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Psp68, A Dead Box Helicase Confers Salinity Tolerance in Transgenic Pigeon Pea

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A B S T R A C T

Legumes are an important part of human diet account for about 27% of global primary crop production. Pigeon pea is the world's sixth most important and second most important legume pulse crop of India after chickpea and mainly cultivated as rain fed crop. Its production is adversely affected due to salinity in arid and semi-arid regions of world. Salt stress reduces water potential, creates imbalance in ion concentration and causes toxicity. Helicases have been shown to play an important role in plants against salt stress. *p68* which is a prototype member of DEAD-box helicase interacts with Ca^{2+}-CaM, thus regulating diverse signalling pathways against salt stress in plants. In the present study, we have developed transgenic pigeon pea plants with marker free gene *Psp68* for salinity tolerance. Since regeneration is prerequisite for transgenic development and pigeon pea is considered to be recalcitrant, the transgenic pigeon pea plants containing *Psp68* gene have been developed using the tissue culture independent transformation method (Patent Application No. 201811012099). The putative T_0 plants were screened by PCR analysis and the PCR positive plants with transformation efficiency of 16% were observed. Transgenic lines in T_1 generation under salt stress condition showed enhanced tolerance to salt stress in terms of various physio-biochemical parameters like relative water content, membrane injury index, MDA content, chlorophyll content, proline and total soluble sugar content, catalase activity and peroxidase activity.

Introduction

Abiotic stress is the principal cause of decreasing the average yield of major crops by more than 50%, leading to the losses worth hundreds of million dollars each year (Rasool *et al.*, 2013; Lamaoui *et al.*, 2018). Among abiotic stresses, high salinity stress is the most severe environmental stress, which impairs crop production on at least 20% of irrigated land worldwide. Out of the 1500 million hectares agricultural land, 32 million (2%) is affected by secondary salinity of varying degrees. Further, problems will be worsened as near about 50% of the arable land will hit salinity by 2050 (Machado and Serralheiro, 2017). Extensive economic losses due to salinity include costs of $27 billion-plus loss of crop value per year (Kumar *et al.*, 2017). Salinity affects various morphological and

physio-biochemical processes involved in plant growth and development (Rahneshan *et al.,* 2018). Soluble salts when present in excess cause ion toxicity and ion imbalance (Munns, 2005) which ultimately lead to plant demise (Zorb *et al.,* 2018). In response to high salinity stress various genes get up regulated, the products of which are either directly or indirectly involved in plant protection (Shivakumara *et al.,* 2017). Overall, the susceptibility or tolerance to high salinity stress in plants is a coordinated action of multiple stress responsive genes, which also cross-talk with other components of stress signal transduction pathways. The complexity and polygenic nature of salt stress are important factors contributing to the difficulties in breeding salt-tolerant crop varieties (Zhu, 2000; Flowers, 2004; Jangra *et al.,* 2017). Understanding these mechanisms of stress tolerance along with a plethora of genes involved in stress signaling network is important to improve high salinity stress tolerance in crops plants. Since long conventional breeding has been widely used to develop stress tolerant and high yielding crop plants through screening of tolerant germplasm and crossing with cultivated varieties but this procedure is time-consuming, cost and labour intensive (Ashraf, 2010; Yu *et al.,* 2016) and suffers from a poor selectivity, due to transfer of unwanted linked traits along with desirable traits. Moreover, reproductive barrier and low level of variations in genetic pool make it a cumbersome technique. To resolve these barriers associated with traditional breeding, biotechnological approaches such as genetic engineering can be employed to obtain better results in shorter time.

Transgenic approach is being effectively pursued by plant scientists these days to impart salinity tolerance in various crop plants. Transgenics for salinity tolerance is mainly focused on introduction of genes that encode ion transport proteins, compatible organic solutes, antioxidants and transcriptional factors for gene regulation (Ashraf *et al.,* 2008). A large number of these genetic processes demand the intervention of several types of essential enzymes including helicases. The helicases are ubiquitous enzymes that catalyze the unwinding of energetically stable duplex DNA (DNA helicases) or duplex RNA secondary structures (RNA helicases) (Tuteja, 1997; Tuteja, 2000; Tuteja and Tuteja 2004; Gustafson and Wessel, 2010; Linder and Fuller-Pace, 2013). Helicases might be playing an important role in stabilising growth in plants under stress by regulating stress-induced transcription and translation. A hallmark of most of the helicases is the existence of a set of highly conserved amino acid sequences called 'helicase-motifs', which are clustered together for helicase function (Tuteja and Tuteja, 2004a; 2004b). One of the important motifs is DEAD (motif II), which stands for Asp-Glu-Ala-Asp. The DEAD-box RNA helicases is the largest family of RNA helicases. In spite of the sequence resemblance of DEAD-box RNA helicases within the core helicase regions, each DEAD-box helicase is believed to play various crucial roles in plant growth and development (Linder and Jankowsky, 2011). Jiechen, (2016) reported that transgenic lines of cotton plants overexpressing *Apocynumvenetum* DEAD-box helicase 1 (*AvDH1*) showed lower membrane ion leakage, along with increased activity of superoxide dismutase thus confering salinity tolerance. In *Arabidopsis*, DEAD-box protein LOS4 (low expression of osmotically responsive genes 4) and RCF1 (regulator of *CBF* gene expression 1) has been validated to be essential in exporting mRNA and pre-mRNA splicing by regulating the expression of CBF (C-repeat binding factor) factor under cold stress conditions (Gong *et al.,* 2005; Guan *et al.,* 2013). Three DEAD-box RNA helicases AtRH5, AtRH9

