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OVEREXPRESSION OF A DEAD BOX HELICASE *Psp68* GENE AND ITS MUTANT CONFERS SALINITY STRESS TOLERANCE IN BACTERIA

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Abstract

Salinity stress adversely affects crop plant growth and productivity resulting in significant yield losses worldwide. Hence, it is essential to develop stress-tolerant species and as well as to understand the mechanisms behind it. The *Psp68* is a DNA and RNA helicase and it is involved in numerous processes including protein synthesis, maintaining the basic activities of the cell, transcriptional activation/repressors, RNA processing, and abiotic stress response. This study evidence that overexpression of *Psp68* provides salinity (NaCl and LiCl) stress tolerance in bacteria *Escherichia coli* bacterium also. Furthermore, the site-directed mutagenesis technique was applied to create three single mutants namely K168A, Q286G and R461Q, and their expression was also checked in response to the above mentioned stresses. Surprisingly, only the overexpression of single mutant R461Q showed high salinity stress tolerance in *E. coli*. Therefore, this study provides a new tool for developing stress tolerant crop plants and bacteria of agronomic importance in the field of agricultural biotechnology.

Keywords: Cellular stress, *E. coli*, *Psp68*, RNA processing, Salinity stress

Introduction

The helicases are ubiquitous enzymes that catalyze the unwinding of energetically stable duplex DNA or duplex RNA secondary structures (Tuteja, 1997; Tuteja, 2000; Tuteja and Tuteja 2004a and b). These enzymes play an essential role in DNA replication, repair, recombination, transcription, translation, RNA metabolism, and therefore, involved in the basic cellular processes regulating plant growth and development (Kammel *et al.,* 2013; Guan *et al.,* 2013). The helicases play an important role in stabilising growth in plants under stressful environment by regulating stress-induced transcription and translation (Banu *et al.,* 2015; Nidumukkala *et al.* 2019). The p68 is an evolutionarily conserved protein. The human p68 RNA helicase was first identified by immunological cross-reaction with the anti-SV40 large T monoclonal antibody (Crawford *et al.,* 1982). Human p68 cDNA (DDX5) have high homology with eIF4A protein, both have nucleic acid unwinding activity (Scheffner *et al.,* 1989).

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Previous studies with purified p68 protein have shown that it exhibits ATP binding, RNA-dependent ATPase and RNA helicases activities *in vitro* (Iggo and Lane, 1989). The amino acid sequence reveals that p68 protein contains multiple conserved motifs that are well known for all DEAD box RNA helicases (Banu *et al.,* 2015). The overall properties of p68 suggest that it could be an important multifunctional protein involved in protein synthesis, maintaining the basic activities of the cell, transcriptional activation/repressors, RNA processing and abiotic stress response (Banu *et al.,* 2015).

To fight against stresses, plants have develop various unique stress adaptive mechanisms however, the basic cellular responses to stresses are almost similar in prokaryotes, lower eukaryotes and as well as in plants (Kultz, 2003). Therefore, overexpression of the plant stress responsive genes may also provide stress tolerance in the bacteria (Joshi *et al.,* 2009; Joshi *et al.,* 2010; Tajrishi *et al.,* 2011). The objective of this study was to examine whether overexpression of *Psp68* provides salinity stress tolerance in *E. coli* bacterium or not using mutagenesis. Furthermore, the developed single mutations in the conserved motifs of *Psp68* gene were also overexpressed and compared with the growth of the bacteria transformed with mutated and non-mutated *Psp68* genes.

Materials and Methods

Site-directed mutagenesis of *Psp68*

The detail of the *PsP68* gene (Accession number: AF271892.1) was described earlier by Tuteja *et al.,* (2014). The desired point mutations were generated in *PsP68* gene using specific designed forward and reverse primers to develop three mutants viz. K168A, Q286G and R461Q. The Quik Change site-directed mutagenesis kit was used to create the desired point mutations in the *Psp68* gene. The 1.8 kb K168A, Q286G and R461Q mutated *PsP68* DNA fragments were prepared by PCR using normal *Psp68* gene (cloned in pGEM-T easy vector) and appropriate primers (K183A- *Psp68*F, Q286G- *Psp68*F, R461Q- *Psp68*F or *Psp68*R) with desired point mutations designed from *Psp68* gene. Finally, the K183A, Q286G and R461Q, mutated 1.8 kb fragments were cloned in pGEM-T easy vector and the sequence was confirmed by sequencing.

