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FACTORS ENHANCING TRANSFORMATION EFFICIENCY OF PIGEONPEA (*CAJANUS CAJAN*) BY *AGROBACTERIUM TUMEFACIENS*

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Some of the factors that modulate the transformation efficiency of pigeonpea (*Cajanus cajan* L.) were examined, using different strains of *Agrobacterium tumefaciens* harbouring binary plasmid with β-glucuronidase gene as a reporter gene under the transcriptional control of CaMV35S promoter. Factors examined were the genotype, *Agrobacterium* strains, cocultivation period, soaking time of seeds and use of acetosyringone. Transformation frequency was calculated by the number of *gus* positives given by embryonal segments. Transformation efficiency was higher when the explants were cocultivated for 3 days with overnight soaked seeds. The pigeonpea genotype ICPL 87 was more amenable to *Agrobacterium* transformation than LRG 30 and ICPL 85063. The *Agrobacterium tumefaciens* strain LBA 4404 with plasmid pBI121 was more superior in facilitating the transfer of *uid*A gene to pigeonpea explants compared to other strains (LBA 4404 with plasmid pAD288 and GV 2260). Addition of acetosyringone to the MS medium gave little higher transformation frequencies than without acetosyringone in the medium.

Keywords : *Agrobacterium*; *Cajanus cajan;* Transformation frequency.

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 is necessary in developing resistant varieties through introduction of resistant genes. Plant genetic transformation has allowed the introduction of specific genes into a variety of crop species, with *Agrobacterium tumefaciens* being the most common vector. The production of transgenic plants using *A.tumefaciens* in legumes has been limited to a few species including pea[1], alfalfa[2], soybean[3], chickpea[4] and pigeonpea[5-10] and peanut[11-14]. The efficiency of transformation and transgenic plant production depends on the establishment of suitable protocols for inoculation and later the direction and regeneration of transformed cells[15]. The factors influencing the efficiency of T-DNA transfer were reported by the type and age of the tissue[16,17], the cocultivation period[17-19] and the type[20] and duration of growth regulates pretreatment before inoculation[21]. *Agrobacterium* genotype and culture conditions were also important factors for efficient transformation[19,22-24].

Here, we report on some parameters that influence the transformation frequency of pigeonpea with *Agrobacterium tumefaciens*.

Materials and Methods

Plant Material: The pigeonpea cultivar ICPL 87 was used for soaking time and cocultivation experiments and three pigeonpea cultivars viz. LRG 30, ICPL 87 and ICPL 85063 were used for acetosyringone and comparison experiments. The seeds were first washed with soap water, then surface sterilized with 0.1% $HgCl_2$ for 10 min and followed by subsequent washes with sterile double distilled water for 5-6 times. Seeds were left for soaking in sterile double distilled water for 3 hrs and overnight for soaking time experiments and overnight soaked seeds were used for all the other experiments.

Bacterial strains and plasmids used: The *Agrobacterium* strain LBA 4404 carrying the plasmid pBI121 and pAD288 and *Agrobacterium* strain GV 2260 were used for transformation studies. They provide resistance to kanamycin, which was used as a selectable marker. *Agrobacterium* strain LBA 4404 carrying plasmid pBI121 contains CaMV 35S promoter-GUS-nos poly A in pBIN 19 β-glucuronidase (*uid*A) genes with intron and *Agrobacterium* strain LBA 4404 carrying plasmid pAD288 contains GS (glutamine synthetase) and peroxidase construct as (GS-TAP1). TAP represents tobacco anodic peroxidase and β-glucuronidase (*uid*A) genes (obtained

from Prof.D.O.S.Verma, Ohio State University, Ohio, USA). *Agrobacterium* strain GV 2260 harboring a binary plasmid pBI121 with β-glucuronidase (*uid*A) genes.

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 OD (50 ml) were pelleted at 4000g for 5 minutes and resuspended in MS medium (50 ml). This MS medium was used for infection of explants.