and AtRH25 also respond to multiple abiotic stresses in *Arabidopsis* (Kant *et al.,* 2007; Kim *et al.,* 2008). A rice DEAD-box RNA helicase OsBIRH1 (*Oryza sativa* BTH-induced RNA helicase 1) was shown to function in defense responses against pathogen and oxidative stresses (Li *et al.,* 2008). All these reports suggest roles of plant helicases in stress tolerance however, the exact role of most plant DEAD-box proteins largely remains unclear and requires further studies.

The p68 is a prototype member of DEAD-box family and it plays a very important role in cell/organ development (Stevenson *et al.,* 1998) and also participates in a variety of biological processes in animal system including pre-rRNA processing (Liu, 2002; Bates *et al.,* 2005; Fuller-Pace, 2006), RNA-induced gene silencing (Ishizuka *et al.,* 2002), transcription initiation (Fuller-Pace, 2006) and alternative splicing processes (Kar *et al.,* 2011). It was also reported that ATPase activity of recombinant p68 in yeast was stimulated by double-stranded RNA and it unwinds RNA in both 3' to 5' and 5' to 3' directions (Huang and Liu, 2002. It has been reported that p68 RNA helicase is phosphorylated on tyrosine, serine, and threonine residues and its helicase and ATPase activities are stimulated after phosphorylation with protein kinase C (Pradhan *et al.,* 2005b) which is a general cascade to cope with abiotic stresses in plants. Wang *et al.,* (2013) reported that p68 also interacts with Ca^{2+}-CaM regulating diverse signalling pathways leading to stress tolerance in plants.

Psp68 DEAD-box protein exhibits ATPase activity in the presence of both DNA and RNA, binds to DNA as well as RNA and shows unique bipolar DNA helicase activity which suggest that it could be a multifunctional protein (Tuteja *et al.,* 2014). *Psp68* provided salinity stress tolerance in transgenic tobacco and transgenic rice by reducing oxidative stress and improving photosynthesis machinery (Banu *et al.,* 2014). However, very little is known about p68 protein in plant system and it has not been functionally or biochemically characterized in detail. The role of p68 and molecular target of this gene in response to stress tolerance in leguminous plants have also not been reported so far.

Materials and Methods

*Psp68*gene, plasmid and *Agrobacterium tumefaciens* strain

Agrobacterium tumefaciens strain LBA4404 containing pCAMBIA1300 harboring *Psp68* gene was used for genetic transformation experiment. This strain with the above mentioned gene was procured from Dr. Narender K. Tuteja, ICGEB, Delhi

Preparation of *Agrobacterium* inoculum harboring pCAMBIA1300-*Psp68* plasmid and *Agrobacterium*- mediated transformation of pigeon pea with *Psp68* gene

A single colony from fresh bacterial culture raised from glycerol stock culture carrying The *Agrobacterium* strain LBA4404 was inoculated in 20 ml LB medium broth supplemented with kanamycin (50 mg/ml), streptomycin (50 mg/ml) and rifampicin (50 mg/ml) and incubated at 28˚C on orbital shaker overnight (100 rpm).

Transgenic pigeon pea plants containing *Psp68* gene were developed using the protocol for which patent has been filed (Patent Application No.-201811012099).Transformation efficiency was calculated based on the PCR analysis of putative T_0 plants.