Cloning of mutated and non-mutated *Psp68* in pET28a expression vector

All the mutated (K168A, Q286G and R461Q) and non-mutated *Psp68* genes which were cloned in pGEM-T easy vector were digested with specific restriction enzymes (*Nde*I and *Bam*HI). The digested reaction mixtures were run on the agarose to elute the 1.8 kb fallout from the gel. The eluted fall out band was further ligated into the digested pET28a vector (*Nde*I and *Bam*HI). Ligation reactions were further transformed in DH5α competent cells. The colonies were confirmed by PCR using the gene-specific primers. The recombinant plasmids were again confirmed by sequencing.

Growth of *E. coli* and salt stress responses (NaCl and LiCl) of mutated and non-mutated *Psp68* in transformed cells

The *E. coli* (BL21 cells) were transformed with mutated K168A, Q286G and R461Q and non-mutated *Psp68*-pET28a using the standard technique. The transformed

BL21 cells were grown to log phase OD_{600} = 0.5 and the equal amount of cells were transferred to sterile culture tubes with 10 ml of LB medium containing 50 µg/ml kanamycin, 1 mM IPTG and 200, 400, 800 mM and 1M NaCl or LiCl. The cells were allowed to grow at 37°C and the growth was observed every hour by measuring the OD_{600} using a spectrophotometer.

Results

Preparation of single mutants

The conserved motifs of *Psp68* along with their sequence of motif I (GSGK), motif II (VLDEADRMLDMGFEPQ) and motif VI (YIHRIGRT) are shown in Fig. 1A. On the basis of the characteristics of the conserved motifs, motif I, II and VI were selected for substitution in *Psp68* to create the desired mutants. By using PCR based site-directed mutagenesis, amino acid lysine (K) at position 168 of the motif I, glutamine (Q) at position 286 of motif II and arginine (R) at position 461 of motif VI were substituted to alanine (A), glycine (G) and glutamine (Q) respectively. Finally, the resulted *Psp68* mutants were named as K168A, Q286G, and R461Q.

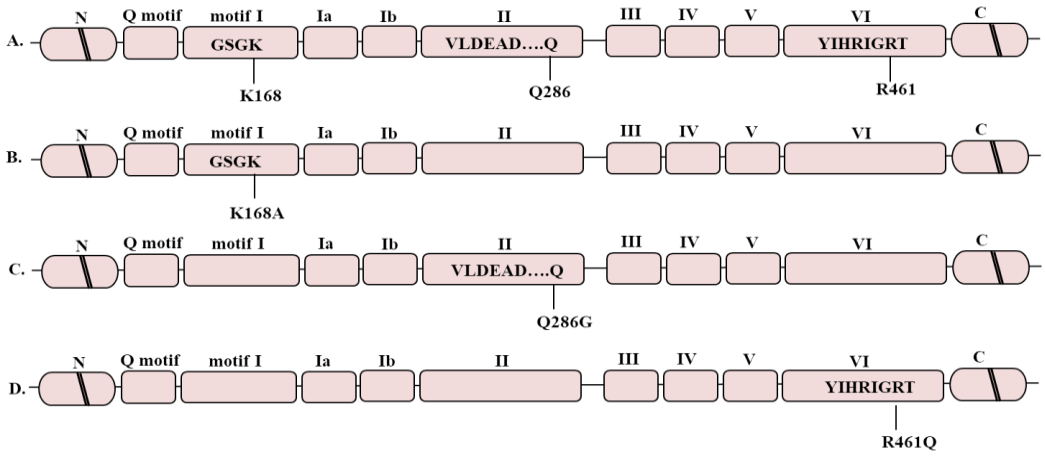

Fig. 1 (A-D). Site-directed mutagenesis in three conserved domains of *Psp68*. A. Schematic diagram showing conserved motifs and the sequence of the three motifs (I, II and VI) on which mutations have been created, B. Schematic diagram of single mutant K168A, C. Schematic diagram of single mutant Q286G, and D. Schematic diagram of single mutant R461Q.

