CHAPTER III

TRANSFORMATION OF EMBRYONIC STRUCTURES OF PIGEONPEA GENOTYPES WITH SALT TOLERANCE GENES

3.1. INTRODUCTION

Pigeonpea (*C. cajan*) is an excellent and crucial grain legume of the Indian sub-continent that is well known for its high dietary protein content. There is prime importance for it to be genetically improved to increase its tolerance against abiotic stress. The abiotic stresses comprising high salt concentration and drought are affecting the growth and productivity of plants. Introduction of genes via genetic engineering offers a good solution to this problem. Development of genetically improved transgenic lines through direct DNA transformation is the promising step towards abiotic stress.

The abiotic stress is the main cause for decreasing the average yield of crop plants by more than 50% (Mahajan and Tuteja, 2005). It imposes both osmotic stress (high solute concentration in the soil) and ion-specific stress (altered ratios of ions). The strategies to overcome abiotic stress include genetic transformation with genes responsible for the synthesis of osmoprotectants, ion transporters and accumulation of osmoprotectants (Qiao *et al.*, 2010). The halophytes can sequester and cause buildup of salt into the cell vacuoles which prevents the upsurge of ions in the cytosol and maintain a high cytosolic ratio of K^+/Na^+. The salinity tolerance mechanisms involve the reduction of Na^+ absorption and increase in its pumping from the cells.

Genetic transformation can be defined as the sequence of delivery, controlled integration into a recipient genome and successful expression of the foreign genes. It can be achieved using techniques such as direct uptake of nuclei, chromosomes, organelles, isolated protoplasts, microinjection and electroporation. With the advent of genetic transformation techniques,

tolerance genes are inserted into a plant genome to enhance resistance towards salinity. Genetic transformation of plants requires markers, which confirm the presence of transformed DNA (Walden, 1989, Klein *et al.,* 1989) and also used for stable transformation, allowing living cells and tissues to grow under conditions that prevent the growth of untransformed tissues (Bowen, 1993).

Gene delivery systems can be divided into two types: 1) Direct gene transfer which is mediated by physical or chemical forces for delivery of the gene into plant protoplasts, cells and even tissues without a vector; and 2) *Agrobacterium*-mediated gene transfer, where *Agrobacterium tumefaciens* can be used as vectors for introducing the gene into the plant cell genome. *Agrobacterium*-mediated gene transfer is the most commonly used as it is less expensive, simple, and well established in a variety of dicotyledonous as well as monocotyledonous plants. It does not require protoplast culture and is relatively easy to obtain high transformation frequencies. The combination, number and activation of *vir* genes of *Agrobacterium* depends on external factors such as temperature, pH (Jeon *et al.,* 1998), growth medium, inoculation concentration and co-cultivation period (Pawan *et al,* 1998) which also influence the rate of infectivity (Gustavo *et al.,* 1998). The size of the plasmid and type of sequences in the plasmid construct also play a central role in its incorporation and expression in the host genome.

A. tumefaciens is a soil bacterium and also called natural plant engineer as it has capability to transfer a specific DNA segment (T-DNA) and a set of expressible *vir* genes of the tumour-inducing plasmid into the genome of infected plants. These *vir* genes are steadily incorporated and transcribed into the host or target genome, which ultimately leads to crown gall disease (Binns and Thomashaw, 1988; Nester *et al.,* 1984). The transferred DNA fragment contain 38 border sequences, 25bp in length, oncogenes and genes coding for opines. These genes are highly homologous in sequence and they are present in a directly repeated orientation (Gelvin, 2003). The foreign DNA kept between the transferred DNA borders can be used for the transformation of plant cells (Zambryski, 1992; Zupan and Zambryski, 1995).

Successful transformation requires regeneration of whole plants from the explants used in the study. Genotypic differences among varieties, *Agrobacterium* strains, regeneration potential of different explants, and time of exposure to bacterial inoculum are the basic factors influencing for the success of genetic transformation. In addition, infection by bacterium, incorporation of gene into host genome and the infection site, bacterial colonization, the activation of the virulence system and subsequent interaction with plant genome, co-cultivation period, the use of antibiotics and selectable markers and culture temperature conditions are also essential (Gustavo *et al.,* 1998). In most of the crop transformation protocols, chemicals such as acetosyringone is recommended for *vir* induction (Hiei *et al.,* 1997; Zhao *et al.,* 2000 and Kumlehn *et al.,* 2006).

