CHAPTER IV

EXPRESSION OF THE *VIGNA ACONITIFOLIA P5CSF129A* GENE IN TRANSGENIC PIGEONPEA ENHANCES PROLINE ACCUMULATION AND SALT TOLERANCE

4.1. INTRODUCTION

Cajanus cajan [L.] Millsp. (Pigeonpea) is the chief and highly proteinaceous grain legume of arid and semi-arid tropics widely cultivated in India. Interest in this crop has been growing rapidly in many countries because of its multiple use as a source of food, cattle feed, firewood and also a restorer of soil nitrogen fertility. Despite of its importance, its productivity level is hampered due to pests, diseases caused by bacteria, fungi, virus etc. Soil salinity, a major abiotic stress is imposing water deficit in plants leading to accumulation of Na^+ and Cl^- ions that are compulsory to plant, thereby decreasing the productivity of crop on a global scale to a greater level which is in direct relation with the needs of the population.

Osmo-protectants protect plant cells from dehydration, they are non-toxic and are accumulated to significant levels without disrupting plant metabolism (Yamaguchi *et al*., 2005). Proline plays a vital role in protection, osmotic balancing and increase of turgor pressure necessary for the expansion of cells (Sairam and Tyagi, 2004; Matysik *et al*., 2002). The amino acid proline is significant as the only osmoprotectant which scavenges reactive oxygen species such as singlet oxygen, hydrogen peroxide and hydroxyl ions. It also serves as redox potential regulator and protects macromolecules such as proteins, DNA and reduces enzyme deactivation caused by NaCl, heat and other abiotic stresses (Matysik *et al*., 2002; Vinay Kumar *et al*., 2010). Kavi Kishor *et al*. (2005) reported the role, accumulation, anabolism and transportation of proline during salinity stress.

The osmotic tolerance nature of many plant varieties is directly associated with the accumulation of high amounts of proline. Several authors reported about higher deposition of proline in the halophytic plants than in salt sensitive plants such as wheat (Sairam *et al*., 2005), tobacco (Gubis *et al*., 2007; Yamchi *et al*., 2007) and sorghum (Jogeswar *et al*., 2006),

mulberry (Kumar *et al*., 2003), green gram (Misra and Gupta, 2005). The rate limiting enzyme pyrroline - 5 - carboxylate synthetase (*P5CS)* is mostly used in proline biosynthesis and its functional expression revealed enhanced stress tolerance in transgenics. The over-expression of *P5CS* gene deliberates salt tolerance in a number of transgenic crop plants including chickpea (Kiran Kumar Ghanti *et al*., 2011), rice (Anoop and Gupta, 2003; Karthikeyan *et al*., 2011; Su and Wu, 2004), wheat (Vendruscolo *et al*., 2007), tobacco (Yamchi *et al*., 2007), potato (Hmida-Sayari *et al*., 2005) by improving the synthesis and accumulation of proline.

P5CS gene is mutated at 129 position to replace Phe residue with Ala residue using site-directed mutagenesis which is subjected to feedback inhibition (Hong *et al*., 2000). The mutated P5CS gene resulted in more proline accumulation in transgenics with no longer feedback inhibition of the enzyme. The transgenic plants with more proline accumulation showed better protection from salty stress (Hong *et al*., 2000). There have been a few reports of using altered version of *P5CS* gene in overproduction of proline in transgenic citrus rootstock (Molinari *et al*., 2004), chickpea (Bhatnagar-Mathur *et al*., 2009), rice (Vinay Kumar *et al*., 2010).

Several reports are available on transformation of pigeonpea using different explants with different genes from different source probably due to efficient and reproducible transformation techniques being standardized, and the integration of genes being confirmed by the analysis of primary transgenic plants using Southern and Northern blotting techniques (Geetha *et al*., 1999; Lawrence and Koundal, 2001; Dayal *et al*., 2003; Satyavathi *et al*., 2003; Prasad *et al*., 2004; Surekha *et al*., 2005; Kumar *et al*., 2004; Guarav Krishna *et al*., 2011). The present investigation has the following two aims: to transform pigeonpea genotypes with *Vigna aconitifolia P5CSF129A* gene (mutated version); and to evaluate transgenic plants showing stable incorporation and expression of the introduced gene both at molecular and biochemical levels.

4.2. MATERIALS AND METHODS

4.2.1. Plants material

Seeds of pigeonpea cultivars LRG 30, LRG 41 and ICPL 85063 obtained from LAM farms of Guntur, India, were subjected to surface sterilization using detergent solution and 0.1% (w/v) $HgCl_2$ for about 10mins, followed by rinses for 4-5 times with sterile distilled water. The seeds were kept soaking in sterile distilled water for 15-17hrs under aseptic conditions. The embryos were removed from the cotyledons and cut into three segments. The embryonic structures to be used for *Agrobacterium* infection were placed on MS medium containing B_5 vitamins, 3% sucrose, 0.8% agar, 4 mg/l BAP and cultured at 25 ± 2^oC for 16hr under light.

