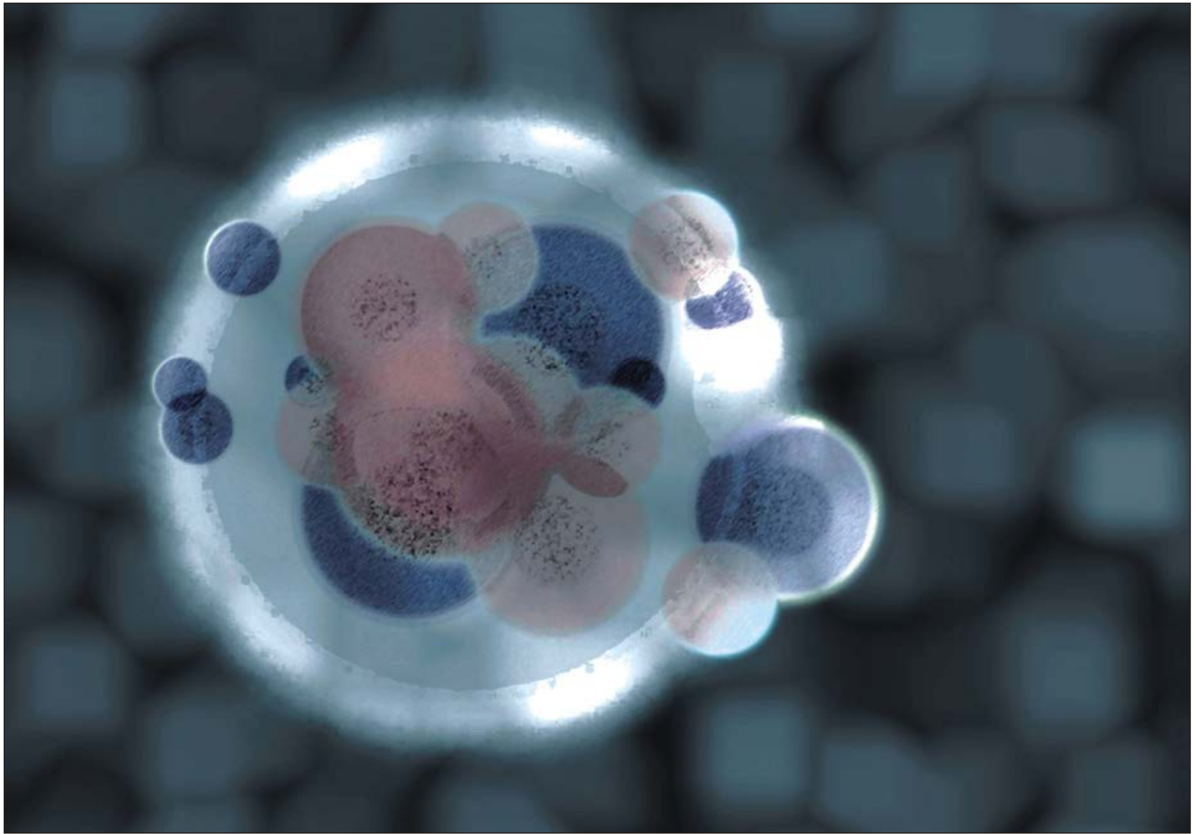

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Differential Response of *Cajanus cajan* Varieties to Transformation with Different Strains of *Agrobacterium*

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Abstract: Stable transformation followed by regeneration and subsequent expression of genes is important in the production of transgenic plants. Plumule and nodal segments of embryo, cotyledons and nodes from 10-12 day old seedlings of pigeonpea genotypes were cocultivated with different strains of *Agrobacterium tumefaciens* containing Ti plasmid and *gus* reporter gene. Transformation frequencies measured as number of explants showing *gus* expression was studied. It was observed that the transformation frequency is explant, genotype and *Agrobacterium* strain dependent. The transformation frequency was higher in ICPL87 and nodal explant in all genotypes studied. *Agrobacterium* strain LBA4404 was more compatible in giving higher frequency of transformants with all explants of all genotypes.

Key words: Pigeonpea, *Agrobacterium*, transformation

INTRODUCTION

Pigeonpea is one of the important protein yielding grain legumes of semi-arid tropics in India. Due to its importance as a pulse crop, pigeonpea improvement in terms of protein quality, stress tolerance and pest tolerance is necessary. Developing resistant varieties is possible by conventional interspecific hybridization or introduction of resistant genes through genetic engineering.