Agrobacterium mediated gene transformation of embryonic structures of pigeonpea genotypes has been successfully done using different genes by several researchers (Lawrence and Koundal, 2001; Mohan and krishnamurthy, 2003; Geetha *et al.,* 1999; Satyavathi *et al.,* 2003; Prasad *et al.,* 2004; Surekha *et al.,* 2005; Verma and chand, 2005; Surekha *et al.,* 2007; Krishna *et al.,* 2011). Based on regeneration experiments conducted in the previous chapter, embryonic structure was selected for transformation experiments as it produced a higher plantlet regeneration rate.

The *P5CSF129A* gene is a salt tolerance gene (Chern *et al.,* 2001; Saharan *et al.,* 2004). The expression of this gene is subjected to feedback inhibition by proline due to the change in the conformation of P5CS protein. The gene was obtained by site-directed mutation by substituting the Phe residue at 129 position with an Ala in the polypeptide of P5CS from *Vigna aconitifolia* (Hong *et al.,* 2000).

VPPase gene encodes for a 80kDa single polypeptide which is a subunit of an inorganic pyrophosphatase (PPi) (Maeshima, 2000). It plays a crucial role in maintaining the proton gradient without consuming energy (ATP). This gene was transformed into *C. cajan* to test its

function in acquiring salt tolerance. Expression of the gene helps maintaining osmotic balance, thus promoting uptake of water and enhancing salt tolerance of plants. It maintains both acidic pH and electrochemical gradient through the vacuolar membrane during the period of stress response in plants. Embryonic structures of *C. cajan* genotypes were transformed using two constructs each harboring of the two salt tolerance genes.

3.2. MATERIALS AND METHODS

3.2.1. Plant material

The genotypes viz., LRG 41, ICPL 85063 and LRG 30 of pigeonpea were utilized for the transformation experiments. Seeds were washed with soap water followed by subsequent washes under running tap water. Seeds were then subjected to surface disinfection in 0.1% aqueous $HgCl_2$ for about 10min and were washed in autoclaved distilled water for 7-8 times. They were soaked for 15-17h in autoclaved distilled water under aseptic conditions.

The embryonic structures were excised aseptically from those 15-17h-soaked seeds on a laminar air flow bench. The explants were infected with *Agrobacterium* strain harboring pCAMBIA1301 and pCAMBIA2300 and left for co-cultivation for 3days. After 3 days, embryonic structures were transferred onto selective MS medium plates supplemented with 250mg/l cefotaxime and 25mg/l hygromycin. Those cultures were carried out at 25±2°C and 16/8hr photoperiod conditions in a tissue culture room.

3.2.2. Chemicals

The sources of the chemicals and related supplies used in this study are as follows: all the sulphates, non-suphates, iron-EDTA, B_5 vitamins, sucrose, agar of MS medium, different concentrations of BAP, cefotaxime, hygromycin, rifampcin, kanamycin, Luria Bertani broth, plant phytohormone such as NAA, IBA and GA_3 were obtained from Himedia.

3.2.3. Bacterial strains and plasmids used

3.2.3.1. *Agrobacterium tumefaciens* (1301 and 2300)

Agrobacterium tumefaciens strain LBA4404 harboring binary plasmids of pCAMBIA1301and pCAMBIA2300 were used. Those transformation vectors consist of reporter gene namely GUS (*uid* A) which encodes for β-glucuronidase under the control of CaMV35S promoter. They also have the hygromycin phosphotransferaseII (*hpt*II) gene for hygromycin sulphate resistance under the regulation of NOS promoter.

3.2.4. Genetic transformation procedure

Disarmed *A. tumefaciens* strains LBA4404 harboring binary plasmids pCAMBIA1301 and pCAMBIA2300 were used for transformation. *Agrobacterium* strains has resistance to rifampicin and kanamycin. Thus, the strains were maintained on LB agar plates containing rifampicin (20µg/ml) and kanamycin (50µg/ml). Strains were subcultured on to fresh medium for every 15 days.

