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Ectopic expression of a LEA protein gene *TsLEA1* from *Thellungiella salsuginea* confers salt-tolerance in yeast and Arabidopsis

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Abstract *Thellungiella salsuginea* is a valuable halophytic genetic model plant in the Brassicaceae family. Based on previous construction of a salt treated *Thellungiella* cDNA library carried by pGAD-GH shuttle vector which could directly express in *Saccharomyces cerevisiae*, a putative salt-tolerance gene *TsLEA1* was identified by large-scale stress-tolerance screen in salt sensitive yeast strain G19. The longest 483 bp ORF of *TsLEA1* cDNA coding a 160 amino acids protein with a predicted conserved pfam domain shared an 89% amino acid sequence similarity to Arabidopsis LEA group 4 proteins. The transcription level of *TsLEA1* gene in *T. salsuginea* seedlings increased upon salt treatment and its transcript accumulated more in roots than in aerial parts. The ability of the *TsLEA1* to facilitate salinity tolerance was analyzed in yeast and transgenic Arabidopsis. It was confirmed that TsLEA1 exhibits conserved salt tolerance in plant as well as in yeast. The results suggested that the TsLEA1 may participate in response to stresses in over expressed circumstance, protecting yeast and plant cells under stress conditions.

Keywords LEA protein · *Thellungiella salsuginea* · Salt-tolerance

Yiyue Zhang and Yin Li contributed equally to this study.

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Introduction

LEA proteins are a heterogenous group of proteins found in different tissue and cell types in plants, which were first discovered because they were accumulated to high levels during late stages of embryo development. Synthesis of LEA proteins is associated with dehydration in seeds and with water deficit in vegetative tissues [1, 2]. These proteins can be induced by ABA and various water-related stresses including salinity [3, 4]. LEA proteins are also produced in anhydrobiotic plants, animals and microorganisms in which their expression correlates with desiccation tolerance [5]. Under cold, drought, and salt stress, the activation of LEA-type genes represent damage repair pathways and have close relation to ABA signalling [6, 7].

Salt stress is one of the major abiotic stresses limiting plant growth and development which may cause severe disastrous effects in agriculture. Numerous efforts have been made to study the mechanisms by which plants respond to salinity stress and adapt to salt tolerance. Some plant species have developed complex approaches to adapt for the saline environment. The Brassicaceae family halophyte salt cress (*Thellungiella salsuginea*) which can tolerant high salinity and complete life cycle after exposure to extreme up to 500 mM NaCl [8] was used in saline stress studies [9]. As a close relative of *Arabidopsis thaliana* with small genome, the salt cress can serve as a model plant

with similar experimental convenience in researches [8–11].

In previous studies we generated a cDNA library from a salt-treated *Thellungiella* including both aerial parts and roots of plants which carried by pGAD-GH shuttle vector, and described 946 ESTs from this library [12]. The pGAD-GH shuttle vector which carried this library can directly ectopically express proteins in yeast. Based on this system, stress-related genes could be screened and analyzed for their ability to confer tolerance in yeast cells by ectopic expression. This salt cress cDNA library was transformed into the salt sensitive yeast strain G19 to perform salt tolerance screen and salt tolerant transgenic yeast clones were selected. It has been proved that a LEA protein gene (named *TsLEA1*, Accession No. EU365627) in these selected genes had improved survival of salt-sensitive yeast cells when ectopically expressed. Meanwhile, the salt induced gene *TsLEA1* was identified and characterized as a salt tolerance gene in transgenic plant as well as in yeast.

Materials and methods

Yeast strain and spot for salt tolerance assay

The budding yeast *Saccharomyces cerevisiae* strain G19 (*MATα leu2-3*, *2-112*, *trp1-1*, *ura3-3*, *ade2-1*, *his3-11 can1-100*, *15(φ) ena1*:: *HIS3*::*ena4*) [13] was used. The genes encoding plasma membrane Na^+ export pumps have been deleted in the yeast strain G19 and, therefore, it is more sensitive to external Na^+ concentration than wild-type yeast [14].