For production of transgenics, successful regeneration is important. In pigeonpea, there are several successful reports using different varieties and different explants. Regeneration was obtained via callus cultures (Kumar *et al*., 1983), direct differentiation from leaf discs (Eapen and George, 1993; Geetha *et al*., 1998), cotyledons (Geetha *et al*., 1998; Mehta and Mohan Ram, 1980), cotyledonary nodes (Geetha *et al*., 1998; Prakash *et al*., 1994). Regeneration through multiple shoot induction (Geetha *et al*., 1998; Franklin *et al*., 1998, 2000) and somatic embryogenesis (Patel *et al*., 1994; Kulkarni and Krishnamurthy, 1989) was also reported. Protoplast regeneration upto callus stage has also been reported (Sarangi *et al*., 1992; Sagare *et al*., 1997). We successfully obtained regeneration from cotyledons, nodes from 10-12 day old seedlings, plumule and nodal segments of embryo.

Agrobacterium is capable of infecting intact cells and introduces one to several copies of the transformed DNA into the plant genome. Successful introduction of *gus* reporter gene through *Agrobacterium* was reported by using embryo axis (Geetha *et al*., 1999; Lawrence and Koundal, 2001; Prasad *et al*., 2004; Satyavathi *et al*., 2003), cotyledonary nodes (Geetha *et al*., 1999; Prasad *et al*., 2004) and leaf disks (Arundhati, 1999). We report transformation of *gus* reporter gene in segments of embryo, cotyledons and nodes from 10-12 day old seedlings.

MATERIALS AND METHODS

Seeds of different pigeonpea cultivars viz LRG 30, ICPL 87 and ICPL 85063 were obtained from market yard, Guntur. The *Agrobacterium* strain LBA 4404 carrying the plasmid pBI121 and pAD288 and *Agrobacterium* strain GV 2260 were used for transformation studies. All the three plasmids have kanamycin resistance gene and β-glucuronidase gene. *Agrobacterium* strain LBA 4404 harbouring plasmid pBI121 has CaMV 35S promoter-*GUS*-nos poly A in pBIN 19 β-glucuronidase (*uid* A) genes with intron. *Agrobacterium* strain LBA 4404 harbouring plasmid pAD288 contain GS (glutamine synthetase) and peroxidase construct as (GS-TAP1). TAP represents tobacco anodic peroxidase and β-glucuronidase (*uid* A) genes (obtained from Prof. D.P.S. Verma, Ohio State University, Ohio, USA). *Agrobacterium* strain GV 2260 harbouring a binary plasmid pBI121 with β-glucuronidase gene was also used.

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All the explants were inoculated on MSB2 medium (MS medium supplemented with amgli BAP) with 3% sucrose and 0.8% agar and maintained at a photoperiod of 16 h light and 8 h dark at 25±2°C for regeneration experiments. For transformation, the explants viz plumule and nodal segments of embryo and cotyledons were excised from seeds that were surface sterilized with 0.1% mercuric chloride and soaked overnight. Nodes from 10-12 day old seedlings were excised from seeds germinated on MSB2 medium for 10-12 days. The bacterial cultures were grown overnight at 28°C in liquid LB medium (1% tryptone, 0.5% yeast extract and 1% sodium chloride at pH 7.0) containing 50 mg L^{-1} kanamycin. The *Agrobacterium* cells of 0.6 O.D (50 mL) were pelleted at 4000 g for 5 min and resuspended in MS medium (50 mL). The explants were inoculated for infection in 10 mL of bacterial suspension for 10 min.

After 10 min incubation in bacterial suspension, the explants were blot-dried on sterile filter paper and cocultivated 3 days under dark at 25±2°C. After cocultivation for 3 days, explants were washed thoroughly and blot-dried on sterile filter paper and cultured for a week on MSB2 containing 75 mg L^{-1} kanamycin and 250 mg L^{-1} cefotaxime. Half of the explants were used for histochemical assay according to protocol (Hiei *et al*., 1994) and rest half were left for further growth.

DNA was isolated from the leaves of the plants growing in vermiculite as per the modified method (Dellaporta *et al*., 1983) and DNA was used for PCR analysis. The experiment was carried out in two replicates, each time with 30 explants with all the varieties.

