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Research Article

Functional validation of mungbean LEA protein coding gene in bacterial expression system confers salt stress tolerance

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Abstract

Mungbean (*Vigna radiata* R. Wilczek) is a major tropical food grain legume that is widely cultivated in tropical part of the world. Mungbean like other plants, tolerate and survive limited water situation owing to expression of stress associated proteins that offers membrane stability and cell protection. Late Embryogenesis Abundant (LEA) proteins are among the group of low molecular weight proteins, that play diverse roles in stress protection in several species of plants and animals. A LEA protein coding gene *VrLEA2* was isolated from mungbean and its role in stress tolerance has been demonstrated using a bacterial expression system. *VrLEA2* gene isolated was of size 893 bp and characterized as a group 1 LEA protein based on the sequence signature motif with presence of hydrophilic domain and a characteristic 20-mer conserved amino acids motif. *VrLEA2* gene was cloned into a bacterial expression vector, pET 28a (+), transformed into the *E.coli* BL21 (DE3) cells for recombinant protein expression and subsequently subjected to antibiotic selection with kanamycin. Functional validation of the *VrLEA2* for salt stress tolerance with varied concentration of NaCl (0 mM to 600 mM) showed alteration in colony morphology and reduction in the number of colonies in control compared to the transformed cells demonstrating the improved survival rate of cells expressing VrLEA2 protein. These findings indicate the best use of bacterial expression system for functional validation of plant proteins under stressed environments.

Keywords: characterization; *E. coli*; LEA; protein expression; salinity; plant stress

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Introduction

Plants, throughout their life cycle are exposed to vagaries of climate changes and stress situations. In order to alleviate the stress and survive under these unfavorable conditions, they have developed different responses. Plants are exposed to different biotic and abiotic stresses and are major limiting factors for their growth and development (Suzuki *et al*., 2014). Drought, high temperature, freezing and salinity are some of the abiotic stress from which the normal growth and development of plant is affected. In order to withstand these unfavorable conditions, plants adapted different strategies to prevent the damage and/or to protect themselves after the damage (Nawaz *et al*., 2023). Mungbean, a member of fabaceae is a commonly consumed traditional food grain legume for its high protein content (20-25%) and cultivated for more than 3500 years (Ganesan and Xu, 2018). Like other plants, the plant can withstand multiple stresses; however there is considerable yield penalty due to major abiotic stresses (Nair *et al*., 2019).

Several types of proteins occur in seeds and some start accumulating very early following fertilization while others start accumulating in the later stages, close to the desiccation of the mature seed. Late embryogenesis abundant (LEA) proteins or Early-methionine labeled proteins (Em proteins) are accumulated during late stages of seed maturation or embryogenesis (Gaubier *et al.*, 1993). These LEA proteins are said to occur universally (Dure *et al*., 1989) and translatable form of their mRNAs stored in embryo of dry seed (Raynal *et al.*, 1990). The function of many genes induced during dehydration are predicted from their deduced amino acid sequences and are found in a wide range of plant species; still the precise function of some gene remains unknown. Due to lack of complete understanding of molecular basis for plant tolerance to water stress, next step is to characterize the function of dehydration induced genes in order to address the plants' molecular mechanism responding to water stress.

Late embryogenesis abundant proteins have been studied in detail at molecular level in many crop plants and other organisms including wheat (Litts *et al.*, 1987; Zan *et al*., 2020), cotton (Baker *et al.*, 1988), radish (Raynal *et al*., 1990), carrot (Ulrich *et al.*, 1990), maize (Williams and Tsang, 1991), rice (Litts *et al.,* 1992), sunflower (Almoguera and Jordano, 1992), barley (Espelund *et al.*, 1992; Xu *et al.,* 1996), field bean (Colmenero-Flores *et al.*, 1997), mulberry (Ukaji *et al.*, 2001), *Capsicum annum* (Kim *et al.,* 2005, Wang *et al*., 2020), *Medicago sativa* (Boudet *et al.,* 2006; Luo *et al*., 2023), *Pinus tabuliformis* (Gao and Lan, 2016), watermelon (Altunoglu *et al.*, 2017), *Craterostigma plantagineum* (Juszczak and Bartels, 2017), *Gastrodia elata* (Zeng *et al.,* 2018), grapes (Ibrahime *et al*., 2019), *Pyrus communis* (Shibuya *et al*., 2020), *Salvia miltiorrhiza* (Chen *et al*., 2021) Peanut (Huang *et al*, 2022), Persian walnut (Ma *et al*., 2023) and strawberry (Lin *et al*., 2024).

Expression profile investigation demonstrated that *TaLEA4* was profoundly initiated by drought, high and low temperatures (Min *et al.*, 2012). Henceforth, the outflow of LEA proteins helps in expanding the endurance limit of plants under different abiotic stress conditions. Over articulation of *MeLEA*5 and 6 genes enhanced tolerance to drought, ABA, H_2O_2, cold, osmotic stress conditions (Wu *et al.*, 2018). Pepper *Ca*DHN5 showed evidence in salt and osmotic stresses (Luo *et al.*, 2019). Arabidopsis plants overexpressing *AdDHN1* increased resistance against freezing and drought stresses through scavenging of ROS (Mota *et al.*, 2019). Genetically engineered tobacco plants over-expressing *MfLEA3* have increased resistance to cold and dry condition (Shi *et al.*, 2020). A dehydrin from *Chenopodium quinoa* (CqDHN4) was found to have increased gene expression in the seedlings under salt stress conditions (Melgar *et al*., 2024).

