Agrobacterium tumefaciens-mediated transformation of pigeon pea (*Cajanus cajan* L. Millsp.) and molecular analysis of regenerated plants

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Transformation of pigeon pea (*Cajanus cajan* L. Millsp.) was achieved using *Agrobacterium tumefaciens* strain GV2260, containing the construct of isolated cowpea protease inhibitor gene (*pCPI*; Accession no.: AJ271752). The gene was driven by CaMV 35S promoter containing kanamycin resistance as plant selection marker. Embryonic axes excised from seeds germinated on MS basal supplemented with BAP (2 mg/l) were used as explants. Transformed calli were selected on medium containing kanamycin (50 μg/ml) after 3 weeks of incubation. Thereafter, the green calli were kept for differentiation. MS basal medium containing IAA (0.2 mg/l) and different concentrations of BAP was tested for the regeneration frequency of explants (embryo axes and leaves). Rooting ability of differentiated shoots was tested on half MS basal medium containing different concentrations of IBA. Molecular analysis of the transformed plants was done by Northern techniques.

DEVELOPMENT of a reliable regeneration system coupled with efficient transformation using an *Agrobacterium*-based system has been a major limitation in developing transgenic pulse varieties with useful genes[1]. *Agrobacterium*-mediated transformation and regeneration of transgenic plants have been reported in soybean[2], peas[3], chickpea[4], peanut[5] and cowpea[6]. Plant regeneration from different explants of asceptically grown seedlings of pigeon pea[7–9] has also been previously reported, but none of these protocols are ideal because of difficulty in reproducibility and their very low regeneration frequency. In the present investigation the regeneration frequency of embryonic axes and leaf disc were studied and the cowpea protease inhibitor gene (CPI) isolated in our lab from a native variety (V-130) was used to transform pigeon pea using embryonic axes as explants. Molecular analysis was carried out to show the chromosomal integration and expression of the transgene.

Seeds of pigeon pea (*Cajanus cajan* L. var. Pusa 855) obtained from pulse lab, Indian Agricultural Research Institute (IARI), New Delhi were treated with concentrated sulphuric acid for 1 min, and thereafter washed thoroughly in sterile water until the pH of the water was 7.0. The seeds were soaked in sterile water for 2 h and placed on MS basal medium[10] under aseptic conditions in 90 × 15 mm petri dishes, with 15 seeds per plate. The MS basal medium contained 3% sucrose and 0.7% agar (Qualigens, India) and 2 mg/l, 6-benzylaminopurine (BAP). The petri dishes were incubated at 23 ± 2°C under 16 h photoperiod and light was provided by cool fluorescent lamps. After 3 days of seed germination, the embryonic axes were excised, from the cotyledons, decapitated and placed on MS medium containing 2 mg/l BAP for a week. In another set, seeds were allowed to germinate for one week for emergence of the first two leaves in seedlings. These leaves were excised from the hypocotyl region and cut into pieces of ~ 2–3 mm and were placed along their abaxial side on MS medium containing 2 mg/l BAP for a week in culture tubes. Each tube contained 2 pieces of the leaf. For callusing and regeneration of the explants (embryonic axes and leaf), these were placed on MS medium containing 3% sucrose, 0.7% agar, containing 0.2 mg/l indoleacetic acid (IAA) and different concentrations of BAP (1, 2, 3 and 4 mg/l). Observations on their callusing and regeneration frequencies were recorded. For rooting, regenerated shoots were placed on half MS medium containing 2% sucrose, 0.7% agar and varying concentrations of indolebutyric acid (IBA). The rooting frequency, root length and percentage of root induction were recorded after a week.

The binary vector construct (*pCPI*) cloned in *Bin19* (ref. 11) having the cowpea protease inhibitor gene (1997 bp) was mobilized into *Agrobacterium tumefaciens* strain GV 2260 by freeze–thaw method[12]. The co-cultivated explants were transferred to MS medium (0.7% agar) containing 50 μg/ml kanamycin, 2 mg/l BAP, 0.2 mg/l IAA and 125 μg/ml cefotaxime. These were incubated at 23 ± 2°C and 80% relative humidity, under light of 3000 lux with 16 h photoperiod. Only the putative transformed explants started callusing in about a week and remained green, which were subsequently subcultured in the same medium for 3 weeks. These calli were further subcultured in the same medium till their shoots elongated in about 8 weeks. Rooting of the regenerated shoots was accomplished on half MS medium containing 2% sucrose, 0.7% agar and 0.5 mg/l IBA. The roots started growing in a week, with many lateral roots. The plants at this stage were taken for Northern analysis.