Yeast cells were transformed with the *S. cerevisiae*/*Escherichia coli* shuttle vector pGAD GH (Clontech), which carried full- or nearly full-length *Thellungiella* CDS under the control of the constitutive ADH1 promoter. Transformants were selected from SD selection plates and grown in minimal SD medium to late log/stationary phase. Saturated cultures were diluted to 10-, 100- or 1,000-fold. For growth assays, a 2 μl aliquot of cultures was spotted onto SD medium with or without different concentration of NaCl as noted and the yeast growth was recorded after 8 days.

Plant material, growth conditions and stress treatment

Thellungiella salsuginea (Shandong ecotype) was used in the experiments. This ecotype has been cited as *Thellungiella halophila* under a misapprehension [15]. Surface-sterilized seeds were stratified in darkness for 2 weeks at 4°C, germinated and grown on Murashige and Skoog medium containing 2% sucrose and 0.8% agar. Seedlings with two to four true leaves were transferred to greenhouse and grown at 24°C under 16-h photoperiods. Five-week-old seedlings were subjected to stress treatment by watering with 500 mM NaCl daily and sampled at 0, 1, 4, 8, 12 and 24 h.

The *Arabidopsis thaliana* ecotype Columbia was used in this study. Seeds were surface-sterilized with 10% bleach and washed three times with sterile water. Sterile seeds were suspended in 0.15% agarose and plated on MS medium plus 1.5% sucrose. Plates were stratified in darkness for 2 to 4 days at 4°C and then transferred to a tissue culture room at 24°C under 16-h photoperiods. One-week-old plants were transferred to pots in greenhouse with similar temperature and photoperiod condition. For salt stress treatment in pots, 4-week-old Arabidopsis plants were watered every 4 day with increasing concentrations of NaCl, starting from 50, 100, 150, and 200 mM, and twice with 250 mM. The plants were photographed after treatment [16].

For quantitative real-time PCR analysis, *Thellungiella* and Arabidopsis seedlings with four true leaves were transferred into liquid 1/2 MS medium for 8 h pre-treatment, and subsequently transferred to 1/2 MS medium containing 500 mM NaCl (for *Thellungiella*) or 400 mM NaCl (for Arabidopsis). Samples were harvested every 2 h for RNA extraction.

Sequence analysis, multiple alignment and phylogenetic tree construction

The sequences were compared and analyzed at the National Centre of Biotechnology Information using BLAST program and CD-search. Based on BLASTP results of TsLEA1 on NCBI, homologous were aligned using MegAlign (DNASTAR, Madison, WI) to follow the CLUSTAL-W algorithm with default parameter values [17].

RNA isolation and expression analysis

Total RNA were isolated from salt treated leaves and roots of *Thellungiella* and Arabidopsis essentially as described [18]. Samples were previously frozen with liquid nitrogen and grinded to fine powder, the powder was mixed with extraction buffer (50 mM Tris–HCl pH 6.0, 10 mM EDTA, 2% SDS, 100 mM LiCl), together with 65°C heated phenol/chloroform, vortexed and centrifuged at 4°C for 15 min at 12,000 rpm. The supernatant was phenolized twice and precipitated with one volume of 4 M LiCl. After centrifugation, the RNA pellet was resuspended in TE buffer. The supernatant was phenolized again and added 0.1 volume of 3 M NaAc and three volume of pure ethanol for precipitation. The RNA pellet was washed and resuspended in TE buffer. The isolated RNA was treated with DNase I (TaKaRa) and subjected to reverse transcription by using

the M-MLV Reverse Transcriptase (Promega). The relative levels of *TsLEA1* and *AtLEA4-5* were measured by quantitative real-time PCR with primers annealing to the identical sequences from both homologues (forward primer, 5′GGACACGGCACTGGGAC3′; reverse primer, 5′GTCCGACCAGTTCCAGTGTT3′), using *Actin* as an internal standard, which was amplified with the primers annealing to the identical sequences from both *Actin* genes of *Thellungiella* and Arabidopsis (forward primer, CAGT GTCTGGATCGGAGGAT; reverse primer, TGAACAAT CGATGGACCTGA).