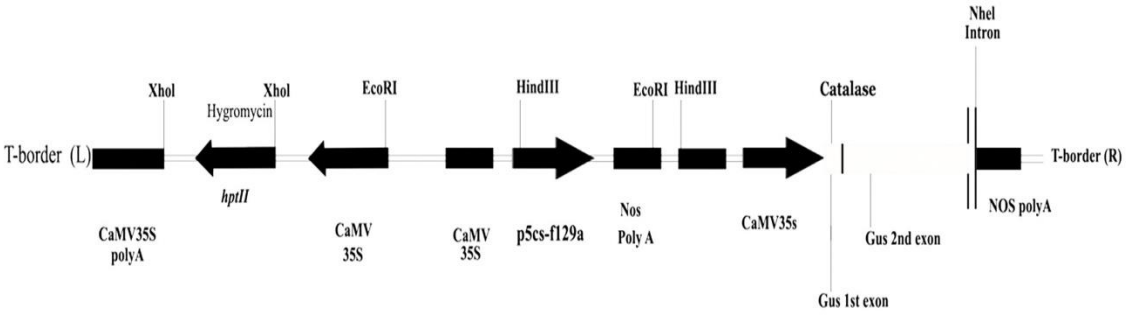
4.2.2. Chemicals

Primers (*hpt*II and *P5CSF129A*) were obtained from Bioserve Pvt Ltd, Hyderabad, India and Southern hybridization kit was obtained Amersham Biosciences, USA.

4.2.3. Bacterial strain and plasmid used

Agrobacterium tumefaciens strain LBA4404 harboring the binary plasmid pCAMBIA1301 vector consisting the mutagenized *V. aconitifolia P5CSF129A* gene under the control of *CaMV*35S promoter was utilized for the transformation of *C. cajan* genome of all three genotypes. The vector also contains the hygromycin phosphotransferaseII (*hpt*II) gene for hygromycin sulphate resistance and reporter gene *GUS* (*uid* A) which encodes for β-glucuronidase (Fig. 4.1).

Fig. 4.1: Construction of T-DNA region of the binary plasmid pCAMBIA1301 that contains mutagenized version of *Vigna aconitifolia* P5CSF129A, *hpt*II for resistance to hygromycin sulphate and gus reporter gene encoding β-glucuronidase.



4.2.4. Genetic transformation procedure

A. tumefaciens strain LBA4404 harboring the binary plasmid pCAMBIA1301 vector was utilized for transformation, it has resistance to rifampicin, and kanamycin. The strain was maintained on LB agar plates containing 20μg/ml rifampicin and 50μg/ml kanamycin and subcultured regularly on to fresh medium every 15 days. The maintainance of bacterial culture and selection of transformants were followed. The embryonic structures from three pigeonpea genotypes were infected with *Agrobacterium* strain LBA4404 harboring the binary plasmid pCAMBIA1301.

T_0 generation plants were transferred to polyhouse. Genomic DNA was isolated and used for both PCR amplification and the leaves of these plants were used for proline estimation. Seeds obtained from T_0 generation were again sown in sterile soil to get T_1 generation and the integration of gene was confirmed by performing molecular analysis such as PCR, Southern blotting, salt tolerance analysis and biochemical analysis such as lipid peroxidation, proline content, chlorophyll content, relative water content (RWC) estimation.

4.2.5. Analysis of T_0 Generation Plants

4.2.5.1. DNA isolation and analysis of gene incorporation

The DNA was isolated from the fresh leaf of both transgenic and non-transgenic (NT) T_0 plants by following Doyle and Doyle (1990)'s procedure. For materials see appendix no.11.

4.2.5.1.1. Procedure

The fresh leaves were collected from plants and frozen in liquid nitrogen. Then, they were ground to a fine powder in a prechilled mortar and pestle. The powdered material was taken in a tube comprising 0.5ml of DNA extraction buffer. The contents of the tubes were mixed well and then incubated for 30min at 65°C.

The aqueous phase was collected by adding phenol: chloroform (1:1) and then chloroform: isoamylalcohol (24:1). The DNA was precipitated with one volume of isopropanol to the aqueous phase. The contents of the tubes are mixed well by inverting for 5 times and left for about 10min at 37^{o}C.

The DNA material was pelleted by centrifuging at 12000rpm for about 10min in a micro centrifuge. After removal of the supernatant, the pellet was rinsed with 70% ethyl alcohol. The pellet was air dried for 1hr after removal of 70% ethyl alcohol. The dried DNA pellet was mixed in 100µl RB_2.

4.2.5.2. Polymerase Chain Reaction

Deoxyoligonucleotide primer pairs and DNA polymerase enzyme are used in PCR reaction in thin walled eppendorf tubes. DNA is amplified by repeating the reaction, which is made possible by regular denaturation of freshly synthesized double stranded DNA at high temperatures and annealing again at low temperatures. In a thermal cycler (Eppendorf master cycler), the process was repeated 30 times to get more copies of DNA in this study.

For materials see appendix no. 12.

4.2.5.2.1. Method

10ng of DNA was added to a 0.2ml PCR tube and the PCR mixture was prepared using the following primer sequences:

P5CS - 5'-ACA TTA TAC TCG TCT CCT CTG G-3'

5'-AAT TTT GTT TCC TTT CCT CTG A-3'

*hpt*II - 5'- GAG GCT ATT CGG CTA TGA CTG- 3'

5'-ATC GGG AGC GGC GAT ACC GTA-3'.

