sterility mosaic disease as other major impediments on the yield. The most important yield constraint on pigeon pea is from the lepidopteran pest, *Helicoverpa armigera*. No insect resistant varieties have been developed with conventional breeding techniques mainly due to the non-availability of insect resistant germplasm.

Correspondence and Reprint requests : K. Sankara Rao

To provide a solution to the constraints that limit production and quality of this important leguminous crop, it is imperative that there is a need to widen its genetic base by using transgenic technology for incorporating desirable traits.

Development of transgenics in pigeon pea remains dogged by poor regeneration *in vitro* and low frequency transformation, although efforts have gone into developing efficient protocols of *Agrobacterium*- and microprojectile bombardment-based genetic transformation of this crop (Geetha *et al.*, 1999; Lawrence and Koundal, 2001; Prasad *et al.*, 2004; Satyavathi *et al.*, 2003; Kumar *et al.*, 2004; Surekha *et al.*, 2005; Sharma *et al.*, 2006; Dayal *et al.*, 2003) and plant regeneration from *in vitro* cultured explants via organogenesis as well as by somatic embryogenesis (Naidu *et al.*, 1995; George and Eapen, 1994; Eapen *et al.*, 1998; Geetha *et al.*, 1998; Patel *et al.*, 1994; Dayal *et al.*, 2003; Sharma *et al.*, 2006). Plants were also regenerated via callus (Satyavathi *et al.*, 2003; Thu *et al.*, 2003) and by direct differentiation from leaves (Eapen and George, 1993; Dayal *et al.*, 2003). Multiple shoots were induced on explants of cotyledonary nodes (Shiva Prakash *et al.*, 1994; Singh *et al.*, 2002; Satyavathi *et al.*, 2003; Sharma *et al.*, 2006) and epicotyls (Naidu *et al.*, 1995). However, the *in vitro* regeneration conditions so described are available for only a few of the many cultivars/genotypes of pigeon pea. Further, the morphogenetic response of this crop is known to be a genotype-specific phenomenon (Mohan and Krishnamurthy 1998).

Successful transformation of pigeon pea, for these reasons, will be greatly aided by genotype/variety-specific determination of critical parameters on improving *in vitro* regeneration or by developing transformation methods that do not involve tissue-culture-dependent plant regeneration. Protocols as those demonstrated for other legumes, *Medicago truncatula* (Trieu *et al.*, 2000) and *Arachis hypogaea* (Rohini and Sankara Rao 2000) will be of tremendous utility to research on pigeon pea as well. This article presents a non-tissue culture-based transformation protocol for pigeon pea using the *Agrobacterium tumefaciens*-mediated transformation of injured embryonal axes followed by growth into a whole plant transformant through acclimatization to greenhouse conditions. The protocol ensured generation of transgenic plants with considerable ease in a short time and is not genotype- or variety-dependent.

MATERIALS AND METHODS

Plant material

Seeds of pigeon pea cultivar TTB7 were procured from a local seed dealer at Bangalore. Following overnight soaking in distilled water, the seeds were surface sterilized, first with 1 % (W/V) Bavastin for 10 mins, and later with 0.1 % (W/V) HgCl₂ for 5 seconds, rinsed with distilled water following treatment with each sterilant. Seeds were later germinated in petriplates at 30 °C. Appropriate developmental stage at which infection to be carried out was determined. Two-day old seedlings were found suitable for *Agrobacterium* infection.