Soybean PM30 and mangrove CCTα provided evidence for its role in salt conditions (Yamada *et al.*, 2002). Salt tolerance of tomato LE25, a group 4 protein in transgenic yeast cells was observed in 1.2 M NaCl media. *E.coli* expressing recombinant PM2, from soybean (group 4) showed tolerance to salt at 0.5M NaCl or KCl and also cells expressing modified recombinant *PM2* provides enhanced salt tolerance than cells with single repeat region which indicate it as an functional domain of that LEA protein (Liu and Zheng, 2005). In

yeast, tolerance to oxidative stress was provided by *AtLEA5* under H_2O_2 stress condition and tolerance to UV radiation provided by LEA gene from Tamarix (Mowla *et al.*, 2006). Resistance to salt and heat stresses was observed in *PgLEA* overexpressed in *E.coli* cells (Reddy *et al.*, 2012).The enhanced resistance to low temperature was observed in *E.coli* transformed with *GeLEA* (Zeng *et al.*, 2018). Yeast showed increased cell growth in high salt media due to accumulation of tomato LEA25, which recommends its protective role under dehydration, salinity and chilling stresses condition (Imai *et al.*, 1996). Transgenic yeast overexpressing *TaLEA2* and *TaLEA3* gene from wheat provided enhanced tolerance to hyper osmotic, salt and cold stress conditions (Yu *et al.*, 2005). *Chlorella vulgaris* HIC6 and HIC12, group 3 class of proteins have reduced the damage caused during cold stresses (Wise and Tunnacliffe, 2004). The recombinant *E. coli* cells expressing short peptide of group 3 protein increased cell viability under UV stress (Huwaidi *et al.*, 2018). *E. coli* expressing group 4 BnLEA4-1protein from *Brassica napus* showed heat and salt tolerance (Dalal *et al.*, 2009). *Zmo0994*, a LEA protein from *Zymomonas mobilis* when screened in the tolerance assay in *E. coli* was found to be overexpressing the LEA protein under multiple stress conditions (Yang *et al*., 2020). In the present study, a LEA protein coding gene from mungbean was isolated and transformed into the *E. coli* cells for bacterial expression of the protein and the functional validation studies for salt stress tolerance was carried out with different concentrations of Sodium chloride (NaCl) to demonstrate the role of this gene in imparting stress tolerance.

Materials and Methods

Plant material and genomic DNA isolation

The seeds of popular mungbean cultivar TNAU-Co(GG) 8 was obtained from the Centre for Plant Breeding and Genetics, TNAU, Coimbatore and sown in the soil filled pots and plants were raised and maintained under the greenhouse conditions of Centre for Plant Molecular Biology and Biotechnology. Plant genomic DNA was isolated from the young leaves of three week old plants using modified CTAB method (Doyle and Doyle, 1990). The samples were ground using preheated CTAB buffer without the use of liquid nitrogen. The increased concentrations of CTAB (2.5%), PVP (2%), β-mercaptoethanol (2%) along with the RNAse treatment was performed to isolate quality genomic DNA. The DNA quality was ascertained by the observation of intact bands of genomic DNA separated on a 0.8% Agarose gel. The sample was quantified and the DNA purity of the samples were determined using Biophotometer® (Eppendorf, Germany).

Mungbean LEA gene isolation

The mungbean LEA gene (designated as *VrLEA2*) was isolated through amplification of the genes using specific primers designed based on the draft genome of mungbean from our previous studies. Primers were designed using Batchprimer3 software (You *et al*., 2008). Primers were designed to have the recognition site for the restriction enzymes, BamHI (GGATCC) and SacI (GAGCTC) for ease in cloning.

Polymerase chain reaction was performed in a reaction volume of 25 µl consists of 2.0 µl of 50 ng/µl mungbean genomic DNA template, 1.25 µl of 0.25mM forward primer (5' TAAGCAGGATCCACATTCCATTACCTTCGTCAAC 3'), 1.25 µl of 0.25mM reverse primer (5' TGCTTAGAGCTCTTAACGATAGAATCGCGTAGTG 3') and 12.5 µl of 2X Master-mix (smART Prime®) and 3.0µl sterile water with 35 cycles of denaturation at 94 °C for 1 min, annealing at 61 ° C for 1 min 30 sec. and extension at 72 °C for 1 min 30 secs followed by final extension at 72 °C for 8 mins. The amplified products were visualized on 1% agarose gel and documented.

Molecular cloning and sequencing of mungbean LEA genes

The amplified gene product was eluted from the agarose gel, purified and sequenced at the Eurofins Genomics Private Limited, Bangalore for confirmation. A portion of the eluted product was used for cloning and was subjected to restriction enzyme digestion using *Bam* HI and *Sac* I restriction enzymes (New England Biolabs®) in appropriate buffer by incubating at 37 °C for 1 hr. The amplicon after comfirmation through sequencing was cloned into a pET 28 a (+) bacterial expression vector. The vector was isolated from the *E.coli* DH5α strain host cells following modified Alkaline-Lysis method (Bimboim and Doly, 1979). The vector was also digested with same pair of restriction enzymes as that of insert. Cloning was performed by ligating the digested gene and vector using T4 DNA ligase by incubating the ligation mixture at 16 °C for 1 hr, followed by incubation at 65 °C. Competent cells of expression host *E. coli* BL21 (DE3) was prepared according to Dagert and Ehrlich (1979), using $CaCl_2$ method and the bacterial cells were and stored in aliquots at -80 °C until further use. The ligated pET28-VrLEA2 product was transformed to the competent cells of *E. coli* BL21 (DE3) by heat shock method by incubating at 42 °C for 90 secs followed by incubation at 37 °C for 1 hr. The transformed colonies were propagated by plating them on LB media containing Kan 50 (1 µl/ml) and incubated overnight at 37 °C.

Confirmation of bacterial transformants

Colony PCR was performed for confirmation of transformants harboring the pET28a-VrLEA2 using the gene specific primers. The overnight grown bacterial colonies were lifted using the sterile toothpick and mixed in 50 µl of sterile double distilled water. This was used as the DNA template for PCR. PCR was performed as described before to check for the desired size of amplicon. Plasmid DNA was isolated and restriction digestion analysis of transformed vector was carried out to confirm the presence of *VrLEA2* gene insert in the clones.

Protein expression and screening for salt stress tolerance

Salt stress tolerance ability of mungbean LEA protein was screened using bacterial expression system as described by (Gao and Lan, 2016). *E. coli* cells harboring VrLEA2 gene in a pET28a expression vector was used. A preculture was initiated using the transformed colonies of *E coli* BL21 (DE3) and inoculated in LB broth containing 1 µl/ml of Kanamycin 50 and incubated overnight at 37°C under shaking condition. To this overnight grown culture, 5 µl of 1M IPTG was added and again incubated in the shaker at 37 °C for 3-4 hrs to induce the bacterial expression of the cloned *VrLEA2* gene. After induction of recombinant protein expression, 10 µl of the culture was plated on the LB medium containing six different concentrations of sodium chloride *viz*., 100, 200, 300, 400, 500 and 600 mM along with the untreated control. The plates were then incubated overnight at 37 °C and screened for salt tolerance by observations of colony growth.

