International Journal of Innovative Research and Advanced Studies (IJIRAS)
Volume 4 Issue 8, August 2017

ISSN: 2394-4404

Genetic Transformation Of Pigeonpea Through Particle Gun And Agrobacterium Using Cry1ac Transgene

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Abstract: Genetic transformation of pigeonpea (Cajanus cajan L. Mill sp.) variety PAU 881 was achieved using two different gene transfer methods (particle gun, Agrobacterium). cry1Ac transgene driven by ubiquitin/CaMV 35S promoter and nos terminator was introduced into mature embryos (with one cotyledon attached) excised from overnight soaked seeds. The putative transgenic plants so developed were analyzed through polymerase chain reaction to check presence of the transgene. High transformation frequency was obtained by particle gun-mediated transformation as compared to Agrobacterium-mediated transformation.

Keywords: Cajanus cajan L., genetic transformation, embryo axes

I. INTRODUCTION

Pigeonpea (*Cajanus cajan* L. Millsp., family Fabaceae), also known as red gram, is a short-lived perennial and an important pulse crop in the tropical and subtropical countries of the world, especially in Asia, Africa, Latin America and Australia. It is an often cross-pollinated diploid crop (2n=22) and has a genome size of 833.07 Mb (Varshney *et al*. 2012). Pigeonpea is cultivated for its high protein content (44%) and is also used as fodder, fuelwood, green manure and for construction of roofs, hedges and windbreaks. Being a drought resistant crop, it is suitable for dry land farming and predominantly used as an intercrop with other crops like cotton, sorghum, groundnut, black gram etc. to maintain soil fertility and increase the yield. Pigeonpea is the second most important food legume of India after chickpea. The crop was grown on an area of 3.90 million ha in the states of Andhra Pradesh, Gujarat, Karnataka, Madhya Pradesh, Maharashtra, Punjab and Uttar Pradesh, with a production of 3.17 million tonnes and productivity of 813 kg/ha during 2013-14 (ICAR-Indian Institute of Pulses Research, Kanpur). Commonly known as arhar or tur in India, the country contributes to 92 % of total pigeon pea production in the world (Reddy *et al*. 2016). In Punjab, pigeon pea was cultivated on an area of 45.4 thousand ha with a production of 39.6 thousand tonnes and productivity of 872 kg/ha during 2013-14 (ICAR- Indian Institute of Pulses Research, Kanpur).

The crop production is most affected by biotic stresses, which include more than 300 species of insects and mites. More than 200 insect species have been reported to feed on pigeonpea from germination to harvest (Rao and Shanower 1999). The major insects causing heavy yield losses are considered to be the pod borers (*Helicoverpa armigera* and *Maruca vitrata*). *Helicoverpa armigera* is the most devastating pest and cause extensive economic crop losses to the tune of US$ 350 million annually (Sharma 2005). Efforts to produce insect-pest resistant genotypes of pigeonpea by conventional methods have not been fruitful due to inadequate genetic variation in the cultivated germplasm and incompatiblity of cultivated pigeonpea with wild species (Nene and Sheila 1990). Development of resistant/tolerant cultivars against the pod borers would provide an environment friendly solution to tackle these insect pests.

Genetic transformation is an alternative method to introduce desirable insect resistance gene(s) in pigeonpea. A number of direct and indirect methods are available to transfer desirable foreign gene(s) into crop plants. There are a few reports on genetic transformation in pigeonpea through *Agrobacterium*, particle gun and *in planta* gene transfer methods (Lawrence and Koundal 2001, Dayal *et al*. 2003, Thu

et al. 2003, Surekha *et al*. 2005, Sharma *et al*. 2006, Rao *et al*. 2008, Ramu *et al*. 2012, Kaur *et al*. 2016). Tissue culture and genetic transformation of legumes suffers from a major problem of difficult plant regeneration and low regeneration frequency (Geetha *et al.* 1999, Lawrence and Koundal 2001, Krishna *et al*. 2010, Atif *et al*. 2013, Mahale *et al*. 2014). A number of different explants have been investigated in pigeonpea for *in vitro* regeneration through callus as well as direct organogenesis (Geetha *et al*. 1998, Kaur *et al*. 2012, Dev 2013). However, their applicability for genetic transformation is partial due to genotypic differences (Mohan and Krishnamurthy 1998, Atif et al. 2013) and lengthy *in vitro* shoot differentiation period (Kaur et al. 2016). In the present investigation, genetic transformation of mature embryos (with one cotyledon attached) of pigeonpea variety PAU 881 was conducted through particle gun and *Agrobacterium* methods using *cry1Ac* gene (for pod borer resistance), and the efficacy of transformation was compared.