The description of the preparation of single mutants, K168A, Q286G and R461Q were given in the materials and method section. The mutated and non-mutated *Psp68* genes were amplified by using specific primers and first cloned in pGEM-T easy vector. The sequencing results of the cloned single mutants of *Psp68* genes were confirmed the substitution of AAG (code for amino acid K) to GCT (code for amino acid A), CAA (code for amino acid Q) to GGT (code for amino acid G) and AGA (code for amino acid Q) to CAA (code for amino acid Q). Schematic representations of all the single mutants were shown in Fig. 1B-D.

Cloning and restriction analysis of single mutants in pET28a expression vector

All the single mutants of the *Psp68* gene were cloned in *Nde*I and *BamH*I restriction sites of pET28a expression vector under control of T7 promoter and T7 terminator (Fig. 2A). Transformed cells containing recombinant plasmids were identified by colony PCR (Fig. 2B-D). A single colony was re-suspended in 10 µl of water and then boiled for 5 min at 95ºC. After boiling, 2 µl supernatant was used as a template for PCR using gene specific primers. Colony PCR positive clones were further confirmed by restriction analysis (Fig. 2E-G) using *Xho*I restriction enzyme (internal cutter of *Psp68*).

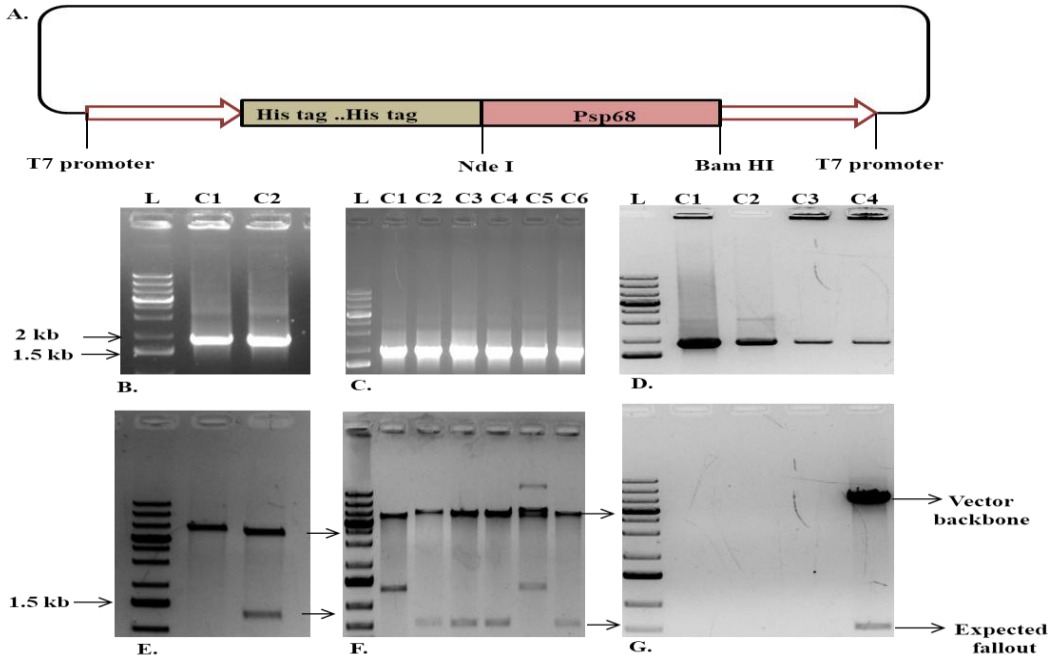

Fig. 2 (A-G). Cloning and restriction analyses of mutants in pET28a. A. Diagrammatic representation of *Psp68*-pET28a construct for expression of *Psp68*. B-D. Confirmation of K168A-*Psp68*-pET28a, Q286G-*Psp68*-pET28a and R461Q-*Psp68*-pET28a respectively by colony PCR. E-G. Restriction analysis of K168A-*Psp68*-pET28a, Q286G-*Psp68*-pET28a and R461Q-*Psp68*-pET28a, respectively by using *Xho*I restriction enzyme**.**

