

Overexpression of *SmLEA* enhances salt and drought tolerance in *Escherichia coli* and *Salvia miltiorrhiza*

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Abstract Salinity and drought are important abiotic stresses limiting plant growth and development. Late embryogenesis abundant (LEA) proteins are a group of proteins associated with tolerance to water-related stress. We previously cloned an LEA gene, *SmLEA*, from *Salvia miltiorrhiza* Bunge. Phylogenetic analysis indicated that *SmLEA* belongs to Group LEA14, which is involved in the dehydration response. To determine its function in detail, we have now overexpressed *SmLEA* in *Escherichia coli* and *S. miltiorrhiza*. The logarithmic increase in accumulations of *SmLEA* proteins in *E. coli* occurred earlier under salinity than under standard conditions. *SmLEA*-transformed *S. miltiorrhiza* plants also showed faster root elongation and a lower malondialdehyde concentration than the empty vector control plants did when cultured on MS media supplemented with 60 mM NaCl or 150 mM mannitol. Moreover, *SmLEA*-overexpressing transgenics experienced a less rapid rate of water loss. Under either salinity or drought, overexpressing plants had greater superoxide dismutase activity and a higher glutathione concentration. These results suggest that *SmLEA* may be useful in efforts to improve drought and salinity tolerance in *S. miltiorrhiza*. Our data also provide a good foundation for further studies into the stress resistance mechanism and molecular breeding of this valuable medicinal plant.

Keywords Drought and salt tolerance · *Escherichia coli* · Late embryogenesis abundant protein · *Salvia miltiorrhiza* Bunge

Introduction

Abiotic and biotic stresses, such as salt, drought, cold, pests, and disease, greatly affect the growth and development of plants, and they present significant obstacles to crop production in large areas of the world (Munns 2005; Mahajan and Tuteja 2005). Drought and salinity are the main factors reducing growth and yields and, under severe conditions, leading to plant death (Taylor et al. 2009). Plants utilize many physiological and molecular mechanisms to cope with an unfavorable environment and minimize possible damage. Those mechanisms generally include the accumulation of osmolytes and reactive oxygen species (ROS) scavengers and the production of putative osmoprotective proteins, such as late embryogenesis abundant (LEA) proteins (Mahajan and Tuteja 2005).

First identified in cotton (*Gossypium hirsutum*), LEA proteins are highly accumulated in the embryos during the late stage of seed development (Dure et al. 1981). Many LEA proteins or their genes have been characterized from different species, and their accumulation has been positively correlated with stress tolerance (Xu et al. 1996; Babu et al. 2004; Sivamani et al. 2000). LEA proteins are divided into nine subgroups, each having a special function under water stress because of differences in sequences and structure (Bray et al. 2000; Wise 2003). Most LEA proteins are cytosolic and hydrophilic and contain random coil or α -helices (Soulages et al. 2002). By contrast, one group of LEA proteins, LEA14, has hydrophobic residues and is likely to function differently from hydrophilic proteins (Wise 2003). LEA14 proteins such as tomato ER5 (Zegzouti et al. 1997), soybean D95-4 (Maitra and

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Cushman 1994), cotton *Lea14-A* (Galau et al. 1993), hot pepper *CaLEA6* (Kim et al. 2005), and sweet potato *IbLEA14* (Park et al. 2011) are highly expressed under stress conditions, e.g., drought, UV radiation, high salt, low temperatures, ethylene, or abscisic acid. However, only a few studies have reported the analysis of transgenic *LEA14* gene expression. Overexpressing *CaLEA6* enhanced tolerance to dehydration and salt stress in tobacco as defined by leaf fresh weights and chlorophyll contents (Kim et al. 2005). Transgenic sweet potato non-embryogenic calli overexpressing *IbLEA14* showed enhanced tolerance to drought and salt stress, whereas RNAi calli exhibited increased stress sensitivity, as evidenced by water contents and lipid peroxidation analyses (Park et al. 2011). However, overexpression of *Craterostigma plantagineum* pcC27-45, which encodes hydrophobic LEA protein, is not sufficient to increase drought tolerance in transgenic tobacco (Iturriaga et al. 1992). Based on all of these different expression profiles in various plant species, each hydrophobic LEA protein assumes a distinctive role against dehydration stress (Kim et al. 2005). However, the functions of hydrophobic LEA14 proteins are still unclear.