Molecular analysis of the transformants

Total genomic DNA was isolated from young leaves of wild type and transformed plants following the CTAB method (Saghai-Maroof *et al.,* 1984).PCR analysis was performed to amplify fragments of *Psp68* gene using gene specific primers. Reactions were carried out in 20µl reaction mixture containing 50 ng DNA, 2µl of 10 X PCR buffer (G-Biosciences) with $MgCl_2$, 0.5µl of 10 mM of each forward and reverse primer, 0.5µl of 10 mM dNTP and 2.5U *Taq* DNA polymerase. The DNA extracted from wild type plants was used as a negative control, the pCAMBIA 1300-*Psp68* as a positive control while the reaction mix without DNA as water blank. The PCR reaction profile comprised of 35 cycles, with strand separation at 95˚C for 4 min, annealing at 52.5 ˚C for 30 s and extension at 72 ˚C for 1 min. The program was extended for 10 min at 72 ˚C. The products were electrophoresed on a 1.5% agarose gel, stained with ethidium bromide and visualized under ultraviolet light (Sambrook *et al.*, 1989).

Physio-biochemical analysis of transgenic plants under salt stress

Transgenic and non-transgenic pigeon pea plants were raised under pot culture conditions in dune sand and were subjected to 75mM NaCl stress 15 days after sowing. Various physio-biochemical parameters like relative water content, chlorophyll content, electrolyte leakage, lipid peroxidation, proline content, total soluble sugar content, catalase and peroxidase activity were recorded 4 days and 8 days after treatment.

Statistical Analysis

All the experiments were performed in triplicates and statistical analysis was carried out on physiological data recorded on T_1 generation using two factorial CRD (Completely Randomized Design) test in OPSTAT programme (Sheoran *et al.*, 1998).

Results and Discussion

Development of transgenic pigeon pea plants carrying *Psp68* gene transformation of pigeon pea var. Manak using *Psp68* gene

The transgenic pigeon pea plants carrying *Psp68* gene were developed using an efficient *Agrobacterium*-mediated transformation protocol for which a patent has been filed (Kharb *et al.,* 2018 Patent Application No.201811012099) (Fig. 1).

Molecular characterization of transgenic pigeon pea plants carrying *Psp68* gene

The putative transgenic plants were screened for the presence of *Psp68* gene in T_0 generation through PCR using gene-specific primers. An amplified fragment of 1.8 kb confirmed the presence of *Psp68* gene in the plasmid DNA. Out of 100 plants screened for the presence of *Psp68* gene, 16 plants showed a clear and sharp band of 1.8 kb, representing a transformation efficiency of 16% (Fig. 2A).

PCR analysis of T_1 transgenic pigeon pea plants carrying *Psp68* gene

Seeds collected from T_0 generation plants were sown in transgenic greenhouse to raise T_1 generation. T_1 generation plants (ten plants from T_0 each line) were screened through direct PCR kit (Phire plant direct PCR kit) using gene-specific primers. PCR analysis showed the amplification of 540 bp fragment in the transgenic plants (Fig. 2B).

Evaluation of transgene efficacy in salt stress tolerance through physio-biochemical analysis

Healthy PCR positive T_1 generation plants were selected for physio-biochemical analysis

to study transgene efficacy under salt stress. Various stress indices like chlorophyll content, relative water content, electrolyte leakage, lipid peroxidation, proline content, total soluble sugar content, catalase and peroxidase activity were estimated for the selected transgenic plants after 4th and 8thday of 75 mM salt treatment.

Effect of Salinity stress on Chlorophyll content and Relative water content

To access the effect of salinity on Chlorophyll (Chl), Chl a, Chl b, total Chl and Chl a: b was measured in transgenic lines and WT plants. Salinity stress (75 mM NaCl) significantly reduced the Chla, Chlb and total Chl in transgenic lines and WT plants but the extent of reduction was higher in WT than transgenic lines. The minimum chlorophyll content was observed in wild type plants 4 DAT (0.22 mg/g FW) and 8 DAT (0.20 mg/g FW) under 75mM NaCl salt stress whereas chlorophyll content was 0.29 mg/g FW (4 DAT) and 0.24 mg/g FW (8 DAT) in wild type control (non stressed) plants. Transgenic line 53, showed highest chlorophyll content on 4 DAT (0.56 mg/g FW) and 8 DAT (0.55 mg/g FW) under 75mM NaCl salt stress (Fig. 3A) Relative water content too has a significant influence on photosynthesis (Surender *et al.,* 2013), a reduction by 5% in RWC leads to reduction in photosynthesis by 40 to 50% Slatyer (1955).

Relative water content of the transgenic and wild-type plants decreased under stress conditions. The minimum relative water content was observed in wild type plants 4 DAT (42.86%) and 8 DAT (23.09%) under 75 mM NaCl salt stress whereas relative water content was 71.46% (4 DAT) and 69.51% (8 DAT) in wild type control plants. RWC increased 0.72 fold 4 DAT and 1.99 fold 8 DAT in transgenic line 53 over wild-type under stress (Fig. 3B).