Quantitative real-time PCR was performed on each cDNA sample with the SsoFast EvaGreen Supermix (Bio-Rad) and analyzed with a CFX96™ Real-time PCR Detection System (Bio-Rad) following the manufacturer's instructions. The program was 98°C denaturation for 30 s, 40 cycles of 98°C for 5 s, 60°C for 5 s, and 65°C for 20 s. Each data point has three replicates. The data were analyzed using CFX manager 1.5 software (Bio-Rad) by employing an optimized comparative Ct (ΔΔCt) value method. The expression level was calculated as 2^ (−ΔΔCt) to compare the relative expression.

Transformation vectors and construction of transgenic plants

For the overexpression of selected salt-tolerance related *Thellungiella* genes, the cDNA fragment including CDS was cleaved form pGAD GH vector, cloned into pGreen binary vector [19] with modification and the genes were overexpressed in Arabidopsis under the control of double 35S promoter. The constructs were introduced into *Agrobacterium tumefaciens* EHA105 strain containing the plasmid pSoup [19]. The resulting bacteria were used to transform wild-type Arabidopsis (Ecotype Columbia) by in planta vacuum infiltration [20]. Seedlings were grown on MS medium supplemented with 1.5% sucrose, and for selection of transgenic plants 50 mg/ml kanamycin was added to the medium. One-week-old plants were transferred to pots under described conditions until plants formed seeds. To select homozygous lines, T2 generation seeds were analyzed for germination on kanamycin. Only T3 plants with about 3:1 segregation ratio were used.

Results

Isolation and characterization of *TsLEA1* cDNA

A cDNA library for direct expression in budding yeast cells was constructed from salt treated whole plants of *Thellungiella* for identification of salt tolerance-related genes [12]. In this study, those cDNAs of *Thellungiella* were inserted into pGAD GH shuttle vector under the control of yeast ADH1 promoter and transformed into *S. cerevisiae* strain G19 cells. The *ENA1* gene, which encodes plasma membrane Na^+ export pump, is deleted in G19 strain so that this strain can be served as a salt sensitive mutant [13, 14]. For large scale identification of salt tolerance genes, G19 yeast cells were transformed with the Gal4 promoter derived AD-fused *Thellungiella* cDNAs and spread on 400 mM NaCl containing selection medium. Yeast transformants which survived on salt-containing selection plate were selected. Among 106 yeast transformants, more than 100 transformants were selected out for subsequent salt tolerance confirmation and then the insert cDNA was isolated for sequencing. Based on sequences analysis compared with their homologues in other specie, genes from those putative salinity tolerance candidates which presence of complete or near-complete ORFs were selected to confirm their salt-tolerant ability in plant.

A cDNA clone which displayed noticeable tolerance to high salinity captured our most attention. Sequence analysis revealed that the harboured plant cDNA encoded for a LEA-like protein. We named the gene as *TsLEA1* (*Thellungiella salsuginea* Late Embryogenesis Abundant protein 1, Accession No. EU365627) for a novel gene encoding LEA like protein in *T. salsuginea*. The *TsLEA1* cDNA was 701 bp long and had the longest open reading frame (ORF) of 483 bp (Supplemental Fig. 1A). The deduced amino acid sequence of TsLEA1 was composed of 160 amino acids with a predicted molecular weight of 16.3 kDa and an isoelectric point of 9.19. The predicted TsLEA1 protein was a soluble protein and contained a conserved Late Embryogenesis Abundant (LEA) group I domain pfam03760 (E-value, 1.03e-6) located between amino acids 1 and 71.