II. MATERIAL AND METHODS

PLANT MATERIAL

Seeds of pigeonpea variety PAU 881 were obtained from Pulses Section, Punjab Agricultural University, Ludhiana. These were sterilized with 0.1% mercuric chloride for 7-10 minutes, washed thrice in sterile water, then soaked overnight in sterile water and kept on an orbital shaker. The seeds were de-coated and split aseptically, and the exposed mature embryos (with one cotyledon attached) were used as explants for particle gun-mediated transformation. During *Agrobacterium*-mediated transformation, mature embryos (with one cotyledon attached) were pricked gently at the apical meristematic region once with a sterile needle and then used for transformation.

PARTICLE GUN-MEDIATED GENETIC TRANSFORMATION

In this experiment, BioRad Gun (PDS-1000/He system) was used for transformation of PAU 881 embryos. Plasmid (pGEM-4Z) [carrying *gusA*, *hptII* genes, *cry1Ac* (1.8 kb) with maize ubiquitin promoter (2 kb), and nos terminator] was isolated from *Escherichia coli* through alkaline lysis method.

The excised embryos were pre-cultured for 24 h on basal MS (Murashige and Skoog 1962) medium, and then cultured on osmotic medium [MS medium supplemented with mannitol (0.4 M)] for 4 h prior to plasmid bombardment. The plasmid was precipitated onto tungsten microcarriers (1 µm); the method described by Kaur et al. (2007) was followed for preparing tungsten microcarrier particles. The plasmid was bombarded into mature embryos using PDS-1000/He gun. The settings on the gun were as follows: 8 mm distance between the rupture disc and the microcarriers, 10 mm distance between the microcarriers and the stopping screen, and 6 cm distance between the stopping screen and the target. The embryos were bombarded at a pressure of 1100 psi; post-bombardment, embryos were inoculated on osmotic medium and incubated in dark at 28ºC for 16 h. Thereafter, the bombarded embryos were transferred to basal MS medium supplemented with hygromycin (30 mg/l) for three cycles of 2 weeks each for selection and regeneration of putative transformed embryos. The plantlets were transferred on basal MS medium without selective agent for further growth. Plantlets with freshly developed roots were thoroughly washed in running tap water and kept on moist cotton in test tubes. The test tubes were covered by a polythene sheet and kept under light intensity of 5000 lux for 7 days in the incubation room for hardening of the plantlets.

AGROBACTERIUM - MEDIATED GENETIC TRANSFORMATION

Agrobacterium strain EHA105 carrying *nptII* gene, *cry1Ac* gene under the control of CaMV 35S promoter and nos terminator was used for the transformation of embryos. Pre-cultured embryos were treated with bacterial suspension (OD 0.6 at 600 nm) for 10 minutes under aseptic conditions. The treated embryos were blotted dry on sterile filter paper and co-cultivated on basal MS medium for 48 h. After co-cultivation, the embryos were first washed in sterile water, then washed in sterile water containing cefotaxime (500 mg/l), blotted dry on sterile filter paper and transferred to selective shoot regeneration medium (MS + 4 mg/l BAP + 50 mg/l kanamycin + 500 mg/l cefotaxime) for inhibition of agrobacterial growth and selection of putative transformed embryos. The selection medium was replenished for three cycles and each cycle comprised of 2 weeks. Shoots were transferred on basal MS medium for root induction. The plantlets were washed and hardened as described above.

MOLECULAR ANALYSIS OF PUTATIVE TRANSGENIC PLANTS

The putative transgenic plants developed through two different gene transfer methods were analyzed for the presence of *cry1Ac* gene by polymerase chain reaction (PCR). The total genomic DNA from young leaves of putative transgenic plants was isolated following the protocol given by Doyle and Doyle (1960). PCR was carried out using gene specific internal primers, forward (5'-TGGAGAACGCATTGAAACCG-3') and reverse (5'-TGTTGCTGAATCCGGAACGC-3'). The PCR mix for each of 20 µl reaction volume contained 75 ng genomic DNA (3 µl), 10 µM of each primer (1 µl), 1 mM dNTPs (4 µl), 25 mM $MgCl_2$ solution (1.2 µl), 5X PCR buffer (4 µl), 5 units/µl Taq DNA polymerase (0.25 µl) and nuclease-free water (5.55 µl); the reaction was performed in an Eppendorf Thermal cycler. The PCR was programmed at an initial denaturation of 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 56 °C for 1 min, 72 °C for 1 min and final extension at 72 °C for 7 min. The amplicons were separated in 1 % agarose gel at 80 V for 1 h till tracking dye reached the other end. Five µl of 100 bp plus DNA ladder (Thermo scientific GeneRuler) was loaded in a gel well along with the negative control (water). The amplified DNA products were viewed under UV light with the help of transilluminator and the gel was photographed using AlphaImager EC gel documentation system.