For Northern blotting, total RNA was isolated from four transformed plants and one nontransformed plant using 50 mg leaf sample from each individual plant and run on 1.2% formaldehyde gel[13]. The gel was blotted onto Hybond N^+-membrane[14] and probed using the isolated (100 ng) cowpea protease inhibitor gene (insert excised from *Bin19* using *Bam*HI and *Sal*I) labelled with a^{32}P-dCTP (specific activity 3000 Ci/mmole). The labelling was performed using Promega™ nick translation kit. The hybridization and washes were done at 65°C sequentially with (i) 2 × SSC/0.5% SDS, (ii) 1 × SSC/0.1% SDS and (iii) 0.5 × SSC/0.1% SDS in a hybridization oven and autoradiographed.

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Treatment of seeds with concentrated sulphuric acid increased the germination to about 95–100% compared to just 10–15% in untreated ones. This acid treatment of seeds also prevented the accumulation of phenolics in the medium, which is a major germination inhibitory factor. The embryonic axis explants on an average induced 6.5 shoots/explant when cultured on 0.2 ppm IAA and 2 ppm BAP (Table 1). After 2 weeks of incubation, the leaf explants increased in size and induced callus from cut ends. Although induction of multiple shoots occurred after 4–5 weeks, there was no further proliferation of shoots on sub-culture. The medium composition which gave the best result was 0.2 mg/l IAA and 2 mg/l BAP. These results showed better regeneration and shooting capacity of embryonic axes compared to leaf explants. The production of plantlets from callus shows negative correlation of the length of callus phase with that of regeneration ability. When the callus phase is reduced, the regeneration frequency tends to increase. When the embryonic axes was decapitated, the cells of the wounded area were capable of forming meristematic apex with less callus formation under the stimulation of phytohormones and thus provided a favourable condition for *A. tumefaciens*-mediated transformation. The pigeon pea leaf explants are generally found to be recalcitrant as reported by Kumar *et al.*[15] and were about 8–20% from leaf and cotyledonary calli as explants on modified Blayde's medium containing KN (0.2 mg/l) and NAA (0.01 mg/l). Geeta *et al.*[16] found that regeneration capacity of leaf explants was low compared to other explants. The decapitated embryonic axes of chickpea[4] had been used to transfer *GUS* and *npt*II gene by *A. tumefaciens* strain LBA 4404.

The average number of roots/shoots was also more in the embryonic axis and also longer root length was observed when compared to leaf on the same medium (Table 2). The roots formed in this medium were healthy with well-developed lateral roots and percentage of root induction was 78.3. The above results clearly show the better response of embryonic axes for regeneration and rooting compared to leaf discs. The embryonic axes were excised from seeds germinated on MS medium with 2 ppm BAP for three days and precultured for a week on MS medium with 2 ppm BAP. This makes the embryonic axes highly susceptible to *Agrobacterium* infection. Although members of *A. tumefaciens* are considered as wide host range pathogen, the ability of the bacterium to produce a compatible reaction varies widely among host plant species and even among genotypes within a species[17]. Two strains were tested in this study for their virulence (LBA 4404 and GV 2260) following the protocol of Rathore and Chand[18] and strain GV 2260 gave better results with Pusa 855 pigeon pea variety.

The explants precultured for 1 week (Figure 1 *a*) were cut/wounded and decapitated for better infection with *Agrobacterium* and the co-cultivated explants transferred to selection medium (MS + kanamycin) in dark. After one week, these explants which had just started callusing were transferred to MS medium with kanamycin (50 μg/ml) supplemented with 2 mg/l BAP, 0.2 mg/l IAA and 125 μg/ml cefotaxime. The calli resuming within 1 week were kanamycin resistant. These remained green in colour even after 3 weeks (Figure 1 *b*). The calli were allowed to grow for 3 weeks in this medium. The kanamycin-sensitive untransformed calli changed to white and started browning (Figure 1 *c*). Table 3 gives the callus induction and shoot

Table 1. Regeneration of shoots from embryonic axis and leaf explants in pigeon pea (Pusa 855)

IAA phytohormones (mg/l)	BAP phytohormones (mg/l)	Percentage of response		Average no. of shoots/explant	
		Embryonic axis	Leaf	Embryonic axis	Leaf
0.2	1	36 ± 2.22	25 ± 1.23	5.8 ± 0.08	4.4 ± 0.04
0.2	2	59 ± 2.16	30 ± 2.06	6.5 ± 0.04	5.0 ± 0.07
0.2	3	29 ± 1.87	18 ± 0.98	3.8 ± 0.05	2.0 ± 0.04
0.2	4	19 ± 1.01	15 ± 1.03	2.0 ± 0.03	1.5 ± 0.03

Data represent an average of 200 explants tested in quadruplicate.