Each 50µl reaction contained the following ingredients:

10X PCR buffer	-	5.00µl
10mM dNTP	-	1.00µl
Template DNA	-	2.00µl
Forward primer	-	2.00µl
Reverse primer	-	2.00µl
Taq polymerase	-	0.50µl
Sterile DD water	-	37.50µl
Total	-	50.00µl

The final volume for each sample was 50µl and the tubes were kept in thermal cycler Eppendorf master cycler and amplified for 30 cycles using the program given below:

Initial denaturation at 95^0C	-	4 min
Final Denaturation at 95^0C	-	1 min
Annealing at 55^0C (*hpt*II)	-	45 sec

54^0C (*P5CSF129A*)

Elongation at 72^0C	-	1 min
Repeat for 30 cycles		
Final elongation at 72^0C	-	10 min

After the amplification was completed, the samples were run on 0.8% agarose gels by loading 10µl of amplified product + 2µl of tracking dye.

4.2.5.2.2. Agarose Gel

0.24gms of agarose was weighed and was dissolved in 30ml of 1X TAE buffer by boiling for 15min. Ethidium bromide is added to cooled agarose solution (40^oC) at a concentration of 1µg/ml. The cooled agarose solution was poured into the gel tray fixed with a comb and sealed

with cellophane at both ends and is left for solidification. After solidification, the comb and the cellophane were carefully removed. The gel was submerged in the electrophoretic tank filled with 1X TAE buffer. The PCR amplified DNA was mixed with gel loading buffer and was loaded directly into the wells of the gel. The electrophoretic tank was connected to power supply at 5V/cm to run the gel. The samples were run through three fourth of the gel, removed and viewed under a UV transilluminator to observe the bands for positive amplification. For materials see appendix no. 13.

4.2.5.3. Proline content assay:

The leaves of control and each PCR amplified T_0 generation plants were analyzed for proline content by following the standard procedure of Bates *et al.* (1973). Proline content was converted to unit per leaf dry weight of each plant and it was expressed as µg per g dry weight.

4.2.5.3.1. Reagents

For reagent preparation, see appendix no. 14.

- Glacial acetic acid
- 3% Sulphosalicylic acid.
- 6N Orthophosphoric acid.
- Acid ninhydrin.

4.2.5.3.2. Procedure

Fresh young leaves of about 0.5gm are collected and homogenized in 5ml of 3% sulphosalicylic acid. The mixtures were centrifuged at 1000xg and the supernatant was collected for the assessment of proline content. To 2ml of filtrate, after adding 2ml of acid ninhydrin, the reaction mixture was heated in a steaming water bath at 100^0C for 1hr. When a brick reddish colour solution appeared, the mixture was then cooled; after adding 4ml of toluene it was transferred into a separation funnel. After mixing thoroughly, the toluene will

be separated as transparent layer. The absorbance is read against blank at 520nm on a spectrophotometer. The standard curve made from the known concentrations of proline was referred for the estimation of free proline content.

4.2.6. Analysis of T_1 Generation Plants

4.2.6.1. PCR amplification

The presence of genes in T_1 generation plants were analyzed by using specific primers followed the confirmation by Southern analysis. PCR reaction was performed with genomic DNA of T_1 plants following the same procedure as mentioned above with both the primers.

4.2.6.2. Southern Blotting

For materials see appendix no. 15.

4.2.6.2.1. Digestion of genomic DNA

Ten micrograms of total genomic DNA isolated from PCR positive T_1 generation plants and non-transformed plants and pCAMBIA plasmid (positive control) were separately digested with *Eco*RI. The digestion was set up with *ECoR*I in 1.5ml microfuge tubes to a final volume of 50µl (Sambrook *et al*. 2001):

Genomic DNA	40.0µl
Sterile water	4.0µl
10X buffer	4.0µl
*ECoR*I	1.0µl
Rnase	1.0µl
Total volume	50.0µl

The samples were digested at 37^{o}C for overnight.

4.2.6.2.2. Agarose gel electrophoresis of digested genomic DNA

1.6gm of agarose was added to 200ml of 1X TAE buffer. The solution was boiled to dissolve agarose. The solution was cooled to room temperature and poured into the gel tray inserted with a comb and sealed with cellophane tape at both ends. After solidification, the comb and cellophane tape were removed and the gel was placed in the gel tank and 1X TAE buffer was added to cover the gel. 5µl of 10X loading buffer was added to the digested DNA samples and spinned briefly to bring the contents to tube bottom. 50µl of digested samples were loaded into the wells of the gel. The gel was run at 5V/cm to get high-resolution separation of the DNA fragments. The gel was stained for about 20min in 0.5µg/ml ethidium bromide and viewed under a UV transilluminator. Photograph was taken and marker DNA positions were noted by using a ruler.

4.2.6.2.3. Gel pretreatment and Southern blotting

The tray was filled with alkali blotting buffer (0.4M NaOH). Three sheets of whatman 3mm filter paper with wick were saturated with alkali buffer and placed on the platform (glass plate) arranged in the tray. The gel was rinsed twice in double distilled water, and placed on top of the wick without any air bubbles between gel and wick. To reduce the absorbance of alkali buffer by paper towels placed above the gel, the gel was wrapped with parafilm. A piece of Hybond N+ membrane was cut with a width of 1mm more than the gel size. The membrane was washed with 2X SSC and placed above gel. Air bubbles were squeezed out using a pipette. The three whatman 3mm filter paper sheets were resized to match the membrane size and saturated with alkali buffer and placed on the membrane and air bubbles were squeezed out. A stack of ordinary filter papers were resized to gel size and placed on top of the whatman filter papers. A glass plate and a small weight was placed on the top of filter papers and left overnight for transfer to occur. After transfer overnight, the membrane was carefully removed and washed in 2X SSC to remove any agarose sticking to the membrane. The membrane was air dried and kept in oven for 10min at moderate heat (40^{o}C).