Salinity stress (NaCl and LiCl) responses of mutated and non-mutated *Psp68* in *E. coli*

The *E. coli* (BL21 cells) containing *Psp68*-pET28a and three different single mutated constructs (K168A-pET28a, Q286G-pET28a and R461Q-pET28a) were separately exposed to NaCl salt stress. The cells were grown to log phase OD_{600}= 0.5 and then 1mM IPTG was added to the culture medium to induce gene expression. Along with IPTG, cells were also induced with 200, 400, 800 mM and 1M NaCl concentrations (Fig. 3A). At 200 mM NaCl stress, cells containing *Psp68*-pET28a along with K168A-pET28a

and R461Q-pET28a mutants were grown easily while very little growth was observed for Q286G-pET28a mutant (Fig. 3A). No cell growths were detected for K168A-pET28a and Q286G-pET28a mutants at 400, 800 mM and 1M NaCl stress. It is interesting to note that at all the stress treatments (200, 400, 800 mM and 1M NaCl), a high growth was observed in the case of R461Q-pET28a mutant (Fig. 3A). The comparison of the growth curves of the BL21 cells containing wild type *Psp68*-pET28a (non-mutated) along with mutated *Psp68*-pET28a showed that the wild type *Psp68*-pET28a and mutated R461Q-pET28a were able to help *E. coli* tolerate to salinity stress. Whereas, the cells containing K168A-pET28a and Q286G-pET28a mutated proteins were not able to cope up with salinity stress conditions (Fig. 3B).

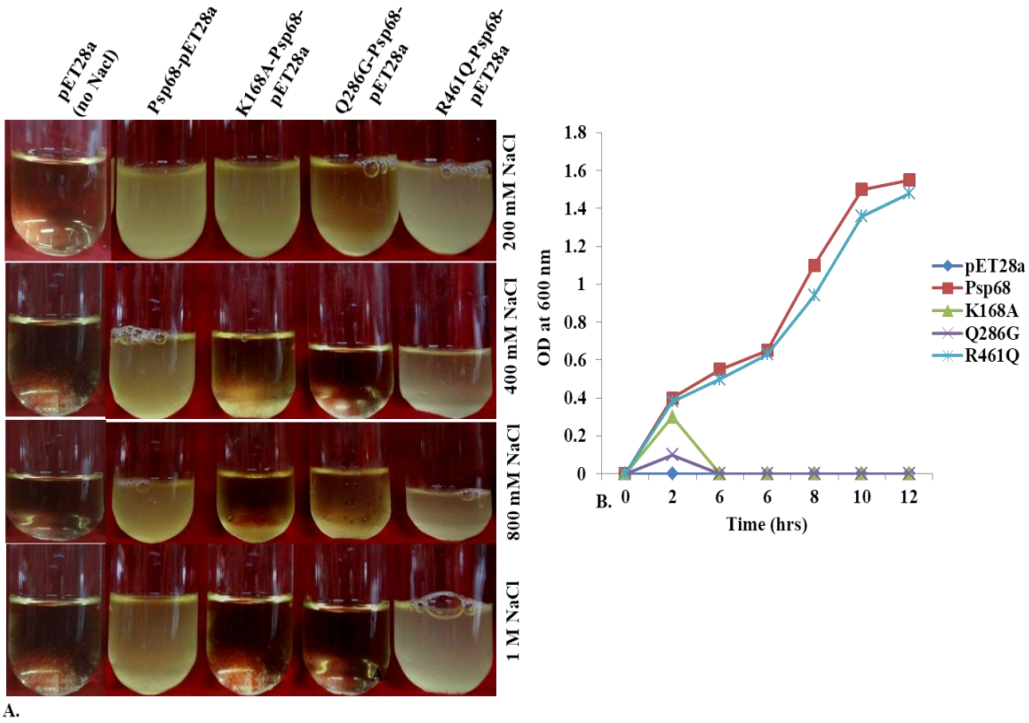

Fig. 3. High salinity (NaCl) stress response of *Psp68* and its mutants in *E. coli*. A. The tubes picture shows the bacterial growth of *Psp68*, single mutants (K168A, Q286G and R461Q), and empty vector after 12 h in the presence of 200 mM, 400 mM, 800 mM and 1M of NaCl. B. Growth curves analysis under salinity stress of *Psp68* and its mutants in *E. coli*.