The explants viz. plumule and nodal segments of embryo were excised aseptically from 3hr and overnight soaked seeds in a laminar flow bench. The explants were infected with different strains of *Agrobacterium* for soaking and cocultivation experiments and *Agrobacterium* strain LBA 4404 carrying plasmid for acetosyringone experiment for 10 min with 0.6 OD and were placed on MS medium containing B_5 vitamins, 3% sucrose, 0.8% agar, BAP, Kn (100mM acetosyringone was supplemented in addition to MS medium for acetosyringone experiments) and cultured at 25 ± 2°C for 16/8 hr under light/dark conditions.

After 10 min co-cultivation with *Agrobacterium* culture (0.6 OD), the explants were blotted dry on a sterile filter paper and inoculated on MS medium. 40 explants (two replicates of each experiment were kept) each were left for 2days, 3days and 4days on medium before transfer on to antibiotic medium (MS medium supplemented with 250 mg l^{-1} cefotaxime and 75 mg l^{-1} kanamycin).

After three weeks on antibiotic medium, half of the explants were used for histochemical *gus* assay according to the protocol[25] and rest half were left for further growth. The leaves of grown plants were collected randomly for PCR analysis of 700bp nptII gene fragment.

Results and Discussion

Plumule and nodal segments treated with different *Agrobacterium* strains and transferred on to antibiotic medium turned green in three days. After three weeks on antibiotic medium, half of the plumules and nodal segments were used for testing *gus* activity (Fig 1) and half were left to grow on antibiotic medium.

Plumules and nodal segments tested for *gus* positive reaction from three genotypes with different *Agrobacterium* strains showed 60-80% *gus* positives (Table 1). Combination of ICPL 87 variety and LBA4404 with plasmid pBI121 gave higher percentage of *gus* positives. This variation in percentage of *gus* positives was observed when one genotype was treated with different *Agrobacterium* strains and there was genotypic variation when different varieties of pigeonpea were treated with one *Agrobacterium* strain.

The effect of cocultivation time on transformation frequencies was studied by using ICPL 87 variety and three strains of *Agrobacterium*. The samples of plumules and nodal segments were cocultivated for two, three and four days, respectively. Three days of cocultivation appears to be optimum from our results with three *Agrobacterium* strains tested on embryonal explants (Table 2). Four days of cocultivation resulted in reduced percentage of *gus* positives. Effect of soaking time was also studied using ICPL 87 and three strains of *Agrobacterium*. Seeds soaked overnight gave higher percentage of *gus* positives than those soaked for 3hrs uniformly using embryonal explants (Table 3) and three *Agrobacterium* strains.

Substitution of acetosyringone in the medium and using LBA4404 with pBI121 was used to study the effect on transformation frequencies. The results (Table 4) indicate once again that embryonal segments gave more number of *gus* positives in 3 days of cocultivation treatments. Comparison of *gus* positive frequencies with and without acetosyringone appears to be marginal from our results on embryonal explants of three varieties and different soaking times.

Half the explants which were left on antibiotic medium for further growth were acclimatized in the polyhouse. PCR analysis was done with DNA isolated from leaves of randomly selected *gus* positive plants from all experiments which showed amplification of a 700bp nptII gene fragment.

Genotypic differences of crop varieties, strain differences of *Agrobacterium*, regeneration potential of different explants, time of exposure to bacterial inoculum and other factors that influence the efficiency of transformation play an important role in production of transgenics.

In the present study, different frequencies of *gus* positives were obtained from different explants of different varieties of pigeonpea and when exposed to different *Agrobacterium* strains.

The genotypic differences in percentage of *gus* positives was uniform in all explants used and on infection with different *Agrobacterium* strains. Of the three varieties tested, the percentage of *gus* positives was highest in ICPL 87, higher in ICPL 85063 when compared to LRG 30 in the explants used (Plumule and embryonal node). These genotypic differences in pigeonpea for percentage of *gus* positives could be explained by factors influencing *Agrobacterium* strain – pigeonpea variety interactions. A strong host genotype and strain interactions exist and

Table 1. Comparative response of explants of Pigeonpea genotypes to different strains of *Agrobacterium tumefaciens* after three weeks of transfer of to antibiotic medium.