4.2.6.2.4. Hybridization with probe

4.2.6.2.4.1. Preparation of labelled probe

100ng PCR amplified *P5CSF129A* fragment was added into a 0.5ml micro centrifuge tube and denatured by heating for 5 min in a boiling water bath. The denatured DNA was immediately cooled on ice for 5 min. The contents were collected to the bottom of the tube by brief spin in a micro centrifuge. The cooled DNA was added to 10µl of reaction buffer and mixed gently. 2µl of labelling reagent was added to above mixture by thorough mixing. 10µl of freshly prepared working concentration of cross linker solution (2µl of 10X stock diluted in 8µl of water to get working concentration) was added and mixed thoroughly. The contents were collected to the bottom of the tube and incubated for 30min at 37^{o}C and were used immediately.

4.2.6.2.4.2. Procedure

After preheating hybridization buffer to 55^{o}C, blots were placed in the buffer and prehybridized for 15min at 55^{o}C with shaking. Labelled probe was added to hybridization buffer and left overnight for hybridization at 55^{o}C with gentle shaking. After hybridization, the blots were carefully removed from hybridization buffer and washed in primary wash buffer twice (initially preheated to 55^{o}C) for 10min each time at 55^{o}C with gentle shaking. The blots were then transferred to secondary wash buffer and washed twice each for 5min with gentle shaking at room temperature. Excess secondary wash buffer was removed from the blots and 30-40µl of detection reagent was pipetted on to blot per square cm (cm^2), and left for 5min. The excess detection reagent was drained and the blots were wrapped in saran wrap with DNA side up. A sheet of autoradiography film was placed on top of the blot in a dark room and exposed for 2hrs. The film was then developed and photographed.

4.2.6.3. Evaluation of tolerance to salinity in transgenic (T_1) plants

Transgenic (T_1) plants were assayed for tolerance to salt shock (sudden exposure to salt treatment) based on the Indian conditions to check for the tolerance. The *in vitro* germinated hygromycin resistant transgenic and five non-transgenic plants were acclimatized to soil mixture (soil and sand, 1:1) with one plant in each plastic cup (length 8.6cm & diameter 9cm).

Each cup had an equal amount (250g) of soil with 4 holes on the bottom to drain water. These plants were used for salt treatments (see appendix no.16).

The acclimatized plants were watered with 50ml of Hoagland solution in greenhouse for the first 2 weeks. Subsequently, the plants were watered every other day for about 7days with 50 ml of Hoagland solution supplemented with 200mM NaCl. Excess solution was drained through the cups, and daily irrigation with salt solution was able to maintain a constant level of salinity. The plant development and biochemical analysis such as proline, chlorophyll, RWC and lipid peroxidation were analyzed after 7 days treatment with 200mM NaCl (Vinay Kumar *et al*. 2010; Karthikeyan *et al*. 2011).

4.2.6.3.1. Proline content assay

The proline content was assessed using leaf tissues from each transformed T_1 plants along with the control plants after salt treatment, following the procedure of Bates *et al*. (1973) as described previously.

4.2.6.3.2. Lipid peroxidation

4.2.6.3.2.1. Reagents

For materials see appendix no. 17.

- 0.1% w/v TCA
- 0.5% TBA diluted in 20% (w/v) TCA

4.2.6.3.2.2. Procedure

Fresh samples of 0.5g were ground in 10 ml of 0.1% trichloroacetic acid. The homogenate was subjected to centrifugation at 10,000xg for about 5min, then 4ml of 0.5% thiobarbituric acid in 20% TCA was added to 2ml of supernatant. The mixture was heated at 95^{O}C for 30min and

then immediately cooled in an ice bath. Precipitations were removed by centrifugation at 10,000xg for 10min. The absorbance of supernatant at 532nm was recorded and the value of non-specific absorption at 600nm was subtracted. The absorption coefficient of 155 $mmol^{-1}$ cm^{-1} was taken as reference for calculating MDA content (Yookongkaew *et al.*, 2007).

MDA ($mM.cm^{-1}$) = 1000 ($Ab_{532\text{-}600}$ nm)/155

4.2.6.3.3. Estimation of Chlorophyll

The total chlorophyll content of leaf material was assayed in acetone extract by using spectrophotometer according to the procedure of Arnon *et al.* (1974).

4.2.6.3.3.1. Procedure

Plant samples (100mg) were weighed individually and ground separately in excess volume of 80% acetone in a mortar and pestle until complete removal of the greenish colour from the tissue. The homogenate was then centrifuged at 5000rpm for 10 minutes at 37°C. The collected supernatant was adjusted to a final known volume of 10ml. Chlorophyll degradation was prevented by wrapping the test tubes with black paper. The wavelength of 663 nm was set for chlorophyll 'a' and 645 nm for chlorophyll 'b' at 100% transmittance on a spectrophotometer. The 80% acetone solution was read as blank before taking the readings of test samples. The optical density was measured and the chlorophyll contents in the original extract was estimated using the formula;

Total chlorophyll (mg/l) = $20.20A_{645}$ + $08.02\ A_{663}$

Chlorophyll 'a' (mg/l) = $12.70A_{663}$ - $02.69\ A_{645}$

Chlorophyll 'b' (mg/l) = $22.90A_{645}$ – $04.68\ A_{663}$

4.2.6.3.4. Relative water content (RWC)

Relative water content was assessed from leaves using the procedure described by Mata and Lamattina (2001) which was converted to percentage of water content of fresh tissue

compared to water content of the respective tissue at full turgor.