Salvia miltiorrhiza, as a well-known traditional Chinese medicine, is widely used for the treatment of cardiovascular and cerebrovascular diseases. This plant has strong environmental adaptability and commonly found throughout China and other Southeast Asian countries. Although *S. miltiorrhiza* is a comparatively drought-tolerant plant, the molecular mechanisms underlying this tolerance are not well-defined. We previously reported the first successful cloning of an LEA gene, *SmLEA*, from *S. miltiorrhiza* (Liu and Wang 2009). Bioinformatics analysis of the 5' flanking region of *SmLEA* showed that this region contains some putative *cis*-elements related to abiotic stress (ABRECE, CGCGBOXAT, GT1GMSCAM4, MYB1AT, MYCCONSENSUSAT) and seed-specific expression (EBOXBNNAPA and SEF4MOTIFGM7S). Real-time PCR analysis demonstrated that its expression is induced by high salinity, cold, drought, and ABA (Liu and Wang 2010). To investigate the biological function of *SmLEA* in greater depth here, we constructed a recombinant plasmid expressing the full length of *SmLEA* and monitored how high-salt conditions might affect the capacity of *SmLEA* to protect transformed cells of *Escherichia coli*. Transgenic plants of overexpressing *SmLEA* were generated and treated with salt and drought. Physiological and biochemical characteristics, including rates of root elongation and water loss, malondialdehyde (MDA) and glutathione (GSH) concentrations, and superoxide dismutase (SOD) activity were compared between the transgenic plants and the controls. These findings provide new information for use in future examinations of stress tolerance mechanisms and molecular breeding of *S. miltiorrhiza*.

Materials and methods

Plant materials, growing conditions, and stress treatments

Tube plantlets of *S. miltiorrhiza* were supplied by Good Agricultural Practice (GAP) for Chinese Crude Drugs Research Center, College of Life Sciences, Shaanxi Normal University, China. Sterile plantlets were cultured on a Murashige and Skoog (MS) basal medium, as described by Yan and Wang (2007). Shoots at the four-leaf stage were removed from overexpressing transgenic and empty vector control plants and were placed on standard MS media for 15 days. Afterward, the plants were moved to stress media for another 15 days to monitor their relative root lengths and MDA concentrations. For salt stress, the MS basal medium was supplemented with 60 mM NaCl; to simulate drought, the MS basal medium contained 150 mM mannitol (Han et al. 2007). Here, mannitol was used as osmotic agent for the characterization and selection of drought-tolerant plants (Mohamed et al. 2000).

Shoots at the four-leaf stage were removed from overexpressing transgenic and empty vector control plants and were incubated on standard MS media for 30 days. Afterward, the tube plantlets were moved from solid MS media to 1/2 Hoagland's nutrient solution and cultured in a growth chamber for 2 weeks. Finally, the plants at uniform developmental stages were chosen for stress treatments, and their leaves were used for monitoring rates of water loss. For stress treatments, the 1/2 Hoagland's solution was supplemented with NaCl to a final concentration of 100 mM or 15 % polyethylene glycol (PEG 6000) (Han et al. 2007). The PEG impact on leaf hydration occurred more rapidly and was more pronounced than that of mannitol (Slama et al. 2007). If an effect of mannitol-mediated osmotic stress is found, PEG treatments were used for further characterization of the response. Under those conditions, the plants were treated for 4 days to monitor changes in SOD activity and GSH concentrations. They were grown at 25 °C, under a 16-h photoperiod (light intensity 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$). All experiments were repeated independently at least three times.