Table 2. Induction of roots in embryonic axis and leaf as explants of pigeon pea (Pusa 855)

IBA (mg/l)	Percentage of response		Average no. of roots/explants		Root length (cm)	
	Embryonic axis	Leaf	Embryonic axis	Leaf	Embryonic axis	Leaf
0.25	5.1 ± 1.61	23.0 ± 1.12	2.8 ± 0.07	1.5 ± 0.04	1.6 ± 0.04	1.4 ± 0.022
0.50	78.3 ± 4.30	37.0 ± 1.31	6.2 ± 0.11	2.2 ± 0.09	3.9 ± 0.08	1.5 ± 0.02
0.75	30.1 ± 1.96	22.0 ± 1.06	4.1 ± 0.09	1.8 ± 0.07	1.2 ± 0.02	1.2 ± 0.03
1.0	21.2 ± 1.07	18.0 ± 0.98	1.0 ± 0.06	1.0 ± 0.03	1.0 ± 0.02	1.1 ± 0.02

Data represent an average of 200 explants tested in quadruplicate.

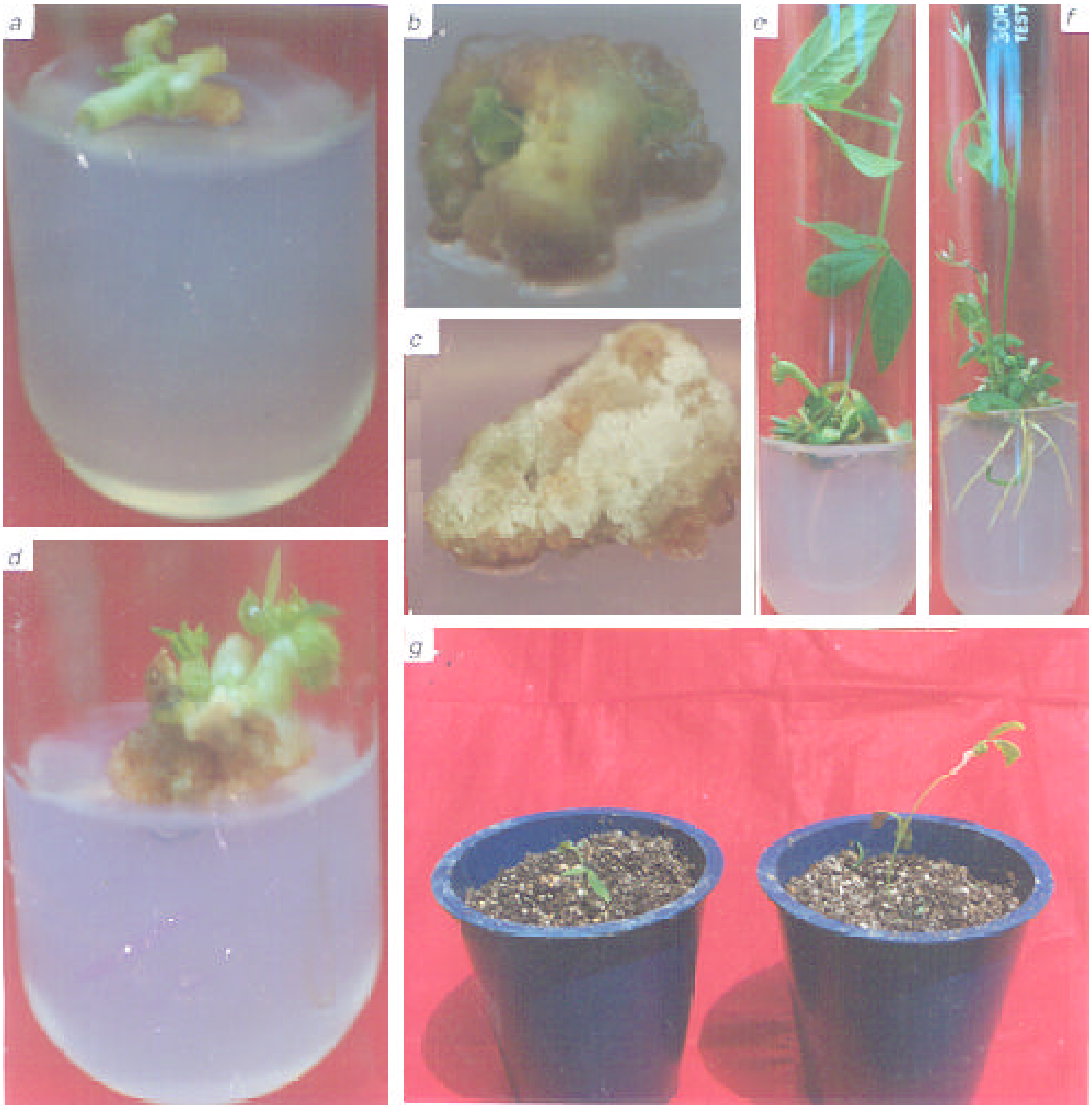

Figure 1. ***a***, Embryonic axis of pigeon pea; ***b***, Transformed callus on MS + kanamycin medium; ***c***, Untransformed callus on MS + kanamycin medium; ***d***, Shoots emerging from transformed callus; ***e***, *In vitro* plant (8 weeks); ***f***, Transformed rooted plant; ***g***, Transformed plants in pots.

bud emergence percentage of putative transformants; about 213 calli (23.8%) were obtained which were green in colour in selection medium. They were further subcultured in the same selection medium and shoot bud initiation started in about 4 weeks (Figure 1 *d*). However, out of 213 calli, only 11 (1.2%) showed shoot bud initiation when placed in MS medium with 2 mg/l BAP, 0.2 mg/l IAA along with 125 µg/ml cefotaxime and 50 µg/ml kanamycin. As shown in Table 3, only 6 calli developed shoots (2 cm) in 2–3 weeks and these were maintained under constant selection pressure of kanamycin (50 µg/ml). Figure 1 *e* shows the eight-week-old *in vitro* cultured transformed plant which has differentiated into shoots and leaves.

Various researchers have developed many methods of shoot regeneration in pigeon pea[8,19,20], but none of the above protocols was reproducible for the variety selected in this study. The standardized regeneration protocol was found consistent with all the batches of explants used in the present study. The calli after 9 weeks of *in vitro* culture (Figure 1 *f*) had differentiated into roots and leaves and were ready to be transferred into pots (Figure 1 *g*) for hardening.