4.2.6.3.4.1. Procedure

Fresh leaf samples (3-4 leaves) were collected and weighed to record the fresh weight (FW). They were wrapped immediately in saran wrap to minimize evaporation and stored in dark. Then the leaves were immersed in deionised water overnight and kept away the samples from physiological activity by physical inhibition of growth and respiration. The samples were placed in a fridge under darkness. The next day the leaf samples were dry blotted on filter paper and reweighed to record the turgid weight (TW). The leaf samples were then placed in the dryer at 60^0C (24hrs) and reweighed to record the dry weight (DW). The RWC is calculated using the formula**:**

RWC = (FW-DW)/ (TW-DW)

Where,

RWC = Relative water content

FW = Fresh weight

DW = Dry weight

TW = Turgid weight

4.2.6.3.5. Statistical analysis

The data was used for the analysis of variance (ANOVA). Each time the experiments were repeated twice with three replicates.

4.3. RESULTS

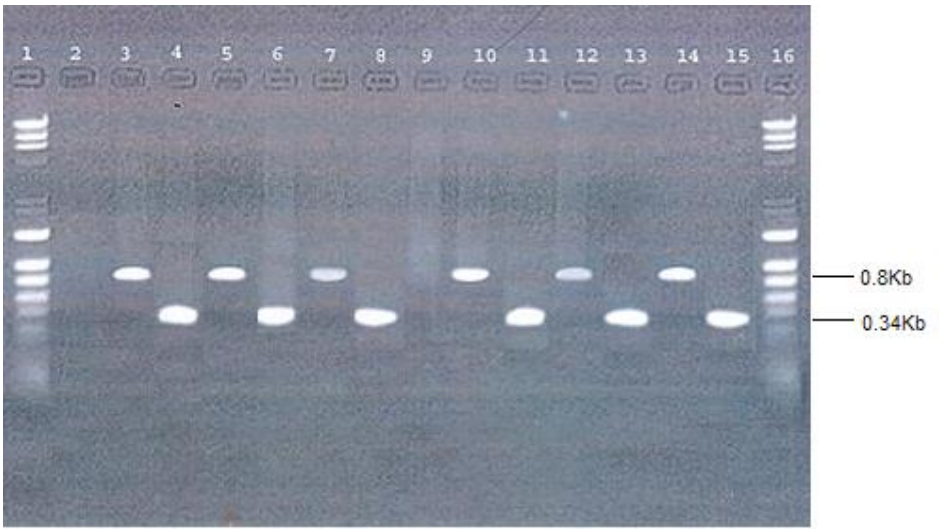
4.3.1. Analysis of T_0 Generation Plants

Among the 225 explants from each genotype that were inoculated, 15%, 18.6% and 17% of the putative transgenic plants grew to the harvest stage. Then analysis was carried out on T_0 plants to confirm the genomic insertion of transgene. The amplification was carried out using gene specific primers (*hpt*II and *P5CSF129A).* Results show that all the hygromycin resistant plants that were acclimatized produced positive amplification products with both pairs of

primers. All the samples ($T_{0\text{-}1}$, $T_{0\text{-}2}$ belonging to LRG 30, $T_{0\text{-}3,}$ $T_{0\text{-}4}$ belonging to LRG 41 and $T_{0\text{-}5}$ and $T_{0\text{-}6}$ belonging to ICPL 85063) amplified a 340bp fragment with *hpt*II primers and a 800bp fragment with *P5CSF129A* primers, while control plants showed no amplification with either primer pair (Fig. 4.2).

The proline content of tissue culture grown T_0 plants was assayed. There was a significant difference between T_0 transgenic and non-transgenic pigeonpea plants (Table 4.1). All the T_0 generation plants showed significantly high levels of (F-1464655, P-2.22E-184) proline content ranging from 4,974.0 ± 3.06 to 7,987.5 ± 0.37µg/gm dry weight in six different plants (PCR positives), which is about 4 times more than non-transformed plants (1,230.7 ± 3.92 to 1653.8 ± 0.26µg/gm proline in dry weight). This data approved the stable integration of *P5CSF129A* gene into host genome and functional expression of the foreign gene in pigeonpea. Significant differences were also found in $T_{0\text{-}6}$ transformant of genotype ICPL 85063 with higher proline accumulation of 7,987.5 ± 0.37µg/gm when compared to the other 5 T_0 transformants.

Fig 4.2: PCR analysis done with putative pigeonpea using *hpt*II and *P5CSF129A* primers showing amplification of 800bp and 340bp fragment



Lanes 1 & 17: 100bp ladder

Lane 2: Positive control showing 800bp fragment amplified with *P5CSF129A* primers

Lane 3: Positive control showing 340bp fragment amplified with *hpt*II primers

Lane 10: Negative control (Untransformed plant)

Lanes 4, 6: Transformed plants of pigeonpea genotype LRG 30 showing 800bp fragment amplified with *P5CSF129A* primers

Lanes 5, 7: Transformed plants of pigeonpea genotype LRG 30 showing 340bp fragment amplified with *hpt*II primers

Lanes 8, 11: Transformed plants of pigeonpea genotype LRG 41 showing 800bp fragment amplified with *P5CSF129A* primers

Lanes 9, 12: Transformed plants of pigeonpea genotype LRG 41 showing 340bp fragment amplified with *hpt*II primers