Transformation and recovery of transformants

Agrobacterium tumefaciens strain LBA4404 harbouring the binary vector pKIWI105 (Janssen and Gardner 1989), was used for transformation. The vector contains the *uidA* reporter gene driven by CaMV 35S promoter and the neomycin phosphotransferase II (*npt II*) gene driven by the nopaline synthase promoter. *Agrobacterium* was grown overnight at 28 °C in 25 ml of LB medium (pH 7.0) containing 50 µg ml⁻¹ kanamycin. The bacterial culture was later resuspended in 100 ml of Winans' AB medium (Winans *et al.*, 1988) (pH 5.2) and grown for 18 h. For *vir* gene induction treatment, wounded tobacco leaf extract was added to the *Agrobacterium* suspension in Winans' AB medium 5 h before infection (Cheng *et al.*, 1996). Tobacco leaf extract was prepared by crushing 8 g of leaf tissue in 8 ml distilled water and added to *Agrobacterium* suspension in Winans' AB medium. The seedlings with plumule just emerging were pricked at the apical meristem of the axis and at the intercotyledonary region with a sterile sewing needle and infected/co-cultivated by immersion in the suspension of *Agrobacterium* for 1 h. Following infection-co-cultivation, the seedlings were washed briefly with sterile water and later placed on autoclaved soilrite (vermiculite equivalent), moistened with water and incubated for germination under aseptic conditions in the growth room in wide mouth, capped glass jars of 300 ml capacity, 5 seedlings per jar.

The growth room was maintained at 26–28 °C under 14 h photoperiod with fluorescent light of intensity 35 mmol m⁻² s⁻¹. After 5 to 6 days of germination and growth on soilrite in glass jars, the seedlings were planted on soilrite in pots for further growth under growth room conditions for at least 10 days before they are transferred to pots containing sand-soil mix and

grown in a containment greenhouse. These putative transformants in pots were initially covered with polythene bags for 2-3 days to maintain high humidity. Upon flowering and pod formation, mature seeds from these T₀ plants were collected. In each experiment 50 embryo axes were infected and the experiments were repeated three times.

Molecular analyses of the putative transgenic plants

Tissues from the progeny plants were analyzed for the presence of the introduced genes. Genomic DNA was isolated following the procedure of Dellaporta *et al.* (1983) from fresh leaf tissue of the greenhouse-grown T₁ and T₂ generations that was used for polymerase chain reaction (PCR) and Southern analysis. PCR was performed to amplify a 450 bp *uidA* gene fragment and a 750 bp *nptII* gene fragment. PCR was initiated by a hot start at 94 °C for 4 min followed by 32 cycles of 1 min at 94 °C, 1 min 30 s at 60 °C and 1 min at 72 °C. Annealing temperature was at 58 °C for the amplification of a 750 bp *nptII* gene fragment. PCR conditions to amplify the 569 bp *virC* region were: 94 °C for 30s, 55 °C for 1 min and 72 °C for 1 min for 30 cycles. The products were run on a 1 % agarose gel. PCR was also performed to amplify the *nptII* gene fragment in the vector. The amplified product was purified, random prime labeled (Amersham Biosciences) for use as a probe for Dot blot and genomic Southern analyses.

Southern analysis

Dot blot and Southern analyses (Southern, 1975) were carried out to confirm the integration and inheritance of the *nptII* gene in the genomic DNA of T₁ pigeon pea transformants. For dot blot analysis, 1 µg of the total genomic DNA was loaded using a dot blot apparatus and transferred on to a nylon membrane (Hybond, Amersham) according to Sambrook *et al.* (1989).

For Southern analysis, 10 µg of total genomic DNA from T₁ generation plants, untransformed plants and 10 µg DNA of pKIWI105 were separately digested with the appropriate enzyme. The digested DNA samples were electrophoresed on a 0.7 % agarose gel and transferred on to a nylon membrane. Hybridization was performed at 65 °C in 6X SSC, 5X Denhardt's solution, 1 % SDS and 100 µg ml⁻¹ Salmon sperm DNA for 18 h. Membranes were washed for 30 min each in 2X SSC, 0.1 % SDS; 0.1X SSC, 0.1 % SDS at 65 °C (Sambrook *et al.*, 1989), wrapped, exposed and developed using a phosphorimager.