Nucleic acid isolation and *SmLEA* clone

One-month-old plantlets were used for PCR screening and evaluation of gene expression. Genomic DNA was obtained by the cetyltrimethyl ammonium bromide (CTAB)-based method (Doyle and Doyle 1987). Total RNA was extracted from *S. miltiorrhiza* leaves with BIOZOL reagent (BIOER, China), according to the manufacturer's instructions. The first complementary DNA (cDNA) chain was synthesized with a PrimeScript® RT Reagent Kit (Takara, Japan). Primers *SmLEA*Pro-F and *SmLEA*Pro-R for *SmLEA* (Supplementary Table 1) were designed according to our

sequence previously published in GenBank (accession number AY725206) and were used to amplify the full-length *SmLEA* open reading frame (ORF), with cDNA as templates (Liu and Wang 2009). PCR was conducted in a PTC-200 thermocycler (Bio-Rad, USA), with 20 μ L of solution containing 50 ng of DNA, 10 μ L of 2 \times Taq plus PCR Mix (Runde, China), 0.5 μ M of each primer, and ddH₂O (up to 20 μ L). These mixtures were treated at 94 °C for 5 min, then subjected to 30 cycles of amplification (94 °C/30 s, 63 °C/30 s, 72 °C/1 min), followed by a final elongation at 72 °C for 10 min. The amplified *SmLEA* ORF was cloned into the pMD19-T simple vector (Takara, Japan) to create the recombinant plasmid pMD-*SmLEA*. That plasmid was then introduced into *E. coli* DH5 α for sequencing.

Protein sequence analysis

Sequence identities were determined using Protein BLAST on the NCBI web server. To construct a phylogenetic tree, we applied the neighbor-joining method and Molecular Evolutionary Genetics Analysis (MEGA, version 4.1). The grand average of hydropathy (GRAVY) of deduced proteins was predicted using ExpASY programs (<http://www.expasy.org/tools>) (Kyte and Doolittle 1982).

Vector construction

The correct recombinant plasmid pMD:*SmLEA* was digested by *Bam*H I and *Hind* III. Finally, the fragments of *SmLEA*, with sticky *Bam*H I/*Hind* III ends, were ligated into the digested pET28a vector to create plasmid pET-*SmLEA*. Primers SmLEAP1a-F and SmLEAP1a-R (Supplementary Table 1) were designed to amplify the pMD:*SmLEA*. Sequencing-confirmed PCR products were double digested by *Kpn* I and *Hind* III, then ligated into the pKANNIBAL vector to generate recombinant plasmid pKan-*SmLEA*. An overexpressing box with *SmLEA* was removed with *Not* I (Takara) from pKan-*SmLEA* and cloned into the *Not* I site in vector pART27, thereby generating overexpression plasmid pAK-*SmLEA* (Supplementary Fig. 2a). Likewise, an overexpressing box was removed with *Not* I from the empty pKANNIBAL vector and cloned into the *Not* I site in vector pART27 to create empty vector plasmid (pAK). The vector carried a spectinomycin-resistance gene (*Spe*) as a bacterial selection marker.

Prokaryotic expression

Recombinant plasmid pET-*SmLEA* and the empty pET28a vector were introduced into *E. coli* BL21 strain to create transformed strains of BL/*SmLEA* and BL/pET28 (control), respectively. The bacteria were grown at 37 °C in lysogeny broth (LB) media under kanamycin selection (50 mg L⁻¹),

with vigorous agitation (200 rpm). When the cultures were incubated at OD₆₀₀ \approx 0.6 to 0.7, isopropylthio- β -galactoside (IPTG) was added to a final concentration of 1 mM to induce expression of the inserted gene in our recombinants. After IPTG induction for 0, 2, 4, or 6 h, the *E. coli* cells were boiled for 5 min in the sample buffer according to the standard method before being loaded on 13 % (w/v) SDS-PAGE gels (Sambrook and Russell 2001). The bands were then stained with Coomassie brilliant blue. To determine the effect of salt stress, *E. coli* cells carrying pET-*SmLEA* and pET28a, which had been induced by IPTG for 6 h, were diluted and inoculated (37 °C, with shaking at 200 rpm) into LB media containing kanamycin (50 mg L⁻¹) plus 800 mM NaCl and LB media containing kanamycin (50 mg L⁻¹). Growth of these strains was measured at 2-h intervals by optical density at 600 nm (OD 600). This experiment was performed at least three times.