RESULTS

Different explants of three genotypes when cocultivated with different strains of *Agrobacterium* for 3 days showed different frequencies of *gus* positives. Plumules and embryonal nodes of different genotypes showed 60-80%, nodes from 10-12 day old seedlings showed 40-58.3% and cotyledons showed 36.6-50% *gus* positives (Table 1, Fig. 1a-d). Nodal segments of embryo gave higher number of *gus* positives than the

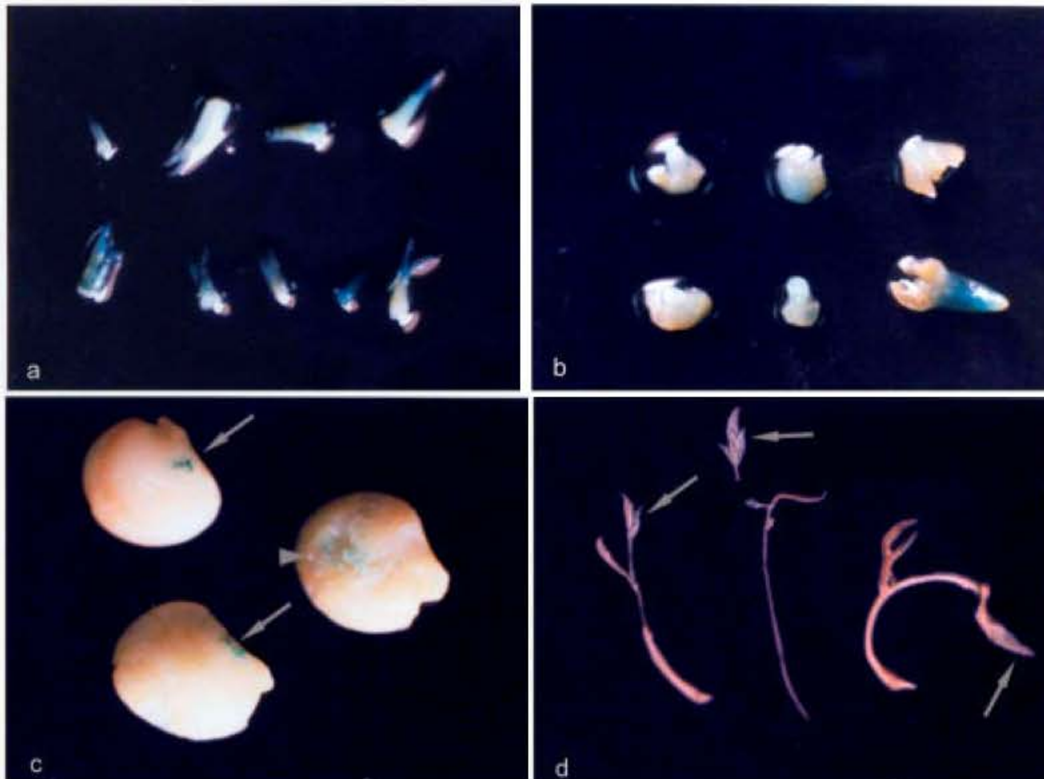

Fig. 1: GUS positives of different explants of pigeonpea. (a) Plumule segment of embryos showing GUS positives of LRG 30, (b) Nodal segments of embryos showing GUS positives of ICPL 85063, (c) Cotyledons showing GUS positives of ICPL 87. Arrows, unwounded cotyledons showing GUS positive regions close to excised regions. Arrow head: Wounded cotyledon showing GUS positive regions in the middle of the cotyledon and (d) Shooting grown from 10-12 day old noodes showing GUS positives of ICPL 87 (arrows)

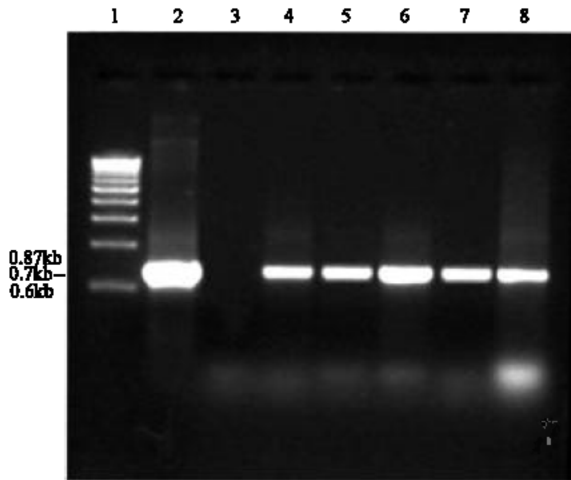

Fig. 2: Gel photograph showing PCR analysis of transgenic shoots of pigeonpea
Lane 1, DNA size marker; lane 2, plasmid pCAMBIA2300 (positive control); lane 3, Untransformed plant (negative control), lane 4, Transformed plant form plumule explant, lane 5, Transformed plant from embryonal nodal explant; lane 6, Transformed plant from 10-12 day old nodlal explant; lane 7, Transformed plant form cotyledon explant; lane 8, Transformed plant from plumule explant