RESULTS

Preliminary experiments have suggested that germination and growth processes of the germinating embryos of pigeon pea remained unaffected in the transformation procedure adopted, namely, pricking the meristem of the apical and intercotyledonary region with needle and infection with *Agrobacterium* by immersing the embryonal axes in the bacterial culture for 1 h. Further steps of hardening for outplanting of the seedling also did not adversely affect the seedlings. It was observed that the seedlings remained fresh and healthy and the germination frequency was as high as 50 % when the infection treatment was prolonged to last for 1 h. However, the seedlings showed symptoms of browning and necrosis when infection was extended beyond 1 h.

The transformation conditions optimized therefore were, infection of the pricked embryonal axes of two-day old germinating seedlings with *Agrobacterium* in 100 ml of Winans' AB medium that was added with wounded tobacco leaf extract, co-cultivation in the same medium for 1 h and transfer to soilrite for further germination and growth. The seedlings were not subjected to a selection regime following infection-co-cultivation. As the primary transformants generated by this *in planta* method are chimeric in nature, it is possible that the transformed sectors are eliminated when untransformed embryo tissues begin to die under kanamycin selection. Instead, the putatively transformed embryonal axes were allowed to grow on soilrite, initially in bottles, and later in pots till they have developed a few normal shoots from the plumular meristem as well as from the meristem in the axils of cotyledons. The T₀ plants were not analysed for the presence of transgenes as they are chimeras. Out of the 22-25 primary transformants that survived infection-hardening treatments from each of the three experiments, 15 plants on the average established on soil in pots under greenhouse conditions, showed slow growth initially, but grew as normal plants, and eventually flowered and set seed (Figs. 1 A and B). Of the several seeds harvested from T₀ plants of all the three experiments, six hundred were sown to obtain the progeny (T₁) plants (Figs. 2 A and B) and 350 of these plants were randomly analyzed for stable integration and transmission of the introduced genes.

PCR was performed on T₁ transformants for both *uidA* and *nptII* genes. Out of the 350 progeny analysed, 48 plants amplified both transgenes (Figs. 3 and 4). Dot blot analysis was carried out to obtain confirmation on

Fig. 1. (A) T_0 plants established in the greenhouse (B) T_0 plants showing flowering and pod formation.

Fig. 2. (A) T_1 plants in the greenhouse (B) T_1 plants showing flowering and pod formation.

the presence of the introduced genes in the plants that have tested positive as being transgenic in the PCR analysis (Fig. 5). Signals were seen in the samples of all 48 plants in the dot blot when probed with *nptII* gene fragment. Eleven of the plants that tested positive in the dot blot hybridization were randomly selected for genomic Southern analysis of *nptII* gene. In the vector

pKIWI105, *Hind* III releases a 4.04 kb fragment that comprises the *nptII* gene. Fig. 6 shows hybridization signal in all the eleven plants (Fig. 6: lanes 1-11) and the vector DNA (Fig. 6: lane 13) except in the wild type (Fig. 6: lane 12), at 4.04 kb position when probed with a 750 bp *nptII* gene fragment confirming integration of the transgene in the pigeon pea genome.

Fig. 3. PCR of the DNA of transgenic plants in the T_1 generation pigeon pea plants using primers for *uidA* (*gus*) gene. Lanes 1-48: DNA from the transgenic plants; Lane PC: positive control (pKIWI105 DNA); Lane NC: DNA from the untransformed control; Lane M: DNA ladder.

Fig. 4. PCR of the DNA of transgenic plants in the T_1 generation pigeon pea plants using primers for *npt II* gene. Lanes 1-48: DNA from the transgenic plants; Lane PC: positive control (pKIWI105 DNA); Lane NC: DNA from the untransformed control; Lane M: DNA ladder.

Fig. 5. Dot blot analysis of the PCR positive T_1 generation transgenic plants using a 750 bp *npt II* gene fragment as a probe. Wells 1-48: DNA from the transgenic plants; Well 49: DNA from the positive control (pKIWI105 DNA); Well 50: DNA from the untransformed negative control.