We have earlier reported the integration of the transgene by PCR amplification and Southern hybridization in the transformed plant[11]. The expression of the transgene to the mRNA level is demonstrated by northern analysis in the present study. The total RNA was isolated from 8-week-old *in vitro* cultured transformed pigeon pea leaves and run on 1.2% formaldehyde agarose gel (Figure 2 *a*). All the four transformed plants showed a signal at about 600 bp (Figure 2 *b*). Considering the fact that the number of nucleotide bases in the predicted coding region is only 504, the hybridizing band obtained in the Northern hybridization could be the mRNA from the coding region. The mRNA isolated form cowpea inhibitor of IV was 583 nucleotides long[21] and this compares well with the present Northern hybridization results. The characterization of Bowman–Birk protease inhibitor mRNA of soybean separated on denaturing agarose gel was found to be 420 nucleotides long[22]. As seen from these results, the coding regions for these protease inhibitors are very small without introns and with the mRNA size ranging from 400 to 600 nucleotides. Newell *et al.*[23], analysed the transformed sweet potato leaves for the expression of cowpea trypsin inhibitor by RNA dot blot hybridization using ^{32}P-dCTP labelled probe comprising the entire coding region of cowpea trypsin inhibitor gene. Though this method can detect whether a particular gene is expressed in certain plant tissues, it may not give the actual size of the mRNA encoded by the gene.

In the present study, the Northern blot analysis showed that all the transformed pigeon pea plants are able to express the cowpea protease inhibitor at mRNA level.

Table 3. Pigeon pea transformation frequency using *A. tumefaciens* (GV 2260) possessing a cowpea protease inhibitor expression cassette

Batch no.	No. of explants co-cultivated	No. of callus explants on MS + kanamycin	Percentage of explants showing callus induction	No. of calli with shoots	No. of shoots per callus
1	108	20	18.5	0	0
2	97	27	27.8	0	0
3	95	23	24.2	1	2
4	96	29	30.2	1	1
5	105	22	20.9	0	0
6	108	19	17.4	2	2
7	93	22	23.6	1	1
8	98	24	24.4	1	3
9	98	27	22.5	0	0
Total	898	213	23.8	6	