This salt tolerant clone had high homology with LEA group I domain containing protein of Arabidopsis (AT5g06760, Accession No. NP_196294) and *Brassica carinata* (Accession No. AAT77224). Based on BLAST results of TsLEA1, we performed a homology alignment analysis between TsLEA1 and other members of LEA group 1 domain containing protein family (Supplemental Fig. 1B). TsLEA1 homologous were conserved with other members of the LEA group I family along the entire coding region, especially within the hydrophobic internal amino acid motif at the *N*-terminus. The primary structure contained rich glycine residues and a preponderance of charged and hydroxylated amino acids such as glutamate and histidine. To show the relation of TsLEA1 and its homologous, a distance tree was shown in Supplemental Fig. 1C. It showed that TsLEA1 protein shared 89% amino acid identity with that of Arabidopsis, 86% with that of Brassica, and had highest identity of 91% with that of *Sisymbrium irio*. The phylogeny trees of RbcL [21] and

Adc [22] had indicated that *Thellungiella* belonged to Brassicaceae and was located close to Arabidopsis and *Brassica* in short distance. It was consistent with previous researches that TsLEA1 locates more closely to members of Brassicaceae family than other family members.

Battaglia et al. grouped the plant LEA proteins into seven groups by distinguishing motifs conserved across species [23]. Based on their classification TsLEA1 and its Arabidopsis homologue AtLEA4-5 (Accession No. NP_196294) could be classified as LEA group 4B proteins which contain the pfam03760 domain and five characteristic conserved motifs [23, 24]. This group of proteins are high hydrophilic and adopt an α-helical structure in *C*-terminal [23]. Comparison of amino acid composition showed that TsLEA1 contain more aliphatic and positively charged amino acid than AtLEA4-5 (Fig. 1).

Transcriptional response of *TsLEA1* and *AtLEA4-5* to salt stress

LEA-type proteins are one of the major types of stress-induced proteins that accumulate upon water, salinity, and extreme temperature stresses [3]. They have been shown to play an important role in cellular protection under stresses [25]. To test the response of *TsLEA1* under salt condition and compare the expression patterns with that of *AtLEA4-5*, we examined the transcription of *TsLEA1* and *AtLEA4-5* gene in seedlings upon salt treatment. As shown in Fig. 2, both *TsLEA1* and *AtLEA4-5* could be induced under salt treatment. The *TsLEA1* did not showed strong induce responses but its transcript accumulates more in roots than in aerial parts while *AtLEA4-5* showed contrary accumulation pattern. This result indicated that *TsLEA1* was a salt low level inducible expression gene. The result of *AtLEA4-5* in Arabidopsis was consistent with previous microarray data from Arabidopsis.

Ectopic expression of *TsLEA1* confers salt-tolerance in yeast

The *TsLEA1* clone was driven by yeast ADH1 promoter in shuttle vector pGAD GH and translated as fusion proteins in yeast. When *TsLEA1* expressed in salt sensitive yeast strain G19 (ENA1 deletion), it conferred improved survival of yeast cells to salt stress (Fig. 3). When salt content in medium reached to toxic concentration for yeast cells, the control yeast with empty vector grew poorly, while transgenic yeast cells showed more salt tolerance and much better growth. It was indicated that TsLEA1 conferred salt tolerance in yeast cells.

Overexpression of *TsLEA1* improved the salt tolerance in Arabidopsis

To confirm the salinity tolerance in plants, *TsLEA1* was cloned into modified binary vector pGreen (Fig. 4a) and over-expressed in *Thellungiella*'s glycophyte relative Arabidopsis for salt tolerance assay. Transgenic plants

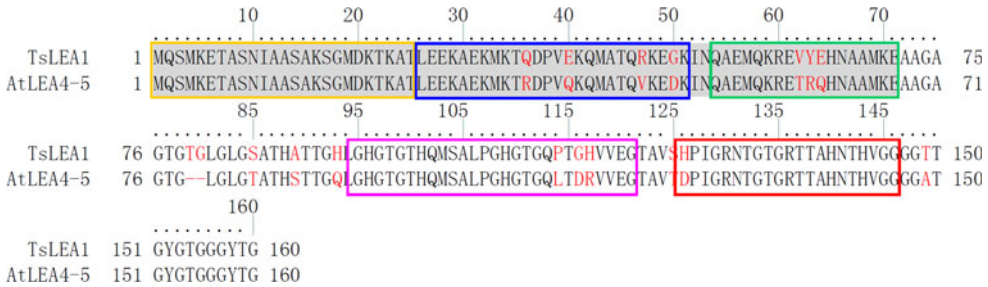

Fig. 1 Amino acid sequences of TsLEA1 from *Thellungiella salsuginea* and AtLEA4-5 from *Arabidopsis thaliana*. *Gray shades* indicate the conserved amino acid sequences of pfam03760 domain. *Boxes* indicate the five conserved motif of plant LEA group 4B in order from *N*-terminal region to *C*-terminal region: motif 2; motif 1; motif 3; motif 4; motif 5