LB medium (see appendix no: 9) contains tryptone 1%, yeast extract 0.5% and 1% sodium chloride. The pH of the medium was adjusted to 7.2 prior to autoclave. For agar plates the liquid medium was solidified with 1.5% agar. Single colonies of the strain were picked and inoculated in 25ml of LB medium (pH 7.2) supplemented with appropriate antibiotics and the colonies were grown for 24h at 28°C on an orbital shaking incubator at 200xg.

Bacterial suspensions (25ml) of 0.6-0.8 OD_{600}nm, was centrifuged at 5000rpm for 10mins and the pellet was separated. The pellet was dissolved in 25ml of ½ MS liquid and this suspension was used for inoculation of explants. The suspension was poured into a sterilized petridish plate to make a thin film. Freshly excised embryonic structures were excised and immediately submerged in the bacterial suspension for 10min. The explants were blotted dry on sterile filter paper, and then they were immediately inoculated on MS plates containing a supplement of

4mg/l BAP. The co-culture lasted for 3 days. The explants were kept in the growth room under aseptic conditions and maintained at 25 ± 2ºC under 16h photoperiod with 3000lux fluorescent light.

After 3 days, the explants were subcultured on selection medium (MS medium supplemented with 17.7mM BAP, 25mg/l hygromycin and 250mg/l cefotaxime). The number of explants survived on selection medium were recorded after 1 month and subsequently transferred to antibiotic elongation medium (MS medium supplemented with 0.86mM gibberellic acid, 0.57mM naphthalene acetic acid and 25mg/l hygromycin) for elongation and on to rooting medium (½ MS medium supplemented with 2.46mM IBA and 25mg/l hygromycin) for rooting. The rooted plants were acclimatized in polyhouse as per the procedure described in Chapter 1.

3.2.5. Histochemical analysis of β-glucuronidase (*GUS*)

Histochemical assay for GUS was done following the protocol by Jefferson *et al.* (1987) (For materials, see appendix no. 10). Histochemical *gus* assay for embryonic structures was performed one week after subculture of the explants on to selection medium. The explants were fixed for 45mins at room temperature in 0.3% formaldehyde in 10mM MES (pH 5.6) and 0.3M mannitol, followed by several washes in 50mM NaH_2PO_4 at pH 7.0. The fixatives and substrate solution were introduced into explants with a brief vacuum infiltration. X-glucanase activity assay was performed on 1mM substrate in 50mM NaH_2PO_4 (pH 7.0) at 37ºC after incubation from 20mins to several hours. The explants were rinsed in 70% ethanol for about 5min, and then observed under microscope.

3.2.5.1. Clearing chlorophyll in *GUS* treated explants

The following procedure was followed to remove chlorophyll from the *gus* treated explants. The explants were removed from X-Gluc staining solution and washed twice in sterilized distilled water and washed once in 70% ethanol. The explants were transferred to a acetone-methanol solution (1:3) to remove chlorophyll, which was kept at 4^{o}C for 1hr. This solution was replaced with fresh solution and were kept at 4^{o}C till chlorophyll was completely depleted. Tissues were washed twice in sterilized distilled water and were transferred to 50% glycerol for long-term storage.

3.3. RESULTS

The embryonic structures of three genotypes viz., ICPL 85063, LRG 41 and LRG 30 of *C. cajan* were infected with *Agrobacterium* strains harboring plasmids pCAMBIA1301and pCAMBIA2300, separately. The embryonic structures commonly turned green, multiple shoot buds (10-20) regenerated in 3 weeks on selection media (Table 3.1 & 3.2). The shoots were developed from the axillary buds of embryonic structures. After a week on antibiotics containing selection media, half of the explants were used for testing GUS activity and half were left on medium to grow. The frequencies of resistance to hygromycin in embryonic structures with pCAMBIA1301 was 94.66 ± 0.72, 94.22 ± 2.04, 95.9 ± 1.32 and for pCAMBIA2300, it was 90.0 ± 0.52, 91.0 ± 1.2, 92.3 ± 1.0 for LRG 30, LRG 41 and ICPL 85063 genotypes (Table 3.1 & 3.2).