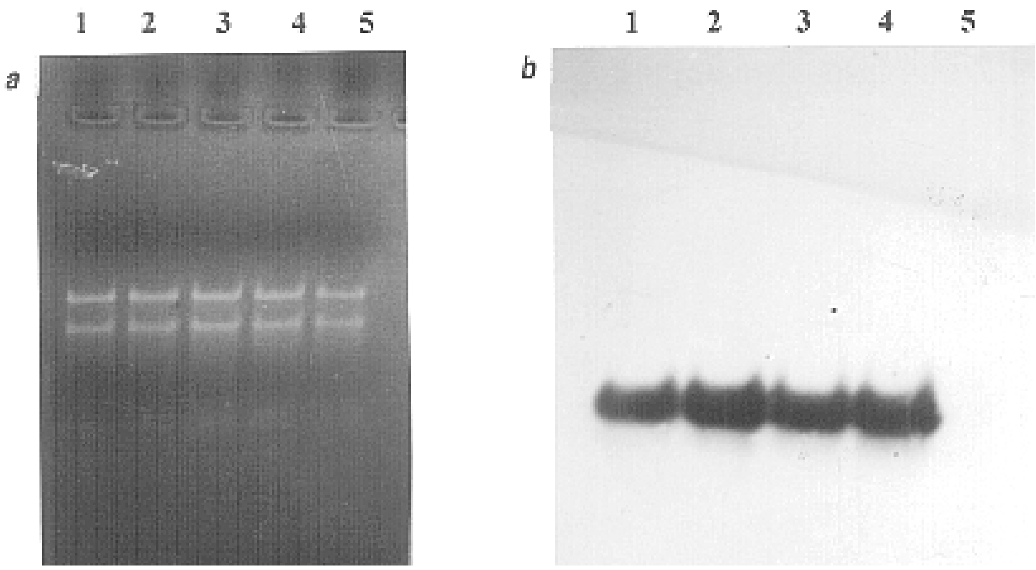

Figure 2. ***a***, Total RNA isolated from *in vitro* cultured plants. Lanes 1–4, Transformed plants; Lane 5, Untransformed plant; ***b***, Northern hybridization of total RNA with cowpea protease inhibitor probe. Lanes 1–4, Transformed plants; Lane 5, Untransformed plant.

Though there have been cases of transcriptional gene silencing[23], we did not observe any gene silencing at the mRNA level. These pigeon pea plants expressing the cowpea protease inhibitor gene should now be brought to field conditions and multiplied by selfing. This is a step forward in developing transgenic pigeon pea resistant to chewing insects, mainly pod borers. Further, construction of expression cassettes containing two or more insect resistance genes with divergent modes of action would give protection against a wide range of pests. Also, a targetted expression of such insecticidal genes by tissue-specific/wound-inducible promoters is expected to provide high levels of insecticidal proteins in the target tissue. The above aspects are under investigation in our laboratory, to develop more practical strategies to use these genes to develop transgenic plants resistant to insect pests for sustainable agriculture.

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New stable isotope records of sediment cores from the SE Arabian Sea – Inferences on the variations in monsoon regime during the late Quaternary

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We reconstruct here the changes in regional hydrography related to the fluctuations in Indian monsoons during the late Quaternary based on the stable isotope composition of the foraminifers and organic matter in three sediment cores from the upper continental slope of western India. The planktic foraminiferal d^{18}O contrast between the Last Glacial Maximum (LGM) and Holocene (Δd^{18}O), after correcting for the global 'ice effect' is relatively high (0.8–1.0‰), suggesting notable changes in the sea surface conditions. This includes a moderate sea surface cooling (~ 2°C) and an enhanced evaporation (increasing salinity by ~ 2.5 p.s.u.) during the LGM and/or increased precipitation during the early Holocene. The diminished Δd^{18}O value of benthic records (~ 1.0‰) appears to be a basin-wide phenomenon along the shallow depths of the upper continental slope and is related to the eustatic sea level fluctuations. Carbon isotope composition of the organic matter suggests that primary productivity was the main source of organic carbon along this margin throughout the late Quaternary.

THE modern surface circulation and hydrography in the northern Indian Ocean and rainfall over Indian subcontinent are intimately related to the seasonal variations in the Indian monsoon system. The strong seasonality in monsoons also leads to large changes in the marine productivity, with highest productivity values observed during the summer monsoons[1,2]. Information on the late Quaternary (past ~ 130,000 years) variations in Indian monsoons is important in assessing the natural environmental changes that have taken place in the past and that may occur in the

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