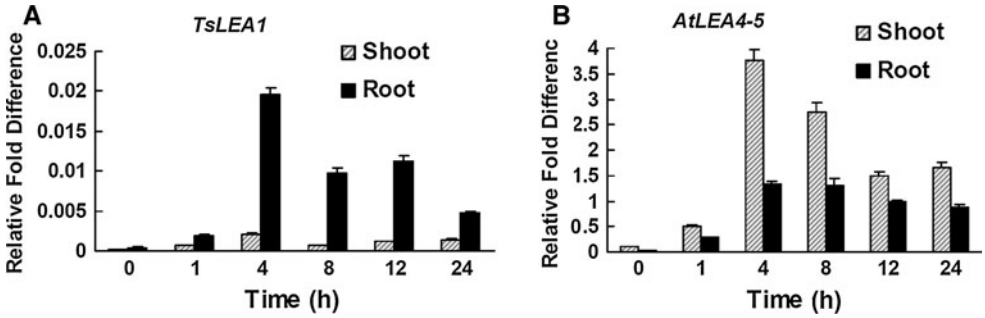

Fig. 2 Expression patterns of *LEA* homologues in salt-treated *Thellungiella* and Arabidopsis seedling. *Error bars* represent standard error of the mean. **a** (expression of *TsLEA1* in *Thellungiella*), root: roots of seedling; shoot: aerial parts of seedling. **b** (Expression pattern of *AtLEA4-5* in Arabidopsis), root: roots of seedlings; shoot: aerial parts of seedlings

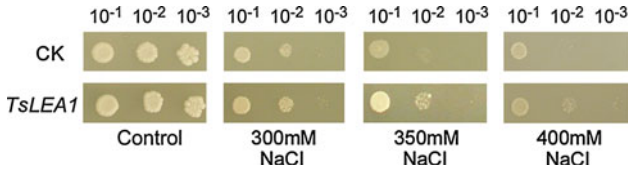

Fig. 3 Tolerance to NaCl of yeast transformants. Transformants with empty vector (CK) and selected *TsLEA1* were grown in liquid SD medium to saturation, and serial dilutions were dropped on SD plates with or without different concentration of NaCl. Growth was recoded for photographs after 8 days

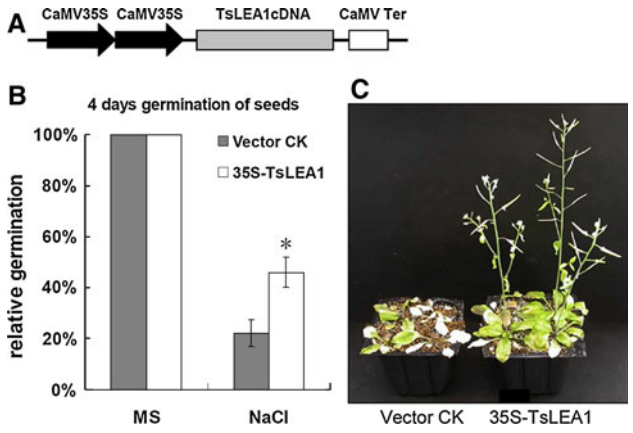

Fig. 4 Salt treated *35S-TsLEA1* transgenic Arabidopsis. **a** Schematic representation of the double 35S-promoter construct used to generate *TsLEA1* overexpress plant. **b** Quantitative analysis of germination on MS medium or MS containing 100 mM NaCl (*Right panel*). Germination (radical emergence) of empty vector control (*gray column*) and 35S-*TsLEA1* plants (*white column*) were calculated at 2 days. Results were normalized to untreated control. Percentages are means ($n = 40$–80 each) of three repeats ±SD. *Asterisk* means extremely significant at $P < 0.01$ (Student's T test). **c** Salt tolerance assay of 4-week-old plants. Representative plants were photographed 24 days after the beginning of salt treatment