Genotype	Explants	LBA 4404 (pBI121)	LBA 4404 (pAD288)	GV2260 (pBI121)
LRG30	Plumule	56/80 (70.0%)	52/80 (65.0%)	48/80 (60.0%)
	Node	88/120 (73.3%)	72/120 (60.0%)	80/120 (66.6%)
ICPL 87	Plumule	64/80 (80.0%)	60/80 (75.0%)	52/80 (65.0%)
	Node	96/120 (80.0%)	92/120 (76.6%)	92/120 (76.6%)
ICPL 85063	Plumule	60/80 (75.0%)	48/80 (60.0%)	48/80 (60.0%)
	Node	84/120 (70.0%)	72/120 (60.0%)	80/120 (66.6%)

Table 2. Frequency of *gus* positives in response to the number of days of cocultivation of ICPL 87 genotype to various strains of *Agrobacterium*.

Number of cocultivation days	Type of explant	LBA 4404 (pBI121)	LBA 4404 (pAD288)	GV2260 (pBI121)
2 Days	Plumule	8/80 (10.0%)	8/80 (10.0%)	8/80 (10.0%)
	Node	16/80 (20.0%)	12/80 (15.0%)	12/80 (15.0%)
3 Days	Plumule	64/80 (80.0%)	60/80 (75.0%)	62/80 (77.5%)
	Node	64/80 (80.0%)	60/80 (75.0%)	62/80 (77.5%)
4 Days	Plumule	60/80 (75.0%)	56/80 (70.0%)	56/80 (70.0%)
	Node	56/80 (70.0%)	52/80 (65.0%)	48/80 (60.0%)

Table 3. Frequency of *gus* positives in response to soaking time of seeds of ICPL 87 genotype to various strains of *Agrobacterium tumefaciens*.

Soaking time of seeds	Type of explant	LBA 4404 (pBI121)	LBA 4404 (pAD288)	GV2260 (pBI121)
3 hrs	Plumule	48/80 (60.0%)	40/80 (50.0%)	44/80 (55.0%)
	Node	52/80 (65.0%)	48/80 (60.0%)	48/80 (60.0%)
overnight	Plumule	68/80 (85.0%)	60/80 (75.0%)	64/80 (80.0%)
	Node	64/80 (80.0%)	62/80 (77.5%)	60/80 (75.0%)

Table 4. Frequency of *gus* positives in different genotypes of pigeonpea in response to acetosyringone (100mM) and cocultivated with *Agrobacterium tumefaciens* LBA4404 (with plasmid pBI121).

Number of cocultivation days	Type of explant	Pigeonpea Genotypes					
		LRG30		ICPL 87		ICPL85063	
		AS-	AS+	AS-	AS+	AS-	AS+
2 days	Plumule	4/40 (10.0%)	12/100 (12.0%)	8/40 (20.0%)	20/100 (20.0%)	4/40 (100%)	12/100 (12.0%)
	Node	4/40 (10.0%)	12/100 (12.0%)	8/40 (20.0%)	20/100 (20.0%)	4/40 (100%)	12/100 (12.0%)
3 days	Plumule	24/40 (60.0%)	64/100 (64.0%)	32/40 (80.0%)	80/100 (80.0%)	24/40 (60.0%)	68/100 (68.0%)
	Node	28/40 (70.0%)	72/100 (72.0%)	32/40 (80.0%)	84/100 (84.0%)	28/40 (70.0%)	72/100 (72.0%)
4 days	Plumule	28/40 (70.0%)	72/100 (72.0%)	28/40 (70.0%)	72/100 (72.0%)	28/40 (70.0%)	72/100 (72.0%)
	Node	28/40 (70.0%)	72/100 (72.0%)	32/40 (80.0%)	80/100 (80.0%)	28/40 (70.0%)	76/100 (76.0%)