Twenty-five embryonic structures of each genotype transformed by the two constructs were tested for GUS expression (*uid A*). About 90-100 % of explants were *Gus* positives (Table 3.1 & 3.2). The explants showed elongation in the three genotypes with an average 5-7 shoots per explant (Table 3.1 & 3.2). The rooting ratio of shoots transformed with pCAMBIA1301 was 42.6 ± 0.5, 60.0 ± 0.7, 69.3 ± 1.2 and for pCAMBIA2300, it was 42.6 ± 0.5, 47.4 ± 1.0, 59.0 ± 0.6 after a week of induction on rooting media (Table 3.1 & 3.2). Finally, 24.0 ± 0.3, 32.0 ± 0.6, 33.3 ± 0.9 % transformed by pCAMBIA1301 and 24.0 ± 0.3, 30.3 ± 0.7, 32.0 ± 0.5 %

transformed by pCAMBIA2300 of putative transgenic plants from the three genotypes were successfully acclimatized. Those transgenic plants flowered and produced mature seeds (Table 3.1 & 3.2).

The percentage of explants that survived initially on selection medium was high. As those explants were sub-cultured every 21 days and hardened to be planted in soil, the percentage of plant survival decreased. Out of a total of 225 explants from each genotype inoculated, there was a maximum of 18, 24 and 25% of pCAMBIA1301 and 16, 18 and 22% of pCAMBIA2300 transgenic plants that grew up to the harvesting stage (Table 3.1 & 3.2).

The ICPL 85063 variety inoculated with LBA4404 carrying the pCAMBIA1301 plasmid produced a better percentage of transgenic plants. Elongation of shoots from antibiotics resistant putative embryonic structures of LRG 30 occurred on selection MS medium supplemented with 0.86mM gibberellic acid, 0.57mM NAA and 25mg/l hygromycin (Fig. 3.1a). Rooting of putative transgenic pigeonpea plantlet of genotype LRG 41 on selection media containing half MS medium supplemented with 2.46mM IBA and 25mg/l hygromycin were observed in 2 months (Fig. 3.1b). Multiple shoot buds developed from embryonic structure of ICPL 85063, they were cultured on selection medium after 1 month (Fig. 3.1c). GUS expression in embryonic structures of genotype ICPL 85063 (endemic blue colour spots) after the first cycle on antibiotics selection medium was detected. The non-transgenic control tissues did not show the blue colour which are pointed by arrows on Fig. 3.1d.

Table 3.1: Frequency of hygromycin resistant and GUS positives plants derived from tissues infected with *Agrobacterium* strain pCAMBIA1301 and the value is the mean of three replicates from the same experiment.

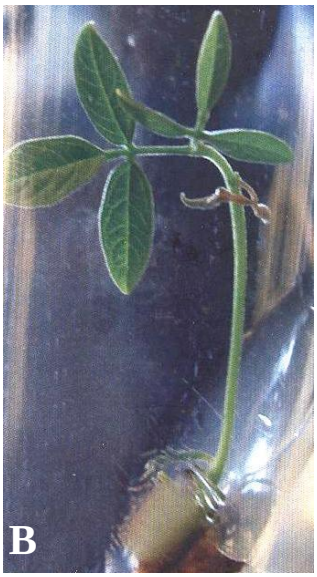
Genotype used	Embryonic structures inoculated	No. of Hygromycin positive explants (%) ± S.E	No. of elongated shoots rooted (Hyg) ± S.E	No. of rooted shoots acclimatized (Hyg) ± S.E	No. of *gus* positives
LRG 30	75	94.66 ± 0.72	42.6 ± 0.5	24.0 ± 0.3	18
LRG 41	75	94.22 ± 2.04	60.0 ± 0.7	32.0 ± 0.6	24
ICPL 85063	75	95.9 ± 1.32	69.3 ± 1.2	33.3 ± 0.9	25

Table 3.2: Frequency of hygromycin resistant and GUS positives plants derived from embryonic tissues infected with *Agrobacterium* strain pCAMBIA2300 and the values are the means from three replicates.