In another experiment, the *E. coli* (BL21 cells) containing non-mutated (*Psp68*-pET28a) and mutated (K168A-pET28a, Q286G-pET28a and R461Q-pET28a) constructs were separately subjected to grow in the presence LiCl salt stress (200, 400, 800 mM and 1M). The results are shown in Fig. 4, which clearly indicate that the transformed BL21 cells with mutated (R461Q-pET28a) or non-mutated (*Psp68*-pET28a) were equally grow well in presence of all the LiCl stress treatments. The other two mutants (K168A-pET28a

and Q286G-pET28a) also showed some growth in presence of 200 and 400 mM LiCl salt stress (Fig. 4).

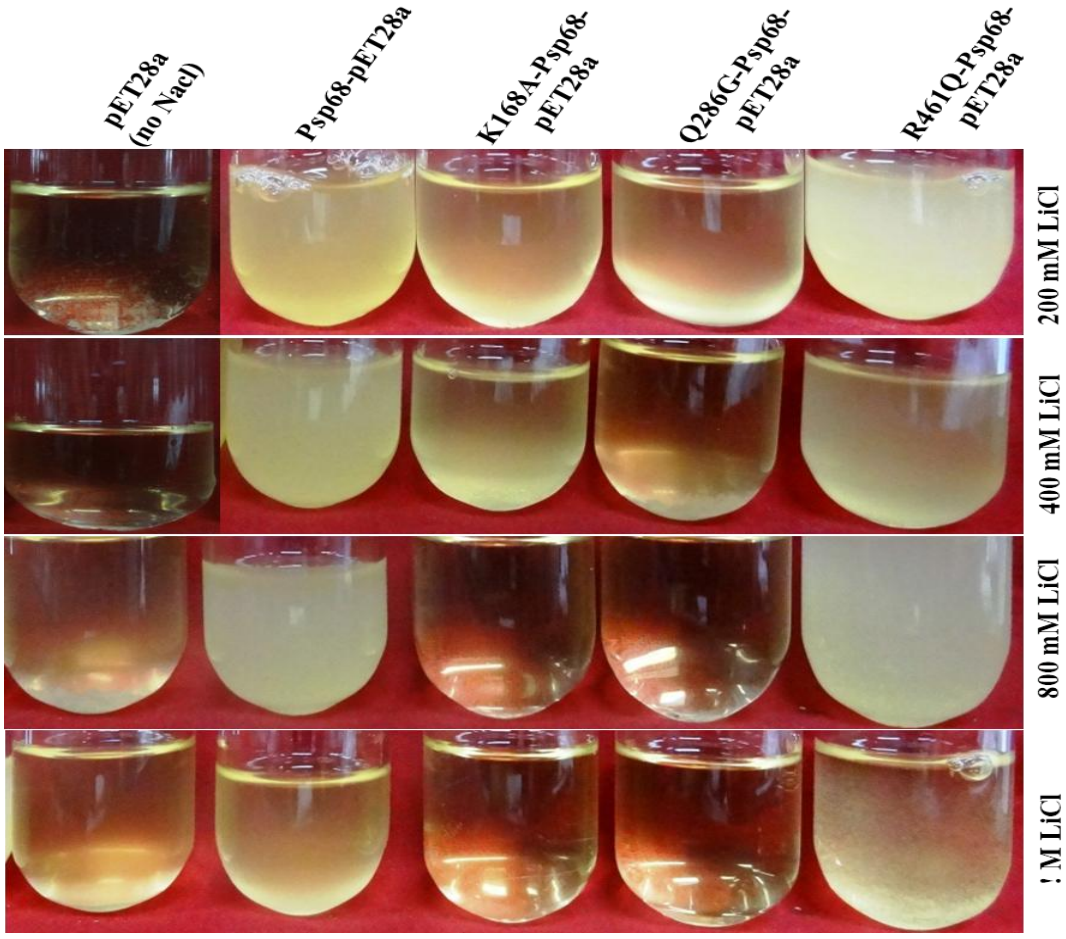

Fig. 4. High salinity (LiCl) stress response of *Psp68* and its mutants in *E. coli*. The tubes picture shows the bacterial growth of *Psp68*, single mutants (K168A, Q286G and R461Q), and empty vector after 12 h in the presence of 200 mM, 400 mM, 800 mM and 1M of LiCl.