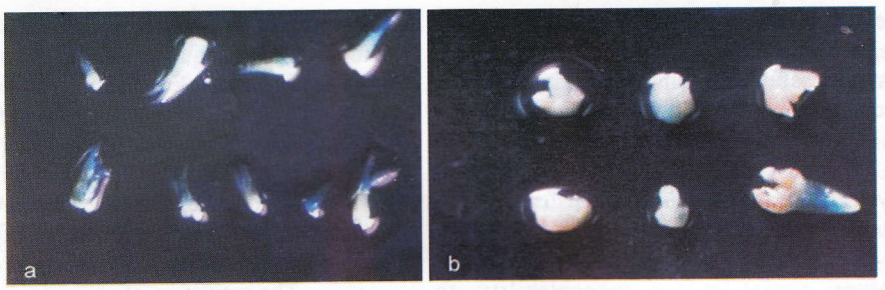

Fig.1. GUS positives of embryonal segments
a) Plumules; b) Embryonal nodes

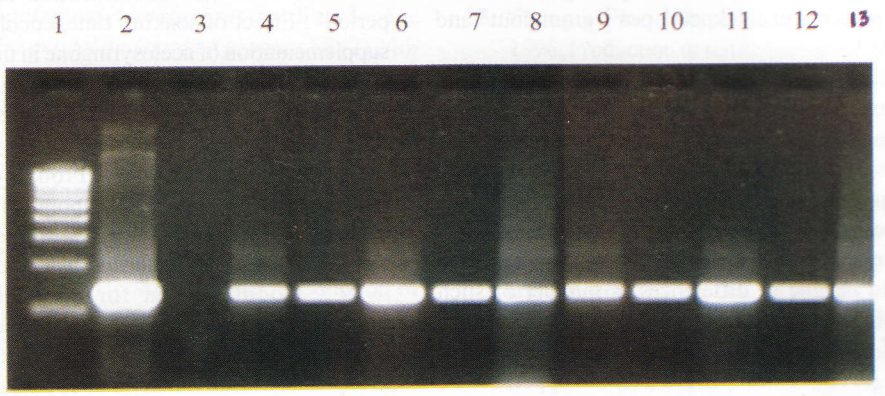

Fig. 2. PCR amplification of 700bp fragment of nptII gene in the genomic DNA of Pigeonpea randomly taken from all experiments.
Lane 1: DNA size marker; Lane 2: Plasmid pCAMBIA2300 (Positive control) ; Lane 3: Non-transformed

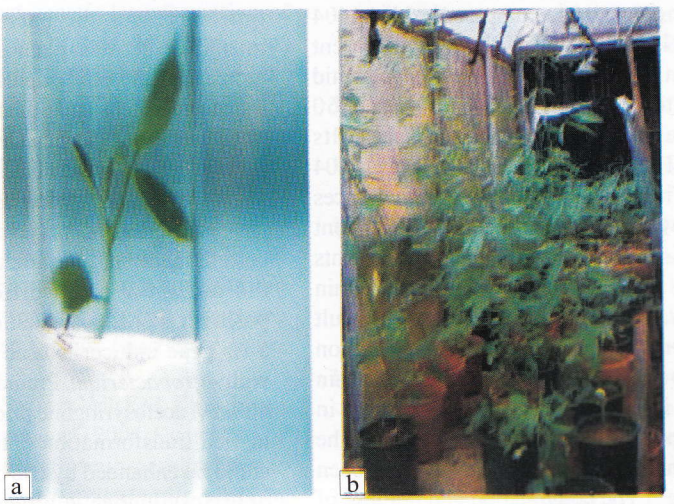

Fig. 3. Regenerated transformed plant.
a) Regenerated plantlet *in vitro*; b) Regenerated plantlet in the polyhouse

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, second step is successful incorporation into host genome and thirdly the sites of incorporation are 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 plant genome are the essential steps for transgenic production[26]. The genotype variation for susceptibility to *Agrobacterium* infection in pigeonpea was reported[27]. Difference in susceptibility of the crop to *Agrobacterium* strains was reported in chickpea[27], pea[22], groundnut[29] and soybean[23, 30,31].