Five T_2 progeny of each of the 11 Southern positive T_1 plants were analyzed. Results of PCR performed (Fig. 7) showed amplification when *nptII* primers were used, indicating the inheritance of *nptII* transgene in the T_2 generation. The absence of residual bacteria was confirmed when amplification was carried out with *virC*-specific primers (Fig. 8). Amplification of *virC* was not detected in the DNA of these plants (Fig. 8: lane 3), but was observed in the bacterial DNA (Fig. 8: lane 2).

DISCUSSION

Pigeon pea is less explored with regard to development of transgenic methods. The generally poor response of the investigated genotypes/cultivars of the crop to tissue culture initiation and plant regeneration has necessitated development of transformation systems that target apical and axillary meristems in the embryonal axis and regenerate plants via meristem culture (Shivaprakash *et al.* 1994; Franklin *et al.* 1998; Geetha *et al.* 1998, 1999; Mohan and Krishnamurthy 1998; Singh *et al.* 2002, 2004; Sharma *et al.* 2006). The transformation

Fig. 7. PCR analysis of the DNA from the T_2 generation pigeon pea plants. Lanes 1-53: DNA from the transgenic plants. Lane PC: DNA from the positive control (pKIWI105 DNA). Lane NC: DNA from the untransformed plant.

study of pigeon pea presented here, similarly makes use of the axillary and apical meristems of the embryonal axis but seeks to retrieve whole plant transformants without having to excise the meristems from the embryonal axis. Transformation was carried out by pricking the apical and the intercotyledonary meristematic regions of two-day-old embryonal axis with a sewing needle, immersing the whole germling in the *Agrobacterium* suspension for infection and by permitting it to grow into a whole plant of the T_0 generation. The main objective was to do away with tissue culture-based plant regeneration in the protocol. The conventional selection step was also not introduced in the protocol. As the *Agrobacterium* infected-cocultivated germlings are chimeric in nature, it is possible that the transformed tissue sectors would be eliminated when the untransformed embryo tissues begin to die under kanamycin selection. The GUS histochemical early detection of transformants, likewise, could not be used in the case of pigeon pea as it gave false positives with the substrate X-gluc. It is likely that the endogenous GUS-like activity exhibited by pigeon pea control (non-

Fig. 6. Genomic Southern blot analysis of the T_1 generation pigeon pea plants. 15 μ g of genomic DNA was digested with *Hind III* and was probed with 750bp *npt II* gene fragment. Lanes 1-11: DNA from the transgenic plants. Lane 12: DNA from the untransformed plant. Lane 13: DNA from pKIWI105 digested with *HindIII*.

Fig. 8. PCR amplification of a 569bp *virC* sequence to ascertain the *Agrobacterium*-free nature of the transformants (Lane 3 in the picture shows no amplification in the plant DNA indicating absence of residual *Agrobacterium* in the transformed plant). Lane 1: marker. Lane 2: positive control (LBA4404/pKIWI105 DNA). Lane 3: DNA from the transformed plant.

transformed) seedlings is brought about by certain other enzymes metabolizing the substrate. Similar endogenous GUS-like activity has been reported earlier in a host of other plants (Sudan *et al.*, 2006) including legumes.

A key component of most gene transfer approaches is the requirement of a high frequency transformation system and efficient plant recovery. In order that the present strategy is rendered efficient, optimization of transformation efficiency for pigeon pea was determined by:

1. varying the duration of exposure of the germlings to *Agrobacterium* infection-cocultivation, and
2. by manipulating virulence induction conditions.