Discussion

To adapt to unfavourable environmental conditions, both eukaryotes and prokaryotes have certain stress tolerance mechanism. Cellular stress response in archaea (Macario and Macario 1999), the eubacteria (Hecker and Volker, 2001) and eukaryotes (Pearce and Humphrey, 2001) were always connected with protein and DNA processing and stability (Kültz, 2003). Several sets of homologous/orthologous stress proteins, including chaperones, different cell cycle regulators and DNA repair proteins are induced by environmental stresses in archaea, eubacteria and eukaryotes. Among the various abiotic stresses, the salinity stress adversely affect plant growth and productivity and it is

well known that functionally analogous stress resistant genes exist both in unicellular organisms and plants (Serrano *et al.,* 2003). As both the prokaryotes and eukaryotes used the similar basic cellular adaptive mechanisms to tackle the stress, it can be assumed that plant stress tolerance genes can be functionally screened in simple prokaryotic organisms (Joshi *et al.,* 2009; Tajrishi *et al.,* 2011; Xu *et al.,* 2020). This study identified the novel function of *Psp68* in salinity stress tolerance in bacteria with an unidentified mechanism.

The p68 is an evolutionarily conserved member of the DEAD-box protein family of helicases and there are at least five DEAD genes in *E. coli* also. The member of this family has been well characterized by its conserved motifs such as: Q motif, Motif I, Ia and Ib and as GG-doublet- Motif II, III, IV, V and VI (Banu *et al.,* 2015). Each of these conserved motifs has important characteristics. The Q motif is responsible for sensing the nucleotide state of the helicase by forming a stable interaction with Walker A box (P-loop) of other helicase motifs (Strohmeier *et al.,* 2011) while the motif I is crucial for the ATPase and helicase activities by interacting with Mg^{2+} ion (Shi *et al.,* 2004). Mutations in this motif abolish the ATPase activity by reducing the affinity of hydrolysis (Cordin *et al.,* 2004). The motif II is also known as 'Walker motif B' (Venkatesan *et al.,* 1982) and has a role in helicase and ATPase activity (Turner *et al.,* 2007). The DE within this motif is highly conserved and plays a role in DNA and RNA replication (Gorbalenya *et al.,* 1989). The motif VI is important both for ATPase activity and RNA binding (Rogers *et al.,* 2002) and mutation of this motif showed leads to defects in the nucleic acid binding (Pause and Sonenberg, 1992), negative impact on ATP hydrolysis and ligand induced conformational changes in *E. coli* (Tuteja and Tuteja, 2006). In plants *Psp68* has been shown to play an important role in salinity stress tolerance (Banu *et al.,* 2015). The aim of this study was to check whether the same gene can provide the salinity stress tolerance in bacteria or not.

One of the interesting findings of this study is that overexpression of *Psp68* within the *E. coli* bacterium significantly enhanced salinity stress tolerance. More importantly, the mutation of the gene in motif VI provided more salinity stress tolerance than the wild-type *E. coli* suggesting that overexpression of the *Psp68* gene could be utilized for the enhancement of salinity tolerance in organisms including the development of engineered beneficial bacteria with plant growth promoting traits. A considerable genera of plant associated bacteria such as Bacillus, Paraburkholderia Pseudomonas, Delftia etc. have been identified as powerful probiotics that significantly promote growth, yield and quality of crop plants (Khan *et al.,* 2017;Rahman *et al.,* 2018,). Genetic improvement of these natural plant growth promoters for higher salinity tolerance would allow them to use in plant growth promotion under higher level of saline soils.