Keeping in view of T-DNA integration, the proposed model for illegitimate recombination[32-34] 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.

Of the *Agrobacterium* strains with different constructs LBA4404 with pBI121 plasmid appears to be the best for transformation from the results obtained with different varieties of pigeonpea. *Agrobacterium* strain LBA4404 with plasmid pAD288 and *Agrobacterium* strain GV2260 with plasmid pBI121 showed relatively low percentage of *gus* positives when compared to LBA4404 with plasmid pBI121. In the present study, two different plasmid constructs in strain LBA4404 were used; plasmid pBI121 incorporated into LBA4404 and GV2260 *Agrobacterium* was also strains used. The results indicated plasmid pBI121 gave better results with LBA4404 than with GV2260. This can be explained as the differences in *vir* gene factors, which increase infection in different *Agrobacterium* strains. In peanut[35] , more transformants were obtained with *Agrobacterium tumefaciens* strain EH101 than *Agrobacterium* strain C58. A similar result was reported in *Vigna mungo* where the transformation frequencies are superior if done with bacterial strain LBA4404 to those infected by EHA105[36]. So also in soybean, the octapine strain C58 and derivatives of the supervirulent succinamopine strain BO542 have been found to be effective[37]. The combination and number of virulent genes involved in transcription influence the rate of infectivity[26]. The *Agrobacterium* LBA4404 with different plasmid constructs pBI121; pAD288 used in which LBA4404 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. Use of *gus* gene with flanking tobacco MARS sequences gave several fold increase in expression of reporter genes in rice[38,39]. 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 *gus* positives.

The activation of *vir* system of *Agrobacterium* depends on external factors: temperature and pH[40], growth medium, inoculum concentration and cocultivation period[41]. Effect of soaking time, cocultivation time and supplementation of acetosyringone in the culture medium was studied in pigeonpea. Overnight soaked seeds showed much better infection by three *Agrobacterium* strains as shown by more number of *gus* positives than the seeds soaked for 3 hrs. Probably the changes in composition of cellular components ie., altered pH, enlarged cell size, activation of cell division in more number of cells are responsible for these differences.

Cocultivation for 2, 3 and 4 days with *Agrobacterium* gave different frequencies of *gus* positives when embryonal plumule and node of ICPL 87 were used as explants. Cocultivation for three days gave more number of *gus* positives than with two days of cocultivation. There is marginal difference between three and four days of cocultivation and slightly more number of *gus* positives in three days of cocultivation. Increase in cocultivation time increases the bacterial population and hence, interaction between plant and bacterial cell increases. But increase in bacterial population also effects the growth of transformed cells and probably resulted in slight reduction in percentage of *gus* positives in four-day of cocultivation. Similar results were obtained in peanut[35,42,43] and mung bean[41] by using *Agrobacterium rhizogenes*.

When acetosyringone was supplemented in culture medium, plumule and nodal explants of embryo did not show much difference in frequency of *gus* positives. For transformation of cereals, acetosyringone, a *vir* gene inducer is used for cocultivation of explants with *Agrobacterium*. Preinduction of *Agrobacterium* with 400mM acetosyringone prior to cocultivation is important in rice transformation[44]. Infectivity of *Agrobacterium* could be enhanced by use of tobacco leaf extract instead of acetosyringone in groundnut[45].

In our experiment, the improvement was not significant with the three genotypes used, when acetosyringone was supplemented in the medium. This may be due to the quantity of acetosyringone and other

phenolic compound produced was optimum for induction of virulence in these varieties. Further, addition of external supply of acetosyringone may not improve the induction of *vir* genes during infection. Leaf explants of pigeonpea variety ICPL 5164 showed increased transformation frequency when acetosyringone was supplemented in media[46]. Genotypic variation may be present for production of phenolic compounds in pigeonpea which may influence infectivity.

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