Results

Plants are often exposed to vagaries of climate changes and infestation by the biotic stress factors. Overtime plants have evolved mechanisms to overcome these stresses and has improved ability to cope with unfavorable conditions through eliciting array of responses at cellular and physiological levels. Mungbean like other grain legumes tolerate desiccation through expression of stress associated proteins. Among them, Late Embryogenesis Abundant (LEA) proteins are low molecular weight proteins which posses multi-various roles for stress tolerance. *In vivo* expression analysis of mungbean *LEA* proteins and its implications for saline stress conditions has been demonstrated in a bacterial expression system.

LEA gene isolation and characterization

Mungbean LEA gene was isolated from genomic DNA by PCR amplification using gene specific primers. The amplified products resolved on 1% agarose gel showed an amplicon of size ~900 bp (Figure 1). Sequencing of the amplicon showed a size of 839 bases. The sequences received were assembled by using CAP3 contig assembly tool and analysis of the sequence revealed it as group 1 LEA protein with signature motif pertaining to hydrophilic domain 'GETVVPGGT' and a 20-mer consensus 'GGQTRKQQ LGSEGYHEMGRK' unique for Group1 Class of LEA proteins (Figure 2). The gene was designated *VrLEA2*.

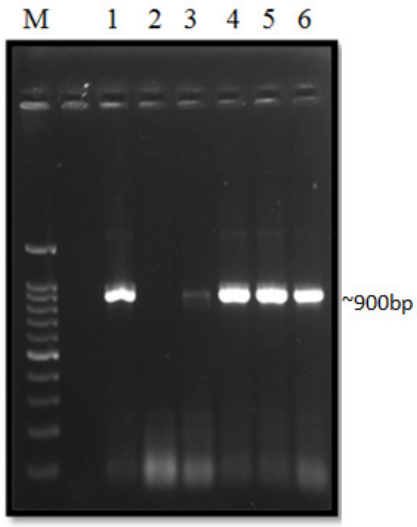

Figure 1. Profile of PCR amplified mungbean *VrLEA2* gene
Lane M: 100 bb ladder; Lane 1-6: PCR amplified *VrLEA2* gene

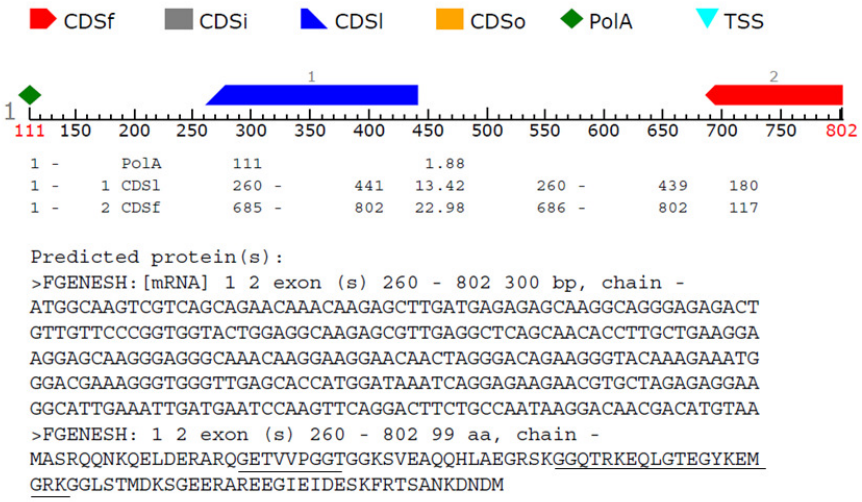

Figure 2. Gene identified from sequences of *VrLEA2* using FGENESH tool
Underlined sequence represents an octameric hydrophilic signature motif and a 20-mer conserved amino acid motif typical to Group 1 LEA protein

Molecular cloning of VrLEA2 gene

For the expression studies, the *VrLEA2* gene was inserted into pET 28a (+) vector and mobilized into competent cells of *E.coli* DH5α strain. The transformants harboring the insert was confirmed by colony PCR

and restriction digestion analysis with *Bam*HI and *Sac*I restriction enzymes. PCR amplification using specific primers confirmed the presence of insert of size ~900 bp in the clones (Figure 3). Similarly the insert presence of same size was verified in the restriction digested products (Figure 4).

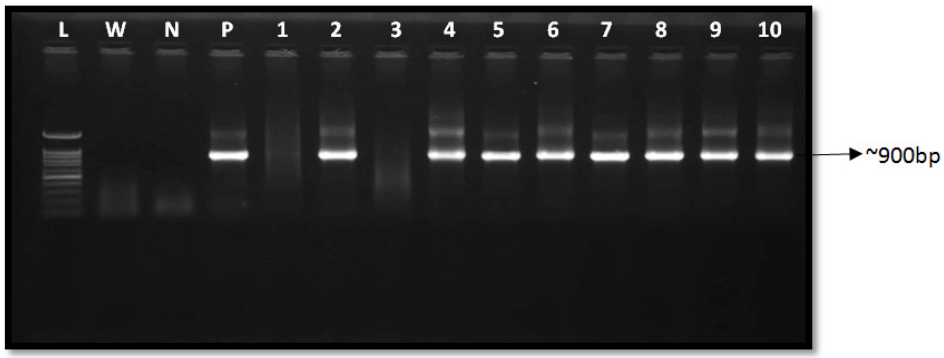

Figure 3. Colony PCR analysis for confirmation of bacterial transformants
Lane L: 100 bp DNA ladder; Lane W- water control; Lane N - pET28a (+) as negative control; Lane P- *VrLEA2* product as Positive control; Lane 1 to 10- Transformed bacterial colonies with *VrLEA2* gene