Salinity stress is one of the most climate change induced abiotic stresses that limit plant growth and productivity. Development of salinity stress tolerant crop plant is an important target to achieve climate-smart agriculture. Our results suggest that overexpression of the *Psp68* gene with mutated motif VI in targeted plant could be an aid for better adaptation of plants to soil salinity (Banu *et al.,* 2015; Nidumukkala *et al.* 2019). Further study is needed to validate this hypothesis by transforming the salinity sensitive crop plants through bioengineering or genome editing.

Conclusion

This study provides a direct evidence that overexpression of *Psp68* and its mutant for motif VI promotes salinity stress tolerance in bacteria. The findings of the current study offer a possibility of the development of salinity tolerant crop plant and also plant growth promoting beneficial bacteria using bioengineering of *Psp68* gene and its motif VI. Further studies are needed to validate this hypothesis that is crucial for the mitigation of soil salinity problem in a large area of cultivated lands.

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Conflicts of Interest

The authors declare no conflicts of interest regarding publication of this manuscript.

References

Banu, M. S. A., K. M. K. Huda, R. K. Sahoo, B. Garg, S. Tula, S. M. S. Islam, R. Tuteja and N. Tuteja. 2015. Pea p68 imparts salinity stress tolerance in rice by scavenging of ROS-mediated H_2O_2 and interacts with Argonaute. *Plant Mol. Biol. Rep.* 33:221-238.

Cordin, O., N. K. Tanner, M. Doere, P. Linder and J. Banroques. 2004. The newly discovered Q motif of DEAD-box RNA helicases regulates RNA-binding and helicase activity. *EMBO J.* 23:2478-2487.

Crawford, L., K. Leppard, D. Lane and E. Harlow. 1982. Cellular proteins reactive with monoclonal antibodies directed against simian virus 40 T-antigen. *J. Viro.* 42:612-620.

Gorbalenya, A. E., E.V. Koonin, A. P. Donchenko and V. M. Blinov. 1989. Two related super families of putative helicases involved in replication, recombination, repair and expression of DNA and RNA genomes. *Nucleic Acids Res.* 17:4713-30.

Guan, Q., J. Wu, Y. Zhang, C. Jiang, R. Liu and C. Chai. 2013. A DEAD box RNA helicase is critical for pre-mRNA splicing, cold-responsive gene regulation, and cold tolerance in Arabidopsis. *The Plant Cell.* 25:342-356.

Hecker, M. and U. Volker. 2001. General stress response of Bacillus subtilis and other bacteria. *Adv. Microb. Physiol.* 44:35-91.

Iggo, R. D. and D. P. Lane. 1989. Nuclear protein p68 is an RNA-dependent ATPase. *EMBO J.* 8: 1827-1831.

Joshi, A., H. Q. Dang, N. Vaid and N. Tuteja. 2009. Isolation of high salinity stress tolerant genes from *Pisum sativum* by random overexpression in *Escherichia coli* and their functional validation. *Plant Signal. Behav.* 4:400-12.

Joshi, A., N. Vaid, H.Q. Dang and N. Tuteja 2010. Pea lectin receptor-like kinase promotes high salinity stress tolerance in bacteria and expresses in response to stress in planta. *Glycoconj J.* 27:133-50.

Kammel, C., M. Thomaier, B. B. Sorensen, T. Schubert, G. Langst and M. Grasser. 2013. Arabidopsis DEAD-box RNA helicase UAP56 interacts with both RNA and DNA as well as with mRNA export factors. *PloS One* 8:e60644.

Khan, M. M. A. E. Haque, N. C. Paul, M. A. Khaleque, S. M. Al-Garni, M. Rahman and M. T. Islam. 2017. Enhancement of growth and yield of rice in nutrient deficient soils by rice probiotic bacteria. *Rice Sci*. 24(5):264-273.

Kültz, D. 2003. Evolution of the cellular stress proteome: from monophyletic origin to ubiquitous function. *J. Exp. Biol.* 206:3119-24.

Macario, A. J. and E. C. de Macario. 1999. The archaeal molecular chaperone machine: peculiarities and paradoxes. *Genetics.* 152:1277-1283.