ISSN: 2394-4404

III. RESULTS AND DISCUSSION

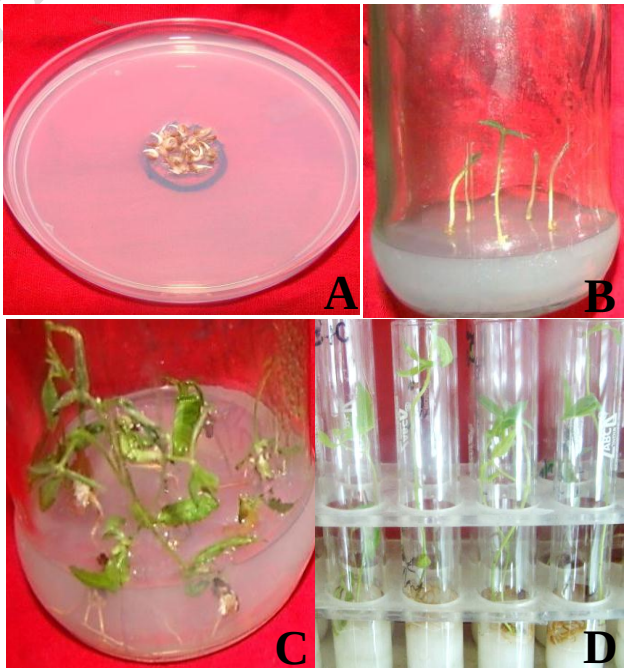
The present study dealt with the development of putative transgenic plantlets of pigeonpea using *Agrobacterium* and particle gun-mediated transformation. Five experiments were performed in each method and a total of 500 mature embryos (with one cotyledon attached) were transformed in each case. Embryos serve as a good system for transformation as these are easily available and highly responsive to direct regeneration as compared to other explants, thus helping in recovery of transgenics. Although callus is also a good system for transformation as in many crop species, transgenics have been developed using callus as the target tissue, however callus of pigeonpea is recalcitrant to regeneration. The callus induced on most of the pigeonpea explants (leaves, epicotyls, cotyledons, embryonal axes) showed no regeneration even when cultured on medium supplemented with high concentration of cytokinin (Dev 2013).

The pre-cultured embryos of PAU 881 were used for *Agrobacterium*-mediated genetic transformation, as pre-culturing has been reported to be crucial to enhance cell ability to uptake T-DNA (Arencibia et al. 1998). During particle gun-mediated genetic transformation, the pre-cultured embryos were grown on osmotic medium both prior to and post-bombardment. Culture of embryos on osmotic medium has been reported to increase transformation efficiency (Vain et al. 1993, Rochange et al. 1995, Brettschneider et al. 1997). This is due to the fact that culture of explants on osmotic medium prior to bombardment causes plasmolysis of cells, and culture of bombarded explants on osmotic medium decreases the probability of protoplasm extrusion from the cells, leading to increased transformation efficiency (Vain et al. 1993). Both particle gun and *Agrobacterium*-mediated transformation have been used for mature embryo transformation in grain legumes (Atif et al. 2013).

The putative transformed embryos of pigeonpea variety PAU 881 were selected by growing them on selective medium containing kanamycin/hygromycin. By either transformation method (particle gun/*Agrobacterium*), DNA is introduced only in a minority of cells. Addition of a gene providing resistance to an antibiotic e.g. kanamycin or hygromycin along with the gene(s) of interest, allows the selection of transformed cells, if the culture medium is supplemented with the antibiotic. Cells that express the resistance gene multiply, whereas the non-transformed cells perish (Higgins and Dietzgen 2000).