Less oxidative stress in T_1 transgenic pigeon pea plants

Abiotic stresses including salinity cause overproduction of ROS, which leads to oxidative stress in plants. Therefore, the indicators of oxidative stress such as lipid peroxidation, electrolyte leakage were studied in *Psp68* expressing transgenic lines and WT plants (Figure 4 A-B). High concentration of salt (75 mM NaCl) significantly increased the extent of oxidative damage and it was significantly higher in WT as compared to *Psp68 carrying* transgenic pigeon pea lines. Electrolyte leakage and lipid peroxidation increased in the transgenic and wild-type plants under stress conditions. The maximum electrolyte leakage was observed in wild type plants 4 DAT (72.55%) and 8 DAT (80.96%) under 75mM NaCl salt stress whereas electrolyte leakage was 32.57% (4 DAT) and 39.06% (8 DAT) in wild type control plants. The minimum electrolyte leakage was observed in transgenic line 53, 4 DAT (18.77%) and 8 DAT (27.67%) under 75mM NaCl salt stress. Under stress transgenic plants were able to maintain lower electrolyte leakage as compared to wild-type plants. The highest lipid peroxidation was observed in wild type plants 4 DAT (3.28µmol/g FW) and 8 DAT (3.88µmol/g FW) under 75 mM NaCl salt stress whereas lipid peroxidation was 1.38µmol/g FW (4 DAT) and 1.49µmol/g FW (8 DAT) in wild type control (non stress) plants. Transgenic plants were able to maintain lower MDA content as compared to wild-type plants under stress conditions. Lipid peroxidation in transgenic line 53 was decreased by 69.40% 4 DAT and 63.61% 8DAT over wild-type under stress conditions.

Effect of salt stress on osmolytes in wild type and T_1 Transgenic pigeon pea plants

All plants produce higher levels of osmolytes in the cytosol and other organelles to

overcome the negative impact of osmotic stress (Ahmad *et al.,* 2016; Latef and Miransari, 2014). Total soluble sugars maintain cell homeostasis under abiotic stresses by acting as osmolytes (Rosa *et al.*, 2009) and accumulation of proline under stress conditions might serve as a sink for excess reductants, providing the NAD^+ and $NADP^+$ necessary for maintenance of respiratory and photosynthetic processes (Kishor *et al.*,, 2005) and has been considered as an acclamatory mechanism of salt stress (Hayat *et al.,* 2012). Both transgenic and wild-type plants showed increase in total soluble sugar and proline content under stress conditions. The minimum total soluble sugar content was observed in wild type plants 4 DAT (56.53 mg/g FW) and 8 DAT (61.20 mg/g FW) under 75mM NaCl salt stress whereas the total soluble sugar content was 51.93 mg/g FW (4 DAT) and 53.73 mg/g FW (8 DAT) in wild type control (non stressed) plants.. Total soluble sugar increased by 22.7% 4 DAT and 21.4 % 8DAT in transgenic line 53 over wild-type under stress. The minimum proline content was observed in wild type plants 4 DAT (1.73 µmol/g FW) and 8 DAT (1.88µmol/g FW) under 75 mMNaCl salt stress whereas the proline content was 0.433 µmol/g FW (4 DAT) and 0.61 µmol/g FW (8 DAT) in wild type control plants. Proline content increased by 2.4 fold 4 DAT and 2.2 fold 8 DAT in transgenic line 53 over wild-type under stress. Under stress conditions, the transgenic line 53 maintained maximum total soluble sugar and proline content (Fig. 5A-B).

Psp68 Enhances ROS Scavenging Capacity in T_1 Transgenic pigeon pea plants

Salinity stress is known to cause ROS induced oxidative damage in plant cells. Therefore, we analyzed the response of enzymatic antioxidants like catalase and peroxidase inT_1 transgenic lines and WT plants under salinity stress. Antioxidant defense machinery protects the plant cells from ROS induced oxidative damage. Catalase and peroxidase activity of both transgenic and wild-type plants increased under stress conditions. The minimum catalase activity was observed in wild type plants 4 DAT (6.34 units/g FW) and 8 DAT (8.11 units/g FW) under 75mM NaCl salt stress whereas the catalase activity was 3.80 units/g FW (4 DAT) and 4.55 units/g FW (8 DAT) in wild type control plants. The highest catalase activity was observed in transgenic line 53, 4 DAT (22.58 units/g FW) and 8 DAT (26.15 units/g FW) under 75mM NaCl salt stress. Catalase activity in the transgenic line 53 increased by 2.5 fold 4 DAT and 2.2 fold 8 DAT over wild-type under stress. The minimum peroxidase activity was observed in wild type plants 4 DAT (0.159 units/g FW) and 8 DAT (0.165 units/g FW) under 75mM NaCl salt stress whereas the peroxidase activity was 0.046 units/g FW (4 DAT) and 0.050 units/g FW (8 DAT) in wild type control (non stress) plants. The highest peroxidase activity was observed in transgenic line 53, 4 DAT (0.50 units/g FW) and 8 DAT (0.55 units/g FW) under 75mM NaCl salt stress. Peroxidase activity increased by 2.14 fold 4 DAT and 2.33 fold 8 DAT in transgenic line 53 over wild-type under stress (Fig.6A-B).