showed enhanced salt tolerance compared with vector control when transgenic plants germinated on salt containing plates or soil-growth plants were treated with NaCl. The germination ratio of 35S-*TsLEA1* transgenic plants on salt containing medium was about 46%, which was much higher than that of control lines (~22%) (Fig. 4b). After continuous salt treatment, the survival rate of 35S-*TsLEA1* transgenic plants was about 38%, while empty vector transgenic plants control could not survival at all (Fig. 4c). These results indicated that ectopic-expression of *TsLEA1* can improve the salt tolerance in Arabidopsis.

Discussion

In previous studies we generated a cDNA library from a salt-treated *Thellungiella* carried by pGAD-GH shuttle vector, which can directly ectopically express proteins in yeast [12]. The yeast expression system provides an efficient platform for identifying and characterizing functionally conserved plant genes in yeast single cells, which is more straightforward than those in higher plants [26, 27]. It provides the possibility for us to discover more salt tolerance genes in *Thellungiella* by large-scale stress-tolerance screen from yeast transformants, and it may further help for revealing the mechanism of salt tolerance in this halophyte. By using fission yeast as a functional and large-scale system, several cDNA clones from the salt cress which confers enhanced salt tolerance are isolated [27, 28].

In this study we found that ectopic expression of salt induced *Thellungiella TsLEA1* gene improved salt tolerance both in Yeast and Arabidopsis. Some progresses in LEA protein function showed similar results in salt-tolerance related functions. Expression of *HVA*1, a LEA homologue from Barley, improves tolerance to drought and salt stress in transgenic rice [29] and mulberry [5]. An Arabidopsis LEA-like protein, AtLEA5, can increase the tolerance in yeast to the oxidative stress [30]. Although no proteins share homologous sequences with plant LEA proteins in yeast, TsLEA1 may protect yeast cells under stress conditions through protection of cellular components.

Although the precise function of LEA proteins has not been elucidated, it is reported that LEA proteins may play as a molecular chaperone to protect proteins from aggregation caused by desiccation, freezing and water-stress tolerance. In plants, LEA proteins show a synergistic effect with trehalose in dehydration [4]. LEA proteins rich in hydroxylated amino acids, the intrinsic flexible nature of LEA allows them to adjust their conformation to a particular microenvironment leads to different conformations under different water availability [23]. These proteins could interact with a variety of cellular components, to serve as a replacement for water as it is lost from the cell during stress [2, 31]. The salt-induced LEA proteins appear to involve in the complex regulation function of damage limitation or repair [7]. We isolated and characterized the full-length *TsLEA1* cDNA, and analyzed its expression patterns in response to salt stress in *T. salsuginea*. The *TsLEA1* was displayed as a salt-induced but very low level expressed gene in *T. salsuginea* comparing with *AtLEA4-5* in Arabidopsis. This may intimate that *TsLEA1* was not an essential gene in *T. salsuginea* for salt-tolerance. It is likely that other LEA homologues in *Thellungiella* may play important role in the process. The higher stress adaptive capacity of the *Thellungiella* species may due to other LEA homologues in *Thellungiella* with other stress-related genes. But the tendency of higher accumulation in root of seedlings suggested a different protective mechanism in the first contact tissues to the salinity environment. The LEA

group 4B genes show responds to ABA, dehydration, and high salinity in vegetative tissues, suggesting a key role under salt and drought stress [23, 32]. Upon ABA and NaCl treatments, the *AtLEA4-5* was shown to be the most responsive gene in Arabidopsis. Constitutive expression of *AtLEA4-5* leads to tolerance to severe drought in Arabidopsis adult plants [33]. In over expressed circumstance the TsLEA1 could confer high salt-tolerance to transgenic yeast and Arabidopsis, and this will provide beneficial approaches to serve for agronomic application.

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