More number of putative transgenic plants regenerated after particle gun-mediated transformation (Fig. 1) as compared to *Agrobacterium*-mediated transformation (Table 1). This is because in *Agrobacterium*-mediated transformation, it is difficult to exterminate *Agrobacterium* from the treated explants after co-cultivation; consequently most of the explants die due to overgrowth of *Agrobacterium*. Out of 250 putative transgenics plants developed after particle gun-mediated transformation, 10 plants showed amplification of *cry1Ac* gene (927 bp, Fig. 2). From 100 putative transgenic plants of pigeonpea regenerated after *Agrobacterium*-mediated transformation, 1 plant showed amplification of *cry1Ac* gene (Fig. 3). Thus, transformation frequency obtained through particle gun transformation method using embryos was higher (4 %) as compared to *Agrobacterium* (1%) [Table 1]. Lawrence and Koundal (2001) developed insect resistant transgenic pigeon pea plants by *Agrobacterium*-mediated genetic transformation of embryonic axes, and reported less than 1% transformation efficiency. Likewise, Mohan and Krishnamurthy (2003) reported transformation efficiency of 1.7-6.7% based on *Agrobacterium*-mediated transformation of mature decapitated embryo axes. A number of diverse explants tested in cowpea (mature embryos, immature embryos, embryonic axes with cotyledons etc.) yielded a transformation frequency of 0.05-0.15 % using *Agrobacterium*-mediated gene transfer method (Popelka et al. 2006). Transformation of mature embryos of faba bean using particle bombardment yielded 2 % transgenic plants (Metry et al. 2007). A transformation frequency of less than 1% was obtained using biolistic transformation of embryonic axes in cowpea [Ikea et al. (2003), Ivo et al. (2008)]. Bhargava and Smigocki (1994) used particle gun to transform mature embryos of different *Vigna* spp., and observed *gus* expression in meristematic regions after 18 h of bombardment. Particle gun transformation method frequently results in higher transformation frequency as compared to *Agrobacterium* (Homrich et al. 2012).

In conclusion, particle gun-mediated transformation resulted in higher transformation frequency as compared to *Agrobacterium*-mediated transformation. The method provided more number of putative transgenic plants with considerable ease in a short time and is not genotype or variety dependent. However, nature of the putative plants developed by either method need to be investigated for chimerism.



A) Mature embryos with one cotyledon attached cultured on osmotic medium
B) Regeneration of bombarded embryos on selective medium
C) Growth of plantlets on basal MS medium
D) Hardening of plantlets

Figure 1: Particle gun-mediated genetic transformation in pigeonpea variety PAU 881 using cry1Ac gene

S. No.	Name of transformation method	No. of mature embryos (with one cotyledon attached) transformed	No. of putative plants	No. of PCR positive plants	Transformation frequency (%)
1	Particle gun	500	250	10	4.0
2	*Agrobacterium*	500	100	1	1.0

Table 1: Comparison of transformation methods for genetic transformation of pigeonpea variety PAU 881 using cry1Ac gene

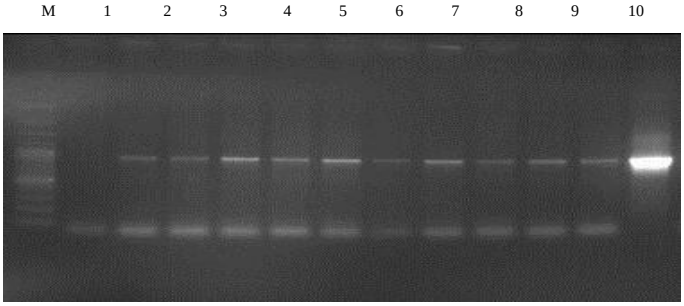


Figure 2: PCR analysis of putative transgenic plants of pigeonpea obtained through particle gun-mediated transformation. M: 100 bp plus DNA ladder, 1-11: DNA of putative transgenic plants, 12: Positive control (plasmid DNA)

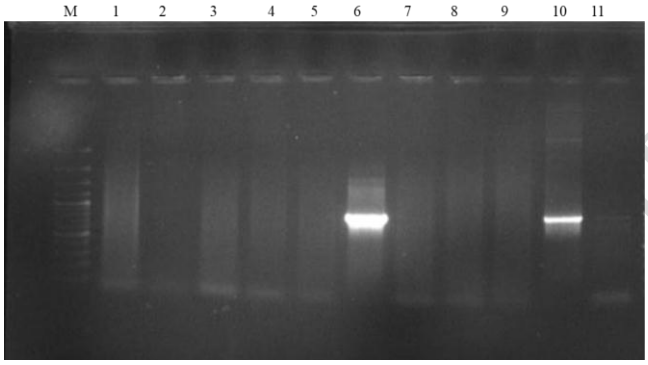


Figure 3: PCR analysis of putative transgenic plants of pigeonpea obtained through Agrobacterium-mediated transformation. M: 100 bp plus DNA ladder, 1-9: DNA of putative transgenic plants, 10: Positive control (plasmid DNA), 11: Negative control (water)

ACKNOWLEDGEMENTS

Financial support from UGC, New Delhi is gratefully acknowledged.

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