Table 1: Frequency of *gus* positives from different explants of pigeonpea genotypes infected by different strains of *Agrobacterium tumefaciens*

Genotype	Explants	LBA 4404 (pBI121)	LBA 4404 (pAD288)	GV 2260 (pBI121)
LRG 30	Plumule	28/40 (70.0%)	26/40 (65.0%)	24/40 (60.0%)
	Node	44/60 (73.3%)	36/60 (60.0%)	40/60 (66.6%)
	Nodes from 10-12 day old Seedlings	30/60 (50.0%)	24/60 (40.0%)	30/60 (50.0%)
	Cotyledons	24/60 (40.0%)	24/60 (40.0%)	22/60 (36.6%)
ICPL 87	Plumule	32/40 (80.0%)	30/40 (75.0%)	26/40 (65.0%)
	Node	48/60 (80.0%)	46/60 (76.6%)	46/60 (76.6%)
	Nodes from 10-12 day old Seedlings	35/60 (58.3%)	30/60 (50.0%)	32/60 (53.3%)
	Cotyledons	30/60 (50.0%)	24/60 (40.0%)	30/60 (50.0%)
ICPL 85063	Plumule	30/40 (75.0%)	24/40 (60.0%)	24/40 (60.0%)
	Node	42/60 (70.0%)	36/60 (60.0%)	40/60 (66.6%)
	Nodes from 10-12 day old Seedlings	30/60 (50.0%)	24/60 (40.0%)	24/60 (40.0%)
	Cotyledons	30/60 (50.0%)	24/60 (40.0%)	24/60 (40.0%)

other explants used. *Agrobacterium* strain LBA 4404 carrying binary plasmid pBI121 showed better response than other strains. Genotype ICPL 87 gave higher transformation frequencies than the other genotypes used.

The explants which showed *gus* positive response were used for DNA isolation. This DNA was then subjected to PCR amplification. Npt gene was amplified from DNA of positive control (plasmid DNA pCAMBIA 2300), negative control (Cajanus non transformants) and from the regenerated plantlets of all strains used (Fig. 2). The explants which displayed gus activity were used for PCR amplification but explants showing blue spots on only roots did not show amplification with npt gene.

DISCUSSION

Genotypes, *Agrobacterium* strains, different explants, time of exposure to bacterial inoculum and other factors influence the efficiency of plant transformation. In the present study, varying frequencies of *gus* positives were obtained from various explants obtained from different varieties of pigeonpea.

The genotypic differences shown as percentage of *gus* positives was uniform in all explants used and infected with different *Agrobacterium* strains. Of the three varieties tested, the percentage of *gus* positives was highest in ICPL 87, followed by ICPL 85063 when compared to LRG 30 in all explants used (plumule, embryonal node, cotyledons and node from 10-12 day old seedling). These genotypic differences in pigeonpea for percentage of transformation could be explained by factors influencing the interactions between *Agrobacterium* strains vis-à-vis pigeonpea varieties. A strong host genotype and strain interactions exist and very few genotypes of the crop are infected by a given *Agrobacterium* strain. For production of transgenics, the first step is successful infection by bacterium and also successful incorporation into host genome. The site of incorporation of the gene is also important for its expression. Bacterial colonization, induction of bacterial virulence system, generation of T-DNA transfer complex, T-DNA transfer and integration of T-DNA into the plant genome are the essential steps for transgenic production (Gustavo *et al.*, 1998). The genotype variation for susceptibility to *Agrobacterium* infection in pigeonpea was reported (Rathore and Chand, 1997). Differences in susceptibility of the crop to *Agrobacterium* strains was also reported in legumes such as chickpea (Islam *et al.*, 1994), pea (Hobbs *et al.*, 1989), groundnut (Lacorte *et al.*, 1991) and soybean (Byrne *et al.*, 1987; Delzer *et al.*, 1990).

Keeping in view of T-DNA integration, the proposed model for illegitimate recombination (Gheysen *et al.*, 1991;

Lehman *et al*., 1994; Puchta, 1998) involves pairing of a few bases known as microhomologies, is required for a preannealing step between T-DNA strand (coupled with vir D2) and plant DNA. The differences in percentage of *gus* positives among different varieties of a crop as observed in the present study and also reported earlier could be due to differences in number of such microhomology sequences in genomic DNA.