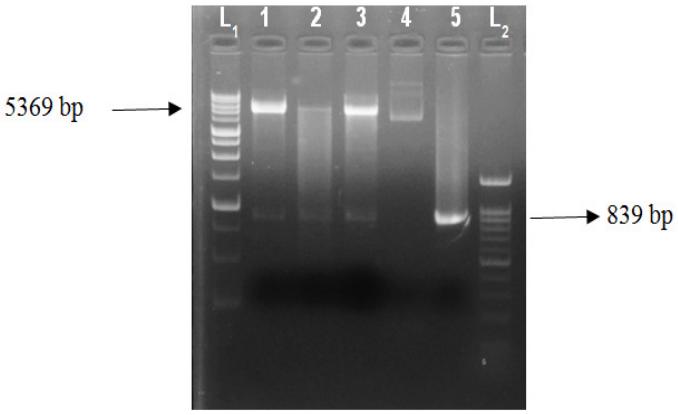

Figure 4. Restriction digested products of pET28a-*VrLEA2* resolved on 1% agarose gel
Lane L_1: 1kb ladder; Lane L_2 – 100 bp ladder; Lane 1to 3 - Recombinants digested with *Bam*H1 and *Sac*; Lane 4- Undigested pET28 a (+) vector; Lane 5- VrLEA2 PCR product

Salinity tolerance assays of E. coli transformants

Functional validation of the mungbean *VrLEA2* gene was carried out by screening the *E.coli* BL21 (DE3) bacterial cells harboring LEA gene through culturing of cells in medium supplemented with different concentration of sodium chloride. Among that, survival of both transformants and non-transformants bacterial cells upto 300 mM concentration of NaCl was observed but the transformed pET28a (+)-*VrLEA2* colonies showed more survival rate than the non transformants (Figure 5).

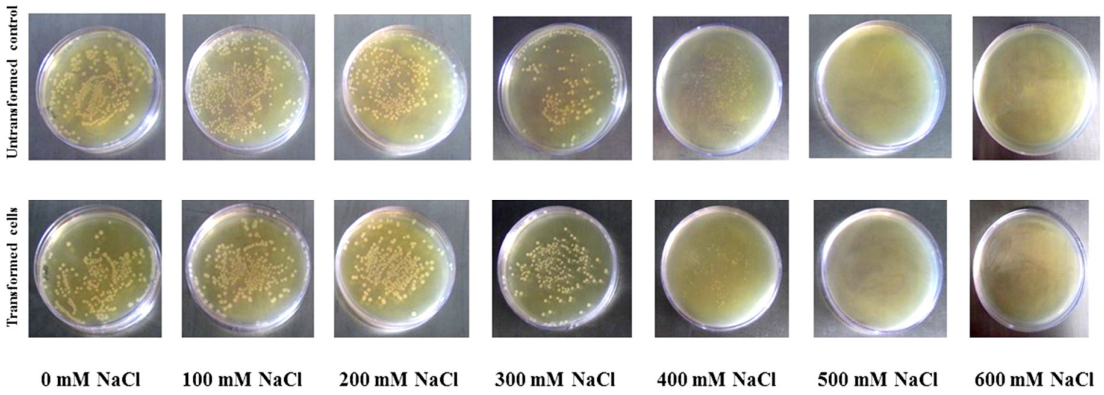

Figure 5. Screening for salt stress tolerance of E.coli BL21 (DE3) cells harboring VrLEA2 gene

Discussion

VrLEA2 gene isolation

Plant DNA isolation free from impurities like polyphenols, polysaccharides is a prerequisite for downstream processing. DNA extraction was performed by modification of the CTAB method described by Doyle and Doyle, 1990 with increased concentration of CTAB, β-mercaptoethanol and PVP resulting in improved quality of the isolated genomic DNA and solved the problem of reproducibility of amplicon. *VrLEA2* gene was isolated from the leaves of mungbean using gene specific primers and the amplicon was sequenced with a size of 839 bases and subsequently subjected to *in silico* characterization. Several LEA genes reported earlier from various crop species are found to be low molecular weight proteins with their average gene sizes around 600bp to 1 Kb with their proteins ranging from 10 to 30 kDa (He and Fu, 1996; Zeng *et al.*, 2018).

Classification of VrLEA2 and prediction of functions

Generally most plant LEA gene families possess group specific domains or conserved sequences and they are classified based on their structural properties. Likewise, Group 1 class of LEA proteins are hydrophilic in nature and are mostly random coiled with few α-helical and beta sheets as observed from their secondary structure. The presence of glycine rich hydrophilic repeat at N-terminus is related to small hydrophilic plant seed protein of Pfam00477 super family. This hydrophilic nature of *Vr*LEA2 protein might help in biological activity during water stress. Even though the role of hydrophilins remains unclear, some evidence supports their involvement in adaptive stress tolerance like heterologous expression of LEA proteins in some plants and yeast that confers tolerance to water deficient conditions (Swire-Clark and Marcotte, 1999; Zhang *et al.*, 2000) and chilling stress conditions (Rinne *et al.*, 1999). Some LEA proteins play cell protective role by improving membrane stability and DNA binding activity like a molecular chaperones (Rajesh and Manickam, 2006). Earlier studies from our lab have identified, isolated few genes coding for these LEA proteins from embryonal axes of mungbean. Subsequent bioinformatics studies on genome wide identification of these LEA genes in the draft genome of mungbean led to identification of 307 such LEA genes in *Vigna radiata*. These genes are designated as *VrLEA* genes and are mapped on to mungbean chromosome using mapping tool (Likhith *et al.*, 2021).

Further the deletion of RMF hydrophilins reduced osmotic tolerance in *E. coli* (Garay-Arroyo *et al.*, 2000). The signature motif and multiple sequence alignment of protein sequences help to validate the phylogenetic relationship with closely related species. The deduced amino acid sequences of the VrLEA2

protein was found to be closely related to the tune of 70% sequence similarity with that of reported *Vigna radiata* Em protein and multiple sequence alignment revealed all such protein shares the common 20-mer motif regions predominantly confirming their classification into Group 1 LEA proteins. The hydrophilic signature motif 'GETVVPGGT' present within the deduced *Vr*LEA2 protein sequences has shown that it flanks typical glycine residues which are conserved with 20 amino acid residues and has a pattern that provides structural flexibility to the protein. The number of such repeating motif suggests that this protein may have a higher water-binding capacity and this observation agrees with the reports of B19 LEA protein from *Hordeum vulgare* (Espelund *et al.*, 1992) and also posses thermal stability as reported by Hong-Bo *et al.* (2005). Presence of this repeat element has been reported upto 8 times in the crustaceous species *Artemia franciscana* (Taxopeus *et al*., 2014). These repeats have presumed to have functional significance to these class of LEA proteins for desiccation tolerance.