Lanes 13, 15: Transformed plants of pigeonpea genotype ICPL 85063 showing 800bp fragment amplified with *P5CSF129A* primers

Lanes 14, 16: Transformed plants of pigeonpea genotype ICPL 85063 showing 340bp fragment amplified with *hpt*II primers

Table 4.1: Amount of proline present in leaves of non-transformed (NT) and transformed (T_0) transgenic line generated via *Agrobacterium*-mediated transformation of pigeonpea genotypes showing significant differences (P<0.0001) in the three genotypes

Genotype	Plant no.	Proline content (µg /gm dry leaf wt)
LRG 30	NT_{0-1}	1230.77 ± 09
	T_{0-1}	4974.00 ± 11
	T_{0-2}	5591.22 ± 02
LRG 41	NT_{0-2}	1553.22 ± 15
	T_{0-3}	4991.77 ± 06
	T_{0-4}	5212.33 ± 05
ICPL 85063	NT_{0-3}	1653.88 ± 14
	T_{0-5}	5269.22 ± 07
	T_{0-6}	7987.55 ± 12

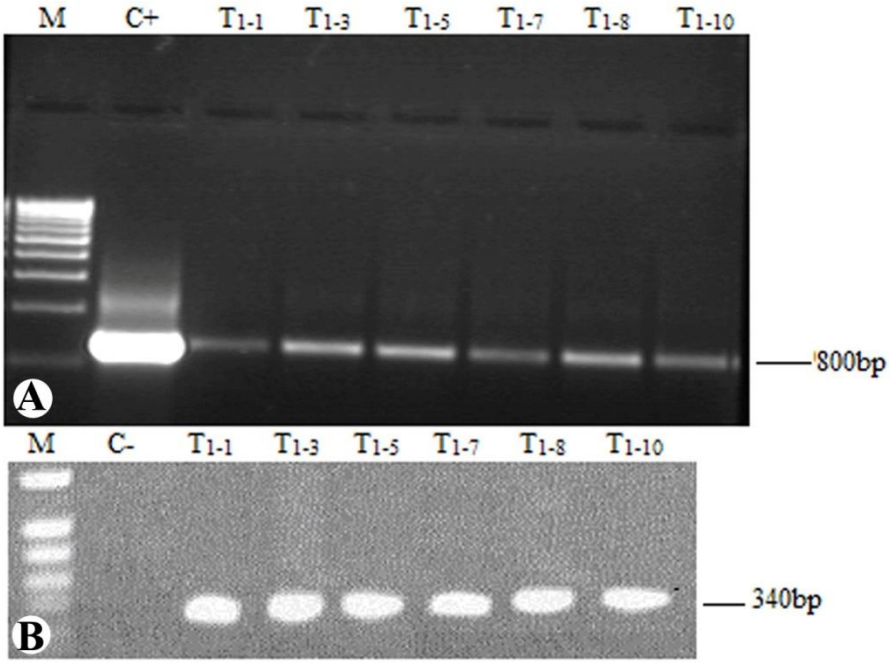
4.3.2. Analysis of T_1 Generation Plants

4.3.2.1. PCR analysis

The stable incorporation of *P5CSF129A* and *hpt*II genes into the pigeonpea genome was confirmed by performing PCR amplification. The amplified products of PCR were separated by electrophoresis on 0.8% agarose gel. The two bands of 800bp and 340bp represent the stable transmission of the transgene to the next generation (Fig. 4.3a & b). The DNA isolated from the leaf tissues exhibiting symptom of hygromycin sensitivity did not display any sign of gene integration (lane II in Fig. 4.3b).

Southern analysis revealed that genomic DNA which were isolated from randomly selected plants of three genotypes (T_1 generation obtained from $T_{0\text{-}1}$ to $T_{0\text{-}6}$) and digested with *Eco*RI and then hybridized with *P5CSF129A* probe, produced a hybridization band matching the size of the PCR amplified fragment. The analysis of each transgenic lines gave the expected 3.8Kb fragment. All the transgenic lines showed the same sized fragment as *Eco*RI enzyme has two internal recognition sites within T-DNA which cuts the genomic DNA at the same location. No signal was detected in the case of non-transformed plants (Fig 4.4). These above results confirmed the incorporation of *P5CSF129A* gene in pigeonpea genotypes.

Fig. 4.3a & b: PCR analysis of T_1 generation using *P5CSF129A* and *hpt*II primers showing amplification of 800 bp and 340 bp fragments.



Lane M: 1Kb marker

Lane C+: Positive control

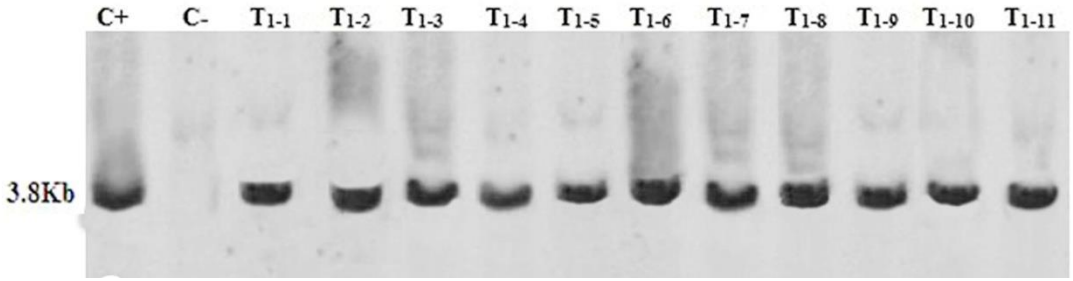
Lane C-: Negative control (Non- transformed plant)