Effect of 75mM NaCl salt stress on wild-type and transgenic pigeon pea plants

Wild-type plants died as they were not able to tolerate salt concentration of 75mM NaCl whereas transgenic plants survived under stressed conditions (Fig.7).

Genetically modified (GM) crop plants are the fastest recognized technology in agriculture (James, 2010) but biosafety issue is a crucial factor for the development of transgenics and global applications of different genetically modified products.

Horizontal transfer of antibiotic-resistance genes to animal and human gut bacteria seem as major biosafety concerns in GM crops (Dale *et al.,* 2002).Therefore, it is required to develop new techniques for the production of 'clean' marker-free transgenic plants. In the present study, marker-free transgenic pigeon pea plants have been developed against salinity stress by introducing *Psp68* gene through a rapid, simple and efficient transformation system which bypasses the tissue culture procedures. Transformation following the characterization using PCR represented transformation frequency of 16.0%.

Salinization is recognized as the main threat to environmental resources and human health in many countries, affecting almost 1 billion ha worldwide/globally (Metternicht and Zinck, 2003). The production and productivity of pigeon pea is adversely affected by salinity suggesting it as a salt sensitive leguminous crop (Tayyab *et al.,* 2016). Advances in molecular and genomic tools have been widely applied to understand the mechanism underlying stress tolerance. Further, the release of pigeon pea genome sequence has paved a way to modify pigeon pea with desired genes to improve salinity tolerance (Varshney *et al.,* 2012). Engineering crop plants with improved salinity tolerance rely on expression of genes that are involved in signaling and regulatory pathways (Wang *et al.,* 2018) or genes that code for proteins involved in stress tolerance (Assaha *et al.,* 2017) or enzymes that regulate pathways involved in synthesis of functional and structural metabolites (Anjaneyulu *et al.,* 2014). It is evident many genes including DEAD-box helicases get triggered by stress, which play a crucial role in various abiotic stresses. Banu *et al.,* (2014) reported that the transcript of *Psp68* is accumulated at a high level and almost equally in every part (roots, leaves, tendrils and flowers) of the pea plant. Therefore, this gene could be a potential candidate for developing stress-tolerant transgenic plants. The *Ps*p68 protein contains all conserved domains that are characteristic of the DEAD-box proteins including 'Q' and 'GG' motif(Tanner *et al.,*) In plant the first report of stress induced helicase gene came by cDNA microarray analysis of 1300 *Arabidopsis* genes where the authors reported a DEAD-box helicase gene (accession number AB050574) as a cold stress-inducible gene suggesting a new role of helicases in stress signalling (Seki *et al.,*). Later, many plant DEAD-box helicases were identified and found to be activated in response to changing environmental conditions (Owttrim, 2006;Vashisht and Tuteja, 2006; Gill *et al.,* 2013; Mahajan and Tuteja,2005) In barley, a salt-responsive transcript HVD1 is induced under salt stress, cold stress, and ABA treatment (Nakamura *et al.,* 2004). AvDH1 is another DEAD-box helicase gene from the halophyte dogbane plant that also strongly upregulated in response to salinity and low temperature (Liu *et al.,* 2008). Under normal growth conditions relatively high level of basal expression of the pea *p68* gene in different plant parts implies its function in growth and/or development processes. Under salt treatment, a single species of pea *p68* mRNA was detected abundantly and constitutively in the tissues examined. This indicated that basic activity of cells might be regulated by pea *p68* under salt stress.

Genome-wide expression analysis of many DEAD-box helicase genes have been identified and suggested that these genes might be stress regulated (Kant *et al.,* 2005). Overexpression analysis in different DEAD-box helicases has been shown to provide multiple abiotic stress tolerance in crop plants by regulating different signalling pathways (Vashisht *et al.,* 2005; Mishra *et al.,* 2005; Tuteja *et al.,* 2013). For example, overexpression of *PDH45* and *OsSUV3* gene

provided salinity stress tolerance in tobacco and rice respectively (Mishra *et al.,* 2005; Tuteja *et al.,* 2013). LOS4 and RCF1 mutant analysis in *Arabidopsis* was found to play an important role in response to cold and heat stress (Gong *et al.,* 2005; Guan *et al.,* 2013). In our study, we showed that marker free *Psp68* provides salinity stress tolerance in pgeon pea.