Function assignments based on stress tolerance assays

Use of heterologous protein expression systems for screening of the cells against stress tolerance has been well documented. The results of these studies suggest possible existence of mechanism for protection of cells against stresses; interestingly these mechanisms are shared commonly by the prokaryotes and eukaryotes alike. Recently, use of *E. coli* as expression system for recombinant protein production with potential use in biochemical, agricultural and pharma applications has been comprehensively reviewed. This system utilized the strategies to manipulate the heat shock proteins for increased expression and improved solubility of recombinant proteins (Ahn and Jung, 2023).

Expression analysis of the *VrLEA2* gene in bacterial expression system in the presence of varied concentrations of salt demonstrated the stress tolerance ability of the mungbean LEA protein. Both bacterial cells of untransformed and transformed colonies survived the salt stress upto 300 mM NaCl concentration; however with reference to the survival rate, transformed cells showed higher tolerability than non-transformed cells. Also, there was significant difference in colony morphology, and there was lowering of cell population with increase in salt concentrations. These observations suggest the cell protective role of mungbean LEA gene to the bacterial cells under salt stress. These findings can be positively corroborated with similar reports of cell protection against stress tolerance in prokaryotic expression system. Reddy *et al*., 2012 reported relative growth advantage of recombinant *E.coli* cells expressing PgLEA protein over the non-transformants under salt stress situations. Similarly, *SiLEA4* gene from the fox tail millet, *Setaria italica* and PtaLEA protein from *Pinus tabuliformis*, expressed in *E.coli* cells under salt stress conditions was reported to play protective role against stresses (Wang *et al.*, 2014 and Gao and Lan, 2016). Despite screening for the salt stress, prokaryotic model system has been demonstrated to be the choice for temperature stress tolerance where heterologous expression of CsLEA7 protein in *E.coli* cells showed resistant role played by the bacterial cells under all stresses including low temperature (Paul *et al.*, 2014). Similarly, functional characterization for multiple stress tolerance with LEA4-1 from *Brassica napus* showed tolerance to low and high temperature stress situations (Dalal *et al.*, 2009). Group 1 and 3 LEA protein from *Artemia sinica* showed tolerance to low temperature stress (Zhao *et al.*, 2016). The *E.coli* expressing *GeLEAs* exhibited tolerance to cold temperature conditions (Zeng *et al.*, 2018). Differing response was observed to the *E. coli* cells that overexpressed a group 4 LEA protein coding gene for improved tolerance to heat, salt, freezing and osmotic stresses. This variation is attributed to the C-terminal region of the LEA protein, which is structurall flexible and play protective role for multiple stresses (Zhang *et al*., 2020). Similarly, *in vivo* analysis of the soybean LOC protein has confirmed the ability of the *E.coli* cells expressing this protein to show protection against osmotic stress (Tan *et al*., 2021).

Prokaryotic expression analysis of a LEA 3 subfamily hydrophilic protein, *CsLEA1* from *Camellia sinensis* showed cold stress tolerance in *E. coli* and also in the yeast cells (Gao *et al*., 2021). Tawzy1-2, a dehydrin coding gene isolated from the wheat, when expressed constitutively was found to demonstrate important role

in protecting the cells of both tobacco and *E.coli* to abiotic stresses. The mechanistic role of the nuclear localized dehydrin protein preserved these cells from damage by the reactive oxygen species and could decrease the oxidation of lipids (Wang *et al*, 2024). Studies with some LEA genes suggest necessary role of some additional cellular regulatory factors which can trigger gene expression under varied stress conditions conferring tolerance to the cells.

Conclusions

Crop productivity is generally affected by changing environmental conditions and poses serious concerns for developing strategies to overcome these challenges and breed crops with improved ability to tolerate various stresses. LEA proteins are among the choicest biomolecules that helps the plants through its survival mechanism to implicate various stress tolerance abilities. These proteins are widely distributed and reported in many crops and other organisms too. LEA proteins protects the cell in various ways through binding of water molecules and other macromolecules during water deficit state, can act like chaperones, bind to metal ion and sequester them. Functional validation of mungbean LEA, *VrLEA2* demonstrated for salt stress tolerance in the present study can be extrapolated for screening against similar other stresses using bacterial expression system. The characteristic hydrophilic nature of this *VrLEA2* protein may contribute to water binding capacity of proteins and could play role as hydration buffer and as stress protectant of macromolecules which reduce protein degradation and aggregation. Further, full length gene isolation can be attempted in future which can be characterized in detail for deployment of these genes in crop improvement to engineer plants that can cope with stressed environments.

Authors' Contributions

Conceptualization: RS, NUP, LRKS; Data curation: NUP, RT, SPL, and LRKS; Formal analysis: RS, NUP and LRKS; Investigation: RS, NUP, LRKS and SPL; Methodology: RS, SPL, SS, NUP; Resources: RS, RJ, BMR; Software: RJ and LRKS; Supervision: RS, RJ; Writing - original draft: RS and NUP; Writing - review and editing: NUP, RT, SR, LSP, SS, BMR and RJ. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

References

Ahn YJ, Jung M (2023). Improved recombinant protein production using heat shock proteins in *Escherichia coli*. Biocatalysis and Agricultural Biotechnology 20:102736. *https://doi.org/10.1016/j.bcab.2023.102736*

Almoguera C, Jordano J (1992). Developmental and environmental concurrent expression of sunflower dry-seed-stored low-molecular-weight heat-shock protein and Lea mRNAs. Plant Molecular Biology 19(5):781-792. *https://doi.org/10.1007/BF00027074*

Altunoglu YC, Baloglu MC, Baloglu P, Yer EN, Kara S (2017). Genome-wide identification and comparative expression analysis of LEA genes in watermelon and melon genomes. Physiology and Molecular Biology of Plants 23(1):5-21. *https://doi.org/10.1007/s12298-016-0405-8*

Baker J, Van dennSteele C, Dure L (1988). Sequence and characterization of 6 Lea proteins and their genes from cotton. Plant molecular biology 11(3):277-291. *https://doi.org/10.1007/BF00027385*

Bimboim H, Doly J (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Research 7(6):1513-1523. *https://doi.org/10.1093/nar/7.6.1513*