Lane T_{1-1} & T_{1-3}: T_1 plants obtained from T_{0-1} & T_{0-2} plants of genotype LRG 30 showing 800bp & 340bp fragments amplified with *P5CSF129A* and *hpt*II primers

Lane T_{1-5} & T_{1-7}: T_1 plants obtained from T_{0-3} & T_{0-4} plants of genotype LRG 41 showing 800bp & 340bp fragments amplified with *P5CSF129A* and *hpt*II primers

Lane T_{1-8} & T_{1-10}: T_1 plants obtained from T_{0-5} & T_{0-6} plants of genotype ICPL 85063 showing 800bp & 340bp fragments amplified with *P5CSF129A* and *hpt*II primers

Fig. 4.4: Southern hybridization analysis of T_1 progeny of primary T_0 transformants obtained from *Agrobacterium*-mediated transformation of pigeonpea



Lane C+- Positive control showing 3.8Kb gene insert obtained from plasmid DNA digested with *EcoRI*, separated and hybridized to a *P5CSF129A* probe

Lane C-- Non-transformed control plant

Lanes 3, 4: T_{1-1} & T_{1-2} – Transformed T_1 plants obtained from T_{0-1} of LRG 30 genotype

Lanes 5, 6: T_{1-3} & T_{1-4} – Transformed T_1 plants obtained from T_{0-2} of LRG 30 genotype

Lanes 7, 8: T_{1-5} & T_{1-6} – Transformed T_1 plants obtained from T_{0-3} of LRG 41 genotype

Lane 9: T_{1-7} – Transformed T_1 plants obtained from T_{0-4} of LRG 41 genotype

Lanes 10, 11: T_{1-8} & T_{1-9} – Transformed T_1 plants obtained from T_{0-5} of ICPL 85063 genotype

Lanes 12, 13: T_{1-10} & T_{1-11} – Transformed T_1 plants obtained from T_{0-6} of ICPL 85063 genotype

4.3.2.2. Evaluation of salinity tolerance in transgenic (T_1) plants

Pigeonpea plants expressing *P5CSF129A* gene showed an increase in plant height with leaves much greener and healthier (Fig. 4.5). Leaf growth was severely affected in non-transgenic lines and after 3days, they started wrinkling and browning. The total chlorophyll concentration maintained higher in transgenic plants over NT under the stress conditions. The total loss of chlorophyll was less in transgenic plants compared to NT plants. The relative water content is considered as a proper measure for both plant water status and osmotic adjustment. The RWC in the leaves of transgenic plants was measured higher than the non-transgenic plants during stress conditions. The entire NT showed lower RWC and varied between genotypes in all transgenic plants (Table 4.2).

The results of free proline levels in both transgenic and non-transgenic lines under saline conditions reinforced the assumption of relation between the upsurge of proline and salt tolerance in pigeonpea plants. The proline accumulation in transgenic lines under salt shocks was 4 times higher than the non-transgenic lines. These results clearly indicate the establishment and representation of *P5CSF129A* gene into the host genome.

The formation of free radicals and level of membrane wounding were assayed by the malonaldehyde content in both transformed and control plants during exposure to 200mM NaCl. The low lipid peroxidation indicates the less membrane damage in *P5CSF129A* transgenic lines under salt shock. These reports clearly indicate that the functional expression of *P5CSF129A* transgene in transgenic plants confers enhanced tolerance to osmotic stress. It was obvious from the above data that the P5CS gene has a role in improving proline biosynthesis and protect plant from damages caused by various stress treatments.

At the same time, the non-transformed plants could not show tolerance to the equivalent range of stress due to low proline content. The present investigation evidently specifies the constructive and direct relation between the tolerance to high salinity and proline accumulation in the transgenic plants. The functional over-expression of an altered version of gene *P5CSF129A* in pigeonpea genotypes resulted in enriched growth rate, which is again in agreement with high proline accumulation and low lipid peroxidation.

Table 4.2: Effect of high salinity on plant growth, relative water content, total chlorophylls, proline accumulation and lipid peroxidation in non-transformed and transformed T_1 plants of pigeonpea (means ± SE, n=3)

T_1 lines	Plant height (cm)	Relative water content (%)	Total chlorophyll content (mg/gm)	Proline content (µg/gm dry weight)	Lipid peroxidation (MDA content) (nmol/g fresh wt)
NT1	11.0±1.1	69.2	1.86±0.14	2,115±03	42.8±0.8
T_{1-1}	25.0±2.9	73.3	2.10±0.19	8,230±09	19.4±1.1
T_{1-2}	23.0±2.1	79.3	2.31±0.13	9,565±11	15.4±1.5
NT2	10.0±0.9	76.0	1.64±0.10	1,995±04	39.2±1.2
T_{1-3}	26.0±1.8	82.0	2.40±0.15	8,563±13	20.6±0.9
T_{1-4}	28.0±2.7	83.0	2.42±0.09	7,890±02	18.6±1.9
NT3	11.5±1.3	73.0	1.89±0.11	2,090±08	41.2±0.7
T_{1-5}	29.0±2.6	82.7	2.46±0.16	8,895±12	19.5±1.2
T_{1-6}	26.0±1.7	85.6	2.42±0.12	7,995± 08	18.9±1.1

NT1 – Non transgenic pigeonpea of Genotype LRG 30

NT2 – Non transgenic pigeonpea of Genotype LRG 41

NT3 – Non transgenic pigeonpea of Genotype ICPL 85063

T_{1-1}&T_{1-2} plants obtained from seeds of T_{0-1}&T_{0-2} putative transformants of genotype – LRG 30.