As the transformation efficiency in terms of the number of primary transformants showing blue sectors following infection-co-cultivation cannot be taken as the realistic measure in the case of pigeon pea, on account of the occurrence of the endogenous GUS like activity, an alternate strategy of observing the physical state of the embryonal axis following the *in planta* procedure was employed. Since the primary requirement of the transformation strategy employed here was to preserve the entire embryonal axis alive and healthy enough for unhampered growth into a plant following pricking and exposure to bacterial culture, the intensity of infection needed careful optimization while determining the duration of infection-cocultivation. It was also necessary that large number of putative transformants be obtained to ensure that at least some of them carry the introduced gene in their genome for transmission to the progeny (T_1 generation). For this reason, the intensity of infection process in terms of duration of infection needed careful monitoring.

In an effort to maximize transformation efficiency, the duration of infection-co-cultivation was increased to a stage when it was no longer safer for the seedling axes. Infection-co-cultivation time upto 1 h did not adversely affect the seedling axis. With infection time beyond 1 h, the embryonal axes turned brown and necrotic. Therefore, this treatment was restricted to 1 h duration.

Our earlier empirical experiments have shown (data not shown) that suspension of bacteria in Winans' (Winans *et al.* 1988) virulence-inducing AB medium fortified with wounded tobacco leaf extract has increased the efficiency of transformation. The bacteria are resuspended in Winans' AB medium, incubated for 18 h, the tobacco leaf extract added to it and the incubation continued for 5 h before the infection.

The optimized protocol involving 1 h infection-cocultivation and the virulence induction treatments facilitated generation of putative primary transformants (T_0) with considerable ease. Similar increase in the transformation frequency was also observed when peanut (Rohini and Sankara Rao 2000a) and safflower (Rohini and Sankara Rao 2000b) were infected with *Agrobacterium* that was treated with wounded tobacco leaf extract. Successive rounds of experiments have produced a number of T_0 plants.

The primary objective of the protocol was to ensure efficient plant recovery following *Agrobacterium* infection-cocultivation. This is achieved by doing away with excision and tissue culture of the target tissue for generating the putative transformants but by allowing the entire embryonal axis to grow into a whole plant with the infected tissue intact. The infection-targeted tissue being the meristems of the shoot apex and cotyledonary axils, it is understandable that the shoot system of the T_0 plant would be chimeric to the introduced genes. Thus, the protocol facilitated generation of putative primary transformants, the T_0 plants, with considerable ease. That some of these plants carry the introduced genes for transmission to the progeny (T_1 generation) is amply demonstrated in the study. Successive rounds of experiments have shown that, on the average, 15 *Agrobacterium* infected-cocultivated seedlings have established as fertile plants. There is every opportunity that the transformation event will be multiplied as pigeon pea produces a large number of seeds, more than once, because it is perennial and polycarpic. Six hundred seeds harvested from T_0 plants were sown to raise the progeny plants (T_1) of which 350 were analyzed by PCR for the presence of both marker genes. Forty eight PCR positive plants were further tested for confirmation of their transgenic nature, all of them showing the presence of the introduced genes. The T_2 plants analyzed for the presence of *nptII* gene have shown that this gene is transmitted to the next generation.

To rule out the possibility of the presence of *Agrobacterium* in the transformants, which might result in artifacts in the PCR, *virC* gene which is specific for the bacterium and not for the T-DNA was included in the experiment. The result of the PCR shows no amplification of *virC* gene in the plant DNA. This confirms the PCR amplification in the transgenic plant DNA is because of the integration of the transgene and not due to the persistence of the *Agrobacterium*.

Using this protocol, the transformability of other pigeon pea cultivars was tested. It was observed that the cultivars Hyderabad-3C and Maruthi were successfully transformed by this method, confirming the credibility of its cultivar-independent application. Further, the use of reporter and marker genes could be optional for transformation has also been demonstrated in this study, the process of transformant detection being deferred to T₁ generation and carried out by PCR analysis. Currently, strategies are being evolved by several researchers to generate marker-free transgenic plants (Veluthambi *et al.*, 2003). The transformation protocol described provides one such strategy.

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