Boudet J, Buitink J, Hoekstra FA, Rogniaux H, Larré C, Satour P, Leprince O (2006). Comparative analysis of the heat stable proteome of radicles of *Medicago truncatula* seeds during germination identifies late embryogenesis abundant proteins associated with desiccation tolerance. Plant physiology 140(4):1418-1436. *https://doi.org/10.1104/pp.105.074039*

Chen J, Li N, Wang X, Meng X, Cui X, Chen Z, Ren H, Ma J, Liu H (2021). Late embryogenesis abundant (LEA) gene family in *Salvia miltiorrhiza*: identification, expression analysis, and response to drought stress. Plant Signaling and Behavior 16(5):1891769. *https://doi.org/10.1080/15592324.2021.1891769*

Colmenero-Flores JM, Campos F, Garciarrubio A, Covarrubias AA (1997). Characterization of *Phaseolus vulgaris* cDNA clones to water deficit: identification of a novel embryogenesis abundant- like protein. Molecular Biology 35:393-405. *https://doi.org/10.1023/A:1005802505731*

Dagert M, Ehrlich S (1979). Prolonged incubation in calcium chloride improves the competence of *Escherichia coli* cells. Gene 6(1):23-28. *https://doi.org/10.1016/0378-1119(79)90082-9*

Dalal M, Tayal D, Chinnusamy V, Bansal KC (2009). Abiotic stress and ABA-inducible Group 4 LEA from *Brassica napus* plays a key role in salt and drought tolerance. Journal of Biotechnology 139(2):137-145. *https://doi.org/10.1016/j.jbiotec.2008.09.014*

Doyle JJ, Doyle JL (1990). Isolation of plant DNA from fresh tissue. Focus 12:13-15

Dure L, Crouch M, Harada J, Ho TH, Mundy J, Quatrano R, Thomas T, Sung ZR (1989). Common amino acid sequence domains among the LEA proteins of higher plants. Plant Molecular Biology 12:475-486. *https://doi.org/10.1007/BF00036962*

Espelund M, Sæbøe-Larssen S, Hughes DW, Galau GA, Larsen F, Jakobsen KS (1992). Late embryogenesis-abundant genes encoding proteins with different numbers of hydrophilic repeats are regulated differentially by abscisic acid and osmotic stress. The Plant Journal 2(2):241-252. *https://doi.org/10.1046/j.1365-313X.1992.t01-46-00999.x*

Ganesan K, Xu B (2018). A critical review on phytochemical profile and health promoting effects of mung bean (*Vigna radiata*). Food Science and Human Wellness 7(1):11-33. *https://doi.org/10.1016/j.fshw.2017.11.002*

Gao J, Lan T (2016). Functional characterization of the late embryogenesis abundant (LEA) protein gene family from *Pinus tabuliformis* (Pinaceae) in *Escherichia coli*. Scientific Reports 6:19467. *https://doi.org/10.1038/srep19467*

Gao T, Mo Y, Huang H, Yu J, Wang Y, Wang W (2021). Heterologous expression of *Camellia sinensis* late embryogenesis abundant protein gene 1 (CsLEA1) confers cold stress tolerance in *Escherichia coli* and yeast. Horticultural Plant Journal 7(1):89-96. *https://doi.org/10.1016/j.hpj.2020.09.005*

Garay-Arroyo A, Colmenero-Flores JM, Garciarrubio A, Covarrubias AA (2000). Highly hydrophilic proteins in prokaryotes and eukaryotes are common during conditions of water deficit. Journal of Biological Chemistry 275(8):5668-5674. *https://doi.org/10.1074/jbc.275.8.5668*

Gaubier P, Raynal M, Hull G, Huestis GM, Grellet F, Arenas C, Delseny M (1993). Two different Em-like genes are expressed in *Arabidopsisthaliana* seeds during maturation. Molecular and General Genetics 238(3):409-418. *https://doi.org/10.1007/BF00292000*

He JX, Fu JR (1996). The research progresses in Lea proteins of seeds. Plant Physiology Communications 241-246.

Hong-Bo S, Zong-Suo L, Ming-An S (2005). LEA proteins in higher plants: structure, function, gene expression and regulation. Colloids and surfaces B: Biointerfaces 45(3-4):131-135. *https://doi.org/10.1016/j.colsurfb.2005.07.017*

Huang R, Xiao D, Wang X, Zhan J, Wang A, He L (2022). Genome-wide identification, evolutionary and expression analyses of LEA gene family in peanut (*Arachis hypogaea* L.). BMC Plant Biology 22(1):155. *https://doi.org/10.1186/s12870-022-03462-7*

Huwaidi A, Pathak N, Syahir A, Ikeno A (2018). *Escherichia coli* tolerance of ultraviolet radiation by *in vivo* expression of a short peptide designed from late embryogenesis abundant protein. Biochemical and biophysical research communications 503 (2):910-914. *https://doi.org/10.1016/j.bbrc.2018.06.095*

İbrahime M, Kibar U, Kazan K, Yüksel Özmen C, Mutaf F, Demirel Aşçı S, Çakır Aydemir B, Ergül A (2019). Genome-wide identification of the LEA protein gene family in grapevine (*Vitis vinifera* L.). Tree Genetics and Genomes 15:1-14. *https://doi.org/10.1007/s11295-019-1364-3*

Imai R, Chang L, Ohta A, Bray EA, Takagi M (1996). A lea-class gene of tomato confers salt and freezing tolerance when expressed in *Saccharomyces cerevisiae*. Gene 170 (2):243-248. *https://doi.org/10.1016/0378-1119(95)00868-3*

Juszczak I, Bartels D (2017). LEA gene expression, RNA stability and pigment accumulation in three closely related Linderniaceae species differing in desiccation tolerance. Plant Science 255:59-71. *https://doi.org/10.1016/j.plantsci.2016.10.003*

Kim HS, Lee JH, Kim JJ, Kim CH, Jun SS, Hong YN (2005). Molecular and functional characterization of *CaLEA6*, the gene for a hydrophobic LEA protein from *Capsicum annuum*. Gene 344:115-123. *https://doi.org/10.1016/j.gene.2004.09.012*

Likhith RK, Alagarasan G, Muthurajan R, Parasuraman B, Subramanian R (2021). Genome wide identification of mungbean (*Vigna radiata* [L.] R. Wilczek) Late Embryogenesis Abundant (LEA) protein gene family. Israel Journal of Plant Sciences 69(1-2):79-86. *https://doi.org/10.1163/22238980-bja10049*