Nidumukkala, S., L. Tayi, R. K. Chittela, D. R. Vudem and V. R. Khareedu. 2019. DEAD box helicases as promising molecular tools for engineering abiotic stress tolerance plants. *Crit. Rev. Biotechnol*. 39(3):395-407.

Tajrishi, M. M., N. Vaid, R. Tuteja and N. Tuteja. 2011. Overexpression of a pea DNA helicase 45 in bacteria confers salinity stress tolerance. *Plant Signal. Behav.* 6:1271-1275.

Pause, A. and N. Sonenberg. 1992. Mutational analysis of a DEAD box RNA helicase: the mammalian translation initiation factor eIF-4A. *EMBO J.* 11:2643-54.

Pearce, A. K. and T. C. Humphrey. 2001. Integrating stress-response and cell-cycle checkpoint pathways. *Trends Cell Biol.*11:426-433.

Rahman, M., A. A. Sabir, J. A. Mukta, M. Khan, M. Alam, M. Mohi-Ud-Din, M. Mia, M. Rahman and M. T. Islam. 2018. Plant probiotic bacteria Bacillus and Paraburkholderia improve growth and content of antioxidants in strawberry fruit *Sci. Rep.* 8(1):1-11.

Rogers, G. W., A. A. Jr., Komar and W.C. Merrick. 2002. eIF4A: the godfather of the DEAD box helicases. *Prog. Nucleic Acid Res. Mol. Biol.* 72:307-331.

Scheffner, M., R. Knippers and H. Stahl. 1989. RNA unwinding activity of SV40 large T antigen. *Cell.* 57:955-963.

Serrano, R., R., Gaxiola, G., Rios, J., Forment, O., Vicente and R. Ros. 2003. Salt stress proteins identified by a functional approach in yeast. *Monatsh Chem.* 134:1445-62.

Shi, H., O. Cordin, C. M. Minder, P. Linder and R. M. Xu. 2004. Crystal structure of the human ATP-dependent splicing and export factor UAP56. *PNAS, USA.* 101:17628-17633.

Strohmeier, J., I. Hertel, U. Diederichsen, M. G. Rudolph and D. Klostermeier. 2011. Changing nucleotide specificity of the DEAD-box helicase Hera abrogates communication between the Q-motif and the P-loop. *Biological Chemis.* 392:357-369.

Tajrishi, M. M., N. Vaid, R. Tuteja and N. Tuteja. 2011. Overexpression of a pea DNA helicase 45 in bacteria confers salinity stress tolerance. *Plant Signal. Behav.* 6:1271-1275.

Turner, A. M., C. F. Love, R. W. Alexander and P. G. Jones. 2007. Mutational analysis of the *Escherichia coli* DEAD box protein CsdA. *J. Bacteriol.* 189:2769-76.

Tuteja, N. 1997. Unravelling DNA helicases from plant cells. *Plant Mol. Biol.* 33:947-952.

Tuteja, N. 2000. Plant cell and viral helicases: essential enzymes for nucleic acid transactions. *Cri. Rev. Plant Sci.*19:449-478.

Tuteja, N. and R. Tuteja. 2004a. Prokaryotic and eukaryotic DNA helicases. Essential molecular motor proteins for cellular machinery. *FEBS.* 271:1835-1848.

Tuteja, N. and R. Tuteja. 2004b. Unraveling DNA helicases. Motif, structure, mechanism and function. *FEBS.* 271: 1849-1863.

Tuteja, N. and R. Tuteja. 2006. DNA helicases as molecular motors: An insight. *Physica A: Statistical Mechanics and its Appli.* 372:70-83.

Venkatesan, M., L. L. Silver and N. G. Nossal. 1982. Bacteriophage T4 gene 41 protein, required for the synthesis of RNA primers, is also a DNA helicase. *J. Biol. Chem.* 257:12426-34.

Xu. L., X. Ji, G. Jin, F. Guan, Q. Luo and F. Meng. 2020. Improvement of salt tolerance on *Escherichia coli* by expression of agglutinin from *Amygdalus Mira*. *Intl. J. Agric. Biol.* 23:333-339.