T_{1-3}&T_{1-4} plants obtained from seeds of T_{0-3}&T_{0-4} putative transformants of genotype – LRG 41

T_{1-5}&T_{1-6} plants obtained from seeds of T_{0-1} - T_{0-2} putative transformants of genotype – ICPL 85063

The values represent an average of 7 plants of transgenic lines and average of 5 plants in case of NT ± SE.

Fig. 4.5: Effect of NaCl application (salt shock) on non-transformed and transformed (T_1) plants: hygromycin resistance T_1 plants after one week of selection in the greenhouse and after 2 weeks, 200 mM NaCl was applied to cups with plants in one step on every alternate day for 7 days.

NT1 – Non-transformed plant of genotype LRG 30

T_{1-1}- T_1 plant obtained from T_{0-1} plant of genotype LRG 30

NT2 - Non-transformed plant of genotype LRG 41

T_{1-3}- T_1 plant obtained from T_{0-3} plant of genotype LRG 41

NT3 - Non-transformed plant of genotype ICPL 85063

T_{1-5}- T_1 plant obtained from T_{0-5} plant of genotype ICPL 85063

4.4. DISCUSSION

Successful production of transformants includes infection by bacterium, incorporation of gene into host genome, and the site into which it is incorporated (Gustava *et al.* 1998). In the present study, we successfully obtained proficient transformation using embryonic structures of three genotypes of pigeonpea. The transformation efficiency based on the number of confirmed T_0 transgenic plants, was 15.6, 19.3 and 17.6% in different genotypes.

The fraction of transgenic plants appeared to be slightly higher than reported by Surekha *et al.* (2005, 15%), while it matched with the prior reports of pigeonpea (46-62%) by Geetha *et al.* (1999) and 31-59% by Lawrence and Koundal (2001). The percentage of transformation was estimated using results from PCR analysis (*hpt*II and *P5CSF129A* specific primers) of T_0 generation plants that set seeds.

The earlier reports were based only on T_0 generation analysis but the present report specifies the steady integration of transferred DNA in T_0 transformed plants by performing PCR analysis. The gene incorporation was further confirmed by Southern blotting in T_1 plants. All the PCR positive transgenic plants have shown significantly high proline levels than the non-transgenic plants. These convincingly demonstrates the steady introduction and expression of *P5CSF129A* gene in the pigeonpea genome. Similarly, the *P5CS*-transgenic wheat plants contain more than ten times higher proline content than non-transgenic control plants (Sawahel and Hassan, 2002).

Recently, Yamchi *et al.* (2007) testified about the transformation of tobacco plants and also observed the production of 26 times higher proline content level in transformed tobacco plants by P5CS gene than non-transformed plants. Ghanti *et al.* (2011) and Karthikeyan *et al.* (2011) observed five times more proline in the transformed chickpea and rice plants than their non-transformed counterparts. Furthermore plants transformed with *P5CS* gene from *Vigna* were able to protect the transgenic plants from impairment caused by salinity treatments. The current results are in close relation with the above reports which justifies the integration and efficient expression of the *P5CSF129A* gene in the pigeonpea genome of various genotypes.

Pileggi (2002) reported the transformation of lettuce by using the mutagenized *P5CS* gene which conferred osmotic tolerance against freezing, extreme temperatures and high saline conditions. Molinari *et al.* (2004) reported the transformation of *Carrizo citrange* by using P5CS gene under the control of CaMV35S promoter and obtained enhanced famine tolerance by the biosynthesis and accumulation of proline. Vinay Kumar *et al.* (2010) produced transgenic indica rice overexpressing *P5CSF129A* gene with 4 to 5 fold increase in proline content than the non-transformed plants which protected those plants against damage caused due to stress. The above results indicate a four fold increase in proline content of T_1 generation plants by the insertion of *V. acontifolia* mutated version of *P5CSF129A* gene.

In addition to the proline enhancement, the level of membrane and cell damage during stress conditions were also assayed by measuring MDA content as a parameter. Plants exposed to salinity produce free radicals but in transgenic plants, the level of free radicals and cell damage were reduced due to high proline accumulation. Our finding are different from results published by Vinay Kumar *et al.* (2010), Vendruscolo *et al.* (2007) and Parvanova *et al.* (2004). Nevertheless the high proline accumulation during the period of salt stress reduced the levels of free radical which was measured by MDA content.

The over expression of *Vigna P5CSF129A* gene in pigeonpea plants showed enhanced tolerance to salt shock which was revealed by accumulation of proline, high levels of chlorophyll content and relative water content. These reports suggest that the salt tolerance in transgenic plants is due to the over-expression of *P5CS* under the control of CaMV35S promoter and by the regulation of the protein turn over under salt shock.

In the above conclusion, the expression of *P5CSF129A* transgene in the T_0 generation and its function resulted in over-production and accumulation of proline in transformed plants when compared with non-transformed plants. The analysis of T_1 generation plants under salt shock revealed proline accumulation, enhanced plant height and lower lipid peroxidation rate compared to non-transgenics with exposure to 200mM NaCl. These results are in agreement with recent reports that the accumulation of osmoprotectants increases salt tolerance of plants and thus makes those compounds highly desirable for agricultural use.