Lin Y, She M, Zhao M, Yu H, Xiao W, Zhang Y, Li M, Chen Q, Zhang Y, Wang Y, He W (2024). Genome-wide analysis and functional validation reveal the role of late embryogenesis abundant genes in strawberry (*Fragaria*× *Ananassa*) fruit ripening. BMC Genomics 25(1):228. *https://doi.org/10.1186/s12864-024-10085-9*

Litts J, Erdman M, Huang N, Karrer E, Noueiry A, Quatrano R, Rodriguez R (1992). Nucleotide sequence of the rice (*Oryzasativa*) Em protein gene (Emp1). Plant Molecular Biology 19(2):335-337. *https://doi.org/10.1007/BF00027357*

Litts JC, Colwell GW, Chakerian RL, Quatrano RS (1987). The nucleotide sequence of a cDNA clone encoding the wheat Em protein. Nucleic Acids Research 15(8): 3607-3618. *https://doi.org/10.1093/nar/15.8.3607*

Liu Y, Zheng Y (2005) PM2, a group 3 LEA protein from soybean, and its 22-mer repeating region confer salt tolerance in *Escherichia coli*. Biochemical and Biophysical Research Communications 331(1):325-332. *https://doi.org/10.1016/j.bbrc.2005.03.165*

Luo D, Hou X, Zhang Y, Meng Y, Zhang H, Liu S, Wang X, Chen R (2019). CaDHN5, a dehydrin gene from pepper, plays an important role in salt and osmotic stress responses. International Journal of Molecular Sciences 20(8):1989. *https://doi.org/10.3390/ijms20081989*

Luo D, Zhang X, Li Y, Wu Y, Li P, Jia C, Bao Q, Zhou Q, Fu C, Liu W, Liu Z (2023). MsDIUP1 encoding a putative novel LEA protein positively modulates salt tolerance in alfalfa (*Medicago sativa* L.). Plant and Soil 487(1):547-66. *https://doi.org/10.1007/s11104-023-05951-6*

Ma J, Zuo D, Ye H, Yan Y, Li M, Zhao P (2023). Genome-wide identification, characterization, and expression pattern of the late embryogenesis abundant (LEA) gene family in *Juglans regia* and its wild relatives *J. mandshurica*. BMC Plant Biology 23(1):80. *https://doi.org/10.1186/s12870-023-04096-z*

Melgar AE, Rizzo AJ, Moyano L, Cenizo R, Palacios MB, Zelada AM (2024). Genome-wide identification and salt stress-expression analysis of the dehydrin gene family in *Chenopodium quinoa*. Current Plant Biology 38:100340. *https://doi.org/10.1016/j.cpb.2024.100340*

Min DH, Zhang XH, Xu ZS, Zhao Y, Chen Y, Li LC, Chen M, Ma YZ (2012). Induction kinetics of a novel stress-related LEA gene in wheat. Plant Molecular Biology Reporter 30(6):1313-1321. *https://doi.org/10.1007/s11105-012-0446-2*

Mota APZ, Oliveira TN, Vinson CC, Williams TCR, Costa MMdC, Araujo ACG, Danchin EG, Grossi-de-Sá MF, Guimaraes PM, Brasileiro ACM (2019). Contrasting effects of wild *Arachis* dehydrin under abiotic and biotic stresses. Frontiers in Plant Science 10:497. *https://doi.org/10.3389/fpls.2019.00497*

Mowla SB, Cuypers A, Driscoll SP, Kiddle G, Thomson J, Foyer CH, Theodoulou FL (2006). Yeast complementation reveals a role for an *Arabidopsis thaliana* late embryogenesis abundant (LEA)-like protein in oxidative stress tolerance. The Plant Journal 48(5):743-756. *https://doi.org/10.1111/j.1365-313X.2006.02911.x*

Nair RM, Pandey AK, War AR, Hanumantharao B, Shwe T, Alam AK, Pratap A, Malik SR, Karimi R, Mbeyagala EK, Douglas CA (2019). Biotic and abiotic constraints in mungbean production—progress in genetic improvement. Frontiers in Plant Science 10:1340. *https://doi.org/10.3389/fpls.2019.01340*

Nawaz M, Sun J, Shabbir S, Khattak WA, Ren G, Nie X, Bo Y, Javed Q, Du D, Sonne C (2023). A review of plants strategies to resist biotic and abiotic environmental stressors. Science of the Total Environment 29:165832. *https://doi.org/10.1016/j.scitotenv.2023.165832*

Rajesh S, Manickam A (2006). Prediction of functions for two LEA proteins from mungbean. Bioinformation 1 (4):133. *https://doi.org/10.6026/97320630001133*

Raynal M, Gaubier P, Grellet F, Delseny M (1990). Nucleotide sequence of a radish cDNA clone coding for a late embryogenesis abundant (LEA) protein. Nucleic Acids Research 18(20):6132. *https://doi.org/10.1093/nar/18.20.6132*

Reddy PS, Reddy GM, Pandey P, Chandrasekhar K, Reddy MK (2012). Cloning and molecular characterization of a gene encoding late embryogenesis abundant protein from *Pennisetum glaucum*: protection against abiotic stresses. Molecular Biology Reports 39(6):7163-7174. *https://doi.org/10.1007/s11033-012-1548-5*

Rinne, PL, Kaikuranta PL, van der Plas LH, van der Schoot C (1999). Dehydrins in cold-acclimated apices of birch (*Betula pubescens* Ehrh.): production, localization and potential role in rescuing enzyme function during dehydration. Planta 209(4):377-388. *https://doi.org/10.1007/s004250050740*

Shi H, He X, Zhao Y, Lu S, Guo Z (2020). Constitutive expression of a group 3 LEA protein from Medicago falcata (MfLEA3) increases cold and drought tolerance in transgenic tobacco. Plant Cell Reports 39:851-860. *https://doi.org/10.1007/s00299-020-02534-y*

Shibuya T, Itai R, Maeda M, Kitashiba H, Isuzugawa K, Kato K, Kanayama Y (2020). Characterization of PcLEA14, a group 5 late embryogenesis abundant protein gene from pear (*Pyrus communis*). Plants 9(9):1138. *https://doi.org/10.3390/plants9091138*