The reduction in leaf chlorophyll content under abiotic stress has been attributed to the destruction of chlorophyll pigments in various crop plants (Tuteja *et al.,* 2012; Zhang *et al.,* 2012; Huda *et al.,* 2013). We observed that stress-induced chlorophyll loss was enhanced in WT plant while transgenic lines retained more chlorophyll. This finding has strong correlation with the previous studies in other DEAD-box helicases (Mishra *et al.,* 2005; Dang *et al.,* 2011; Sahoo *et al.,* 2013). Hence it indicates the expression of *Psp68* gene could have positive effects on the growth and photosynthetic metabolism process. Under salt stress conditions, plants usually adjust their osmotic potential to maintain turgor pressure (Boyer *et al.,* 2008) thus maintaing cellular hydration levels.In present investigation, decrease in RWC was observed in both WT and transgenic plants with salt treatment but decline in RWC was more in wild-type plants under 75 mM NaCl stress as compared to transgenic plants.

Stress also leads to the rapid production of ROS including H_2O_2 in plant tissues that ultimately cause damages to the cell membrane and other cellular components such as plasma membrane, mitochondria and chloroplasts (Gill *et al.,* 2013; Huda *et al.,* 2013). Hence, to avoid any stress-induced injuries plant needs to develop efficient mechanism to remove excess ROS from cells. Enzymatic ROS-scavenging and non-enzymatic antioxidants system are such mechanisms in the plant cells that prevent ROS induced oxidative damage (Gill *et al.,* 2010; Gill *et al.,* 2012; Bhattarcharjee, 2012). Catalase and peroxidase are the major enzymes that are known to be involved in scavenging of cellular production of H_2O_2 (Willekens *et al.,* 1994; Noctor and Foyer, 1998).

Fig.1 Vector map of the binary vector pCAMBIA 1300 carrying Psp68 gene

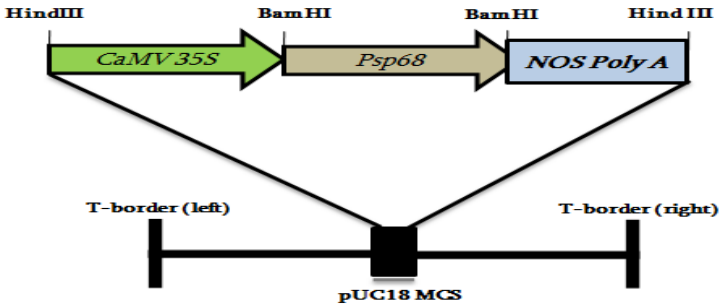

Fig.2 PCR analysis of transformants (A) 1.5 % agarose gel showing amplification of 1.8 kb

fragment of Psp68 gene. (B) 1.5 % agarose gel showing amplification of 540 bp fragment of Psp68 gene in T1 generation plants. Lanes L-1 kb ladder, PC: Positive Control (Plasmid DNA), NC: Negative Control (Genomic DNA of wild-type)

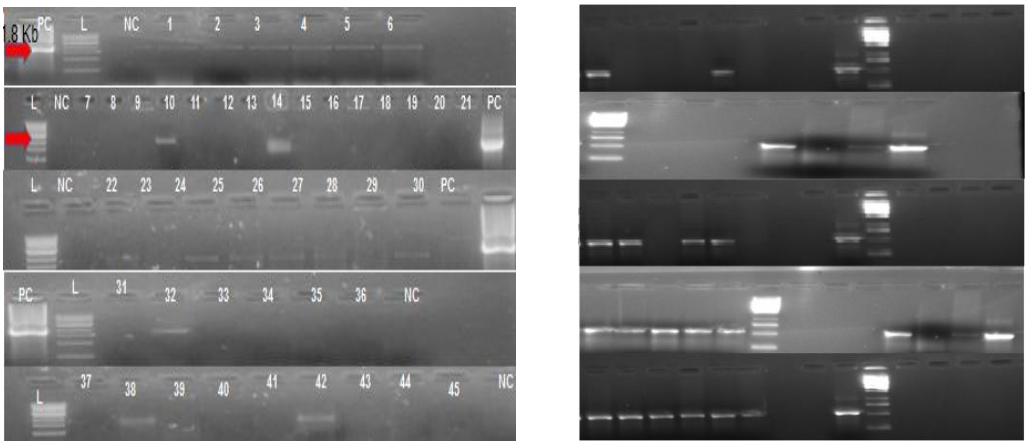

Fig.3 (A) Effect of 75 mM salt stress on chlorophyll content and (B) Relative water content in wild-type and T1 transgenic pigeon pea plants