When *Agrobacterium* strain LBA 4404 with plasmid constructs pBI121 and pAD288 were used for transformation, LBA 4404 with plasmid pBI121 yielded better results than *Agrobacterium* strain GV 2260 with plasmid pBI121 and LBA 4404 with pAD288 plasmid. This could be due to differences in vir gene factors, which influence infection by different *Agrobacterium* strains. In peanut (Egnin *et al*., 1998) more number of transformants was reported with *Agrobacterium tumefaciens* strain EH101 than with strain C58. Similar results were reported in *Vigna mungo* where the transformation frequencies were superior with bacterial strain LBA 4404 than those infected by EHA105 (Karthikeyan *et al*., 1996). So also in soybean, the octopine strain C58 and derivatives of the supervirulent succinamopine strain BO542 were found effective (Chandra and Pental, 2003). The combination and number of virulent genes involved in transcription influence the rate of infectivity (Gustavo *et al*., 1998). The *Agrobacterium* LBA 4404 with different plasmid constructs pBI121; pAD288 was used in the present study. LBA 4404 with pBI121 gave higher percentage of *gus* positives than with pAD288. The size of the plasmid and the type of sequences in the plasmid construct may play an important role in its incorporation and expression in the host genome. Several fold increase in the expression of reporter gene in rice was obtained using the construct with *gus* gene containing flanking tobacco MARS sequences (Allen *et al*., 1996; Cheng *et al*., 2001). Although such incorporation of MARS sequences was not done in the present study, probably the size difference is responsible for the difference in percentage of genetic transformants.

The type of explant used makes a difference for obtaining more number of transformants. Of all the explants used i.e., plumule, embryonal node, cotyledons and node from 10-12 day old seedling, embryonal node produced more number of *gus* positives than the other explants. This may be due to more number of meristamatic cells present in embryonal node for transformation than other explants. Present studies indicated that plumule of pigeonpea is also good for high frequency of transformations. The meristamatic cells at the shoot tip in plumule probably have more transformation capacity than differentiated cells in other explants. The potential for transformation in the embryonal node and plumule may be dependent on the number of dividing cells. Recombination between plasmid DNA and genomic DNA generally takes place during DNA replication (Matthysse, 1986). Embryonal node and plumule directly differentiate into shoot. Node from 10-12 day old seedling has more differentiated cells than dividing cells and hence transformant frequencies is less when compared to embryonal node. The axillary meristems at the junction of the cotyledon and the embryo axes contain cells that are competent for regeneration and could be useful targets for gene integration. *Agrobacterium* mediated transformation of cotyledonary node was reported in soybean (Byrne *et al*., 1987), pigeonpea (Geetha *et al*., 1999; Rathore and Chand, 1997), chickpea (Islam *et al*., 1994), pea (Hobbs *et al*., 1989) and groundnut (Lacorte *et al*., 1991).

Present results are in agreement with the statement (Potrykus, 1991) that tissues are formed by cells with different states of competence for transformation by *Agrobacterium*, which are determined by several factors including genotype, type of organ and growth stage.

Nodal explants from 10-12 day old seedlings and cotyledons gave nearly the same frequencies of transformation. The level of differentiation in these explants i.e., the chromatin state in partially differentiated cells may be responsible for this. Although the general percentage appears to be low, if efficient screening methods are available, the 10-12 day old node and cotyledons are the best explants for use in transgenic production as they can produce more number of shoots as observed from our regeneration studies (Data not shown). Based on these results, it is concluded that genotypic variation exists among *Cajanus* varieties for Ti plasmid incorporation. The types of *Agrobacterium* strain or plasmid construct also influence the infectivity and Ti plasmid incorporation into genome. The potential for incorporation of Ti plasmid is perhaps higher in explants containing more number of dividing cells.

PCR results support that *gus* expression in roots of some *Cajanus cajan* varieties is background expression but not due to *npt*II amplification, whereas *gus* expression in other parts indicated positive transformation event as indicated from *npt*II amplification. The claim that *Cajanus* varieties have native glucuronidase activity is confirmed but its expression is restricted to root meristem but not found in other parts of control plants.

We conclude that ICPL 87 is ideal for genetic transformation using *Agrobacterium* and the explants with more meristamatic cells have more chance for

transformation during cell division. In general genotypic differences play a role in transformation frequencies of *Cajanus cajan* and *Agrobacterium* also influence transformation frequencies.

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