Suzuki N, Rivero RM, Shulaev V, Blumwald E, Mittler R (2014). Abiotic and biotic stress combinations. New Phytologist 203(1):32-43. *https://doi.org/10.1111/nph.12797*

Swire-Clark GA, Marcotte WR (1999). The wheat LEA protein Em functions as an osmoprotective molecule in *Saccharomyces cerevisiae*. Plant Molecular Biology 39(1):117-128. *https://doi.org/10.1023/A:1006106906345*

Tan F, Sun N, Zhang L, Wu J, Xiao S, Tan Q, Uversky VN, Liu Y (2021). Functional characterization of an unknown soybean intrinsically disordered protein *in vitro* and in *Escherichia coli*. International Journal of Biological Macromolecules 166:538-49. *https://doi.org/10.1016/j.ijbiomac.2020.10.211*

Ukaji N, Kuwabara C, Takezawa D, Arakawa K, Fujikawa S (2001). Cold acclimation-induced WAP27 localized in endoplasmic reticulum in cortical parenchyma cells of mulberry tree was homologous to group 3 late-embryogenesis abundant proteins. Plant Physiology 126(4):1588-1597. *https://doi.org/10.1104/pp.126.4.1588*

Ulrich T, Wurtele E, Nikolau B (1990). Sequence of EMB-1, an mRNA accumulating specifically in embryos of carrot. Nucleic Acids Research 18(9):2826. *https://doi.org/10.1093/nar/18.9.2826*

Wang M, Li P, Li C, Pan Y, Jiang X, Zhu D, Zhao Q, Yu J (2014). SiLEA14, a novel atypical LEA protein, confers abiotic stress resistance in foxtail millet. BMC Plant Biology 14 (1):290. *https://doi.org/10.1186/s12870-014-0290-7*

Wang X, Liu H, Li Y, Zhang L, Wang B (2024). Heterologous overexpression of Tawzy1-2 gene encoding an SK3 dehydrin enhances multiple abiotic stress tolerance in *Escherichia coli* and *Nicotiania benthamiana*. Planta 259(2):39. *https://doi.org/10.1007/s00425-023-04328-4*

Wang Y, Chen G, Lei J, Cao B, Chen C (2020). Identification and characterization of a LEA-like gene, CaMF5, specifically expressed in the anthers of male-fertile *Capsicum annuum*. Horticultural Plant Journal 6(1):39-48. *https://doi.org/10.1016/j.hpj.2019.07.004*

Williams B, Tsang A (1991). A maize gene expressed during embryogenesis is abscisic acid-inducible and highly conserved. Plant Molecular Biology 16(5):919-923. *https://doi.org/10.1007/BF00015086*

Wise MJ, Tunnacliffe A (2004). POPP the question: what do LEA proteins do?. Trends in Plant Science 9(1):13-17. *https://doi.org/10.1016/j.tplants.2003.10.012*

Wu C, Hu W, Yan Y, Tie W, Ding Z, Guo J, He G (2018). The late embryogenesis abundant protein family in cassava (*Manihot esculenta* Crantz): Genome-wide characterization and expression during abiotic stress. Molecules 23(5):1196. *https://doi.org/10.3390/molecules23051196*

Xu D, Duan X, Wang B, Hong B, Ho THD, Wu R (1996). Expression of a late embryogenesis abundant protein gene, *HVA1*, from barley confers tolerance to water deficit and salt stress in transgenic rice. Plant physiology 110(1):249-257. *https://doi.org/10.1104/pp.110.1.249*

Yamada A, Saitoh T, Mimura T, Ozeki Y (2002). Expression of mangrove allene oxide cyclase enhances salt tolerance in *Escherichia coli,* yeast, and tobacco cells. Plant and Cell Physiology 43(8):903-910. *https://doi.org/10.1093/pcp/pcf108*

Yang J, Kim HE, Jung YH, Kim J, Kim DH, Walmsley AR, Kim KH (2020). Zmo0994, a novel LEA-like protein from *Zymomonas mobilis*, increases multi-abiotic stress tolerance in *Escherichia coli*. Biotechnology for Biofuels 13:1-5. *https://doi.org/10.1186/s13068-020-01790-0*

You FM, Huo N, Gu YQ, Luo MC, Ma Y, Hane D, Lazo GR, Dvorak J, Anderson OD (2008). BatchPrimer3: a high throughput web application for PCR and sequencing primer design. BMC Bioinformatics 9:1-13. *https://doi.org/10.1186/1471-2105-9-253*

Yu JN, Zhang JS, Shan L, Chen SY (2005). Two new group 3 *LEA* genes of wheat and their functional analysis in yeast. Journal of Integrative Plant Biology 47(11):1372-1381. *https://doi.org/10.1111/j.1744-7909.2005.00126.x*

Zan T, Li L, Li J, Zhang L, Li X (2020). Genome-wide identification and characterization of late embryogenesis abundant protein-encoding gene family in wheat: evolution and expression profiles during development and stress. Gene 736:144422. *https://doi.org/10.1016/j.gene.2020.144422*

Zeng X, Ling H, Yang J, Li Y, Guo S (2018). LEA proteins from *Gastrodia elata* enhance tolerance to low temperature stress in *Escherichia coli*. Gene 646:136-142. *https://doi.org/10.1016/j.gene.2018.01.002*

Zhang L, Ohta A, Takagi M, Imai R (2000). Expression of plant group 2 and group 3 *lea* genes in *Saccharomyces cerevisiae* revealed functional divergence among LEA proteins. The Journal of Biochemistry 127(4):611-616. *https://doi.org/10.1093/oxfordjournals.jbchem.a022648*

Zhang YM, Wang HQ, Liu DM, Liu RJ (2020). Three tandemly aligned LEA genes from *Medicago truncatula* confer differential protection to *Escherichia coli* against abiotic stresses. Biologia Plantarum 64(1). *https://doi.org/10.32615/bp.2019.112*

Zhao W, Yao F, Zhang M, Jing T, Zhang S, Hou L, Zou Z (2016). The potential roles of the G1LEA and G3LEA proteins in early embryo development and in response to low temperature and high salinity in *Artemia sinica*. PLoS One 11 (9):e0162272. *https://doi.org/10.1371/journal.pone.0162272*

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