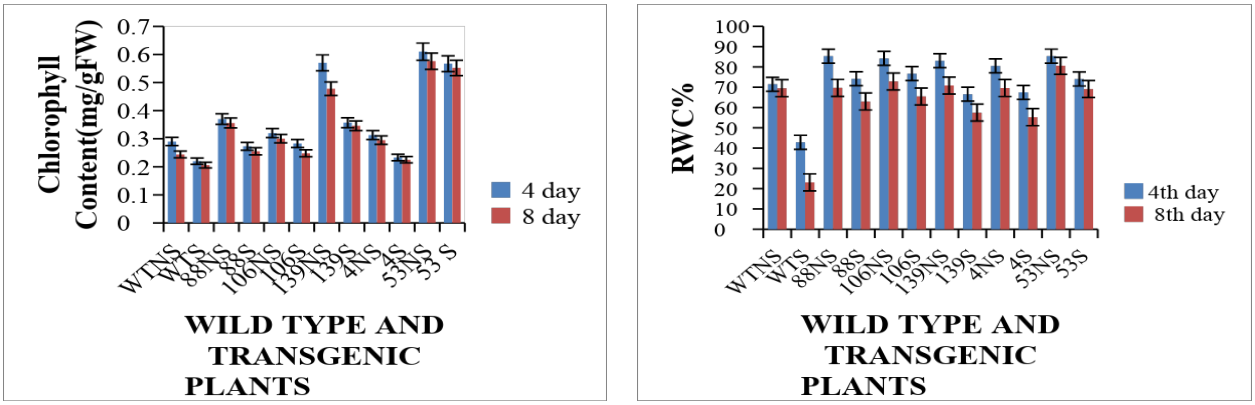

Fig.4 Effect of 75 mM salt stress on (A) electrolyte leakage and (B) MDA content in wild-type and T1 transgenic pigeon pea plants

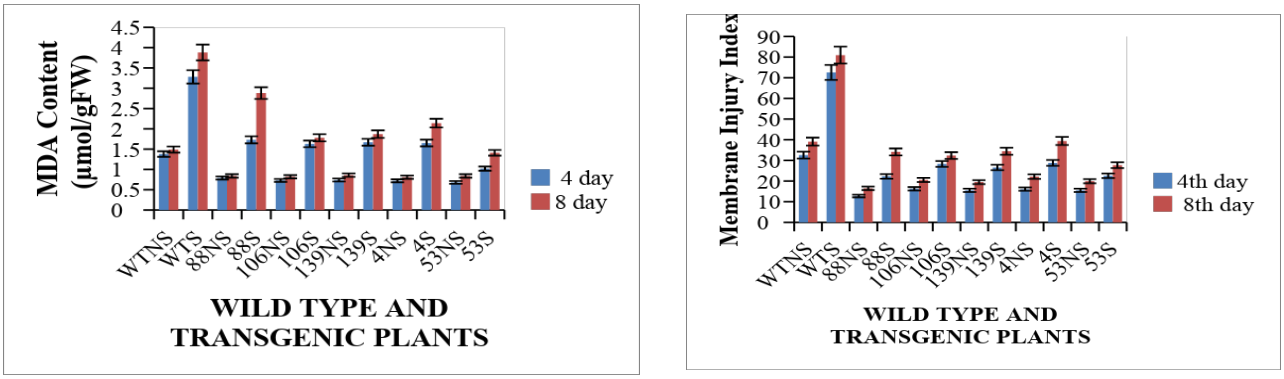

Fig.5 Effect of 75 mM salt stress on (A) Total soluble sugar content (B) Proline content in wild-