Genotype used	Embryonic structures inoculated	No. of Hygromycin positive explants (%) ± S.E	No. of elongated shoots rooted (Hyg) ± S.E	No. of rooted shoots acclimatized (Hyg) ± S.E	No. of *gus* positives
LRG 30	75	90.0 ± 0.52	42.6 ± 0.5	24.0 ± 0.3	16
LRG 41	75	91.0 ±1.2	47.4 ± 1.0	30.3 ± 0.7	18
ICPL 85063	75	92.3 ± 1.0	59.0 ± 0.6	32.0 ± 0.5	22

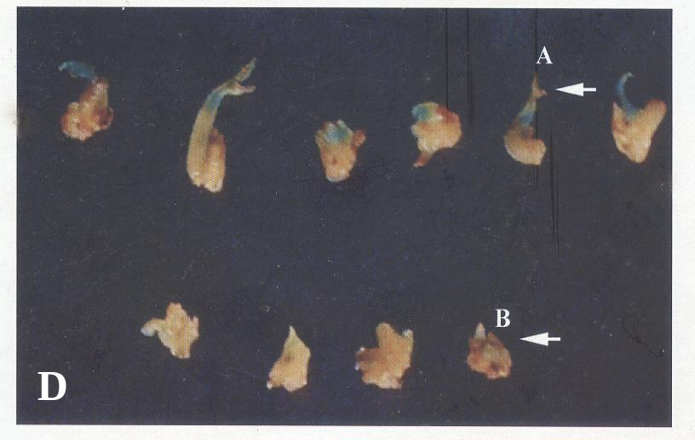
Fig. 3.1: Elongation, rooting and GUS expression of embryonic structures on selection medium.

A.Elongation of shoots from embryonic structures of pigeonpea on selection medium after 2 months.



B. Rooting of pigeonpea plantlet on MS medium

C.Embryonic structures showing initiation of multiple shoots after 1 month



D. GUS expression in embryonic structures of ICPL 85063 on antibiotic medium. Controls did not have blue colour pointed by arrows.

3.4. DISCUSSION

The steps involved in production of genetic transformation include successful infection by bacteria and successful incorporation of foreign genes into host genome. The sites of incorporation is important affecting expression of the foreign genes. In the present study, *Agrobacterium* strains harboring plasmids pCAMBIA1301 and pCAMBIA2300 were utilized for transformation studies.

The *Agrobacterium tumefaciens* strain LBA4404 with pCAMBIA1301 plasmid appears to be the best for transformation from the results obtained with different genotypes of pigeonpea. This may be due to the differences in *vir* gene factors, which increase infection in *Agrobacterium* strain. The changes in composition of cellular components i.e., altered pH, enlarged cell size, activation of cell division producing more cells are also responsible for these differences. Genotypic variation may be present due to production of phenolic compounds in pigeonpea, which may influence the infection efficiency of the agrobacteria.

Transformation efficiency was measured by confirmed plants derived from a number of plants. It is 15.6, 19.3 and 17.6 % in the three genotypes in T_0 generation (Table 3.1 & 3.2). The percentage of confirmed transgenic plants is slightly higher and similar to those reported by Surekha *et al.* (2007, 15%), Geetha *et al.* (1999, 45-62%) and Lawrence and Koundal (2001, 30-59%). The GUS activity of 90-100% of the transgenic explants confirmed the integration of genes into the genome of pigeonpea genotypes and its functional expression.

Pileggi (2002) used pyrrolidine-5-carboxylic synthase to transform lettuce to increase stress tolerance induced by cold, extreme temperature and elevated salt concentration. Molinari *et al.* (2004) testified drought tolerance in *Carrizo citrange* by introducing genes for salt tolerance.

Extending co-culture period increases the bacterial population and hence, interaction between plant and bacterial cells. But bigger bacterial population also affects the growth of transformed cells, which probably resulted in the slight reduction in the percentage of transformants. The type of explant used makes difference for obtaining more transformants. The explant with a higher number of dividing cells and differentiated axillary meristematic cells at the junction of embryonic structures has more potential for the incorporation of Ti plasmid. These cells are capable for regeneration and can be used as an essential targets for gene delivery.

Based on our results, we conclude that genotypic variation exists among *C. cajan* varieties for Ti plasmid incorporation. Variety ICPL 85063 and bacterial strain LBA4404 with plasmid pCAMBIA1301 are recommended for transformation experiments a higher number of multiple transformants were regenerated than from LRG 41 and LRG 30. The embryonic structures of ICPL 85063 developed a higher number of multiple shoot buds on selection MS media supplemented with 250mg/l cefotaxime and 25mg/l hygromycin than the other explants of LRG 41 and LRG 30 varieties.