type and T1 transgenic pigeon pea plants

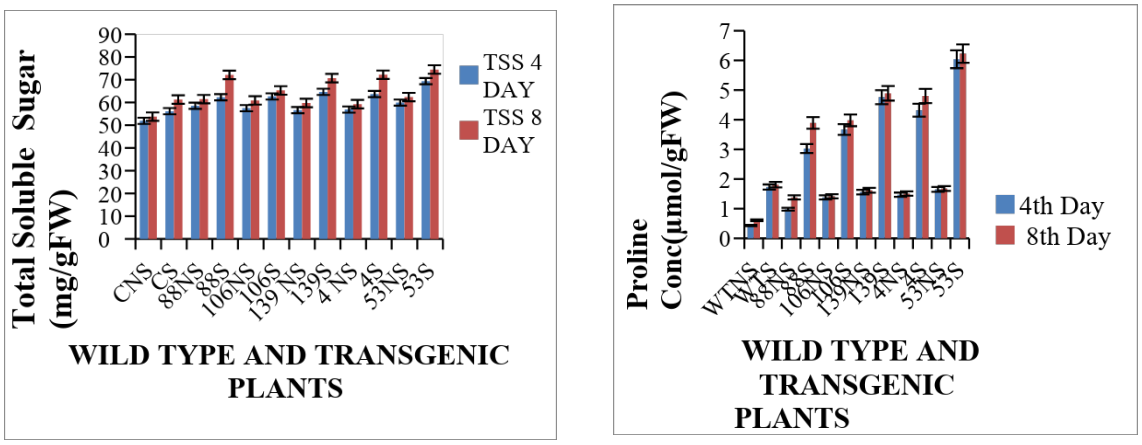

Fig.6 Expression of Psp68 showed less oxidative damage by modulating the ROS machinery under salinity stress. (A) Catalase activity (B) Peroxidase activity in wild-type and T1 transgenic pigeon pea plants under 75mM Salt stress

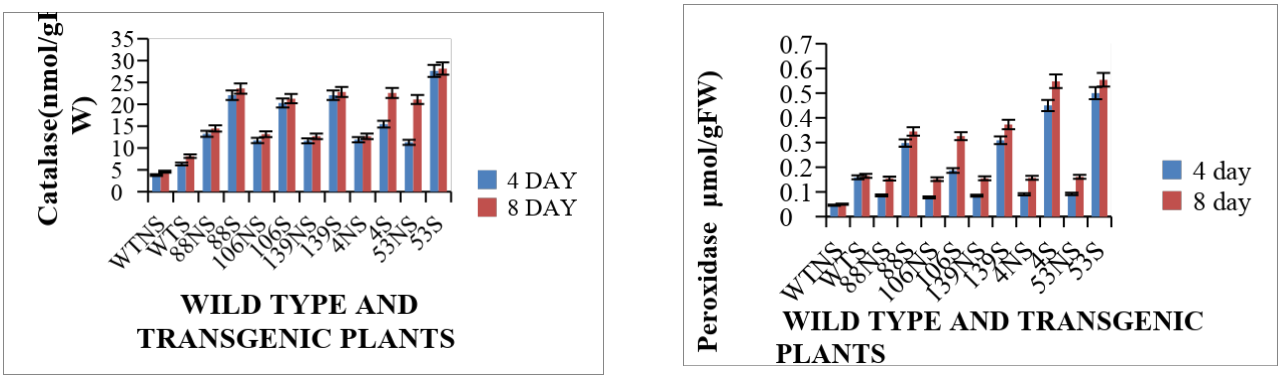

Fig.7 Effect of 75mM NaCl salt stress on wild-type and transgenic pigeon pea plants

Interestingly in this study the activity of these enzymes increased in transgenic lines in response to stress treatment indicating that transgenic lines could readily scavenge H_2O_2 either decomposing it through increased activity of catalase or peroxidase. Previously, a number of overexpression studies have shown an increased activity of catalase and peroxidase in response to abiotic stress treatment (Jiang *et al.,* 2002; Luna *et al.,* 2004; Mhamdi *et al.,* 2010; Gill *et al.,* 2013). Moreover, plants produce higher levels of osmolytes in the cytosol and other organelles to overcome the negative impact of osmotic stress (Ahmad *et al.,* 2016; Latef and Miransari, 2014). Increase in total soluble sugars helps to bring down the osmotic potential of cell sap below that of growing medium, enabling the uptake of water by cells under salt stress (Benzarti *et al.,* 2014) and accumulation of proline under stress has been considered as an acclamatory mechanism of salt stress (Hayat *et al.,* 2012). In present investigation, salt stress resulted in accumulation of proline and total soluble sugars in both wild-type and transgenic pigeon pea plants.

In conclusion, the involvement of DEAD-box helicases in various metabolic processes in plant cells might have general implications. The present study provides new insights into the novel function of marker free *Psp68* gene in conferring salinity stress tolerance in transgenic pigeon pea plant. Salt stress affected the various physio-biochemical parameters resulting in decrease in chlorophyll and relative water content and an increase in electrolyte leakage, peroxidation, total soluble sugar content and proline content. The activity of antioxidant enzymes, catalase and peroxidase increased with salt stress. Among all the transgenic lines, line 53 was found promising for salt tolerance in terms of various physio-biochemical parameters studied under salt stress conditions. This study showed the role of *Psp68* coding for DEAD Box RNA helicase in mitigating salt stress as transgenic plants performed well under salt stress conditions.

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