Plant transformation and molecular detection

Transgenic plants were generated according to the procedure for *Agrobacterium*-mediated leaf disc transformation and were selected on an MS medium containing kanamycin (Yan and Wang 2007). In parallel, plasmid pAK (with the empty vector) was introduced into wild-type *S. miltiorrhiza* as the control (PDK). Primers CaMV35S-F and CaMV35S-R (Supplementary Table 1) were designed to amplify the CaMV35S promoter so that we could evaluate whether the overexpressing box had been integrated in the transgenic plant genome. PCR was conducted in the PTC-200 thermocycler, using 20 μ L of solution containing 50 ng of DNA, 10 μ L of 2 \times Taq plus PCR Mix (Runde, China), 0.5 μ M of each primer, and ddH₂O (up to 20 μ L). These mixtures were treated at 94 °C for 10 min, then subjected to 30 cycles of amplification (94 °C/30 s, 58 °C/30 s, 72 °C/1 min), followed by a final elongation at 72 °C for 10 min. Plasmid pSmLEA-OE was used as the positive control, while genomic DNA from untransformed plants was our negative control. Amplified products were run on a 1.0 % agarose gel containing 0.5 μ g mL⁻¹ ethidium bromide and visualized under ultraviolet (UV) light.

Using SYBR Green II dye (Takara), we detected *SmLEA* expression in transgenic *S. miltiorrhiza* by quantitative real-time PCR in an IQ5 real-time PCR detection system (Bio-Rad). *SmLEA* was amplified with forward primer RLEA-S and reverse primer RLEA-A (Table S1). A housekeeping gene, *SmGAPDH* (glyceraldehyde-3-phosphate dehydrogenase; CV170251), was used as our control and was amplified with GAPDH-S and GAPDH-A (Table S1). *SmLEA* and *SmGAPDH* were amplified under PCR conditions of cDNA-denaturing 95 °C for 5 min, followed by 40 cycles of 95 °C/10 s and 60 °C/20 s. Reverse transcription-polymerase chain reaction (RT-PCR) was repeated at least three times per sample. Expression was quantified by the comparative C_T method (Vandesompele et al. 2002).

Calculation of malondialdehyde concentrations

Malondialdehyde concentration, serving as our index of lipid peroxidation, was determined by the thiobarbituric acid (TBA) method. Trichloroacetic acid (3 mL, 10 %) was mixed with *S. miltiorrhiza* leaf powder (0.2 g) at 4 °C overnight. After centrifugation, the supernatants (2 mL) were added with 2 mL of 0.6 % TBA, heated at 100 °C for 15 min, and centrifuged. The supernatants were measured at 532, 450, and 600 nm, with trichloroacetic acid as the background (Wang et al. 2012). The MDA concentration was calculated by the following formula:

$$C_{\text{MDA}} (\mu\text{mol g}^{-1}) = [6.45 \times (\text{OD}_{532} - \text{OD}_{600}) - 0.56 \times \text{OD}_{450}] / 0.2$$

Determining rates of water loss

Leaves from plants were placed on filter paper, exposed to ambient air in the greenhouse, and collected after 0, 1, 2, 3, or 4 h. All plants had five independent samples, and all data came from three separate repeats.

Measurements of superoxide dismutase activity and glutathione concentrations

Six plants each were selected from those that were exposed for 4 days to either 100 mM NaCl or 15 % PEG. Their leaves were ground into powder with liquid nitrogen. To each 0.5-g powdered sample, we added 5 mL of 50 mmol L⁻¹ phosphate buffer (pH 7.8) and held them for 60 min at 4 °C. After centrifugation, the supernatant was used to monitor SOD activity and reductions in GSH concentrations.

An assay kit (Nanjing Jiancheng Biological Engineering Institute, China) was used to monitor the capacity of SOD to inhibit the photochemical reduction of nitroblue tetrazolium (NBT). Each 3.22-mL reaction mixture contained 75 μM phosphate buffer, 39 μM methionine, 0.225 μM NBT, 0.006 μM riboflavin, 0.03 μM EDTA, and 20 μL of the enzyme extract. The increase in absorbance at 560 nm was monitored following the production of blue formazan for 25 min.

$$\text{SOD} (\text{Ug}^{-1}\text{FW}^{-1}\text{h}^{-1}) = [(A_0 - A_s) \times V_t \times 60] / [(0.5 \times A_0 \times \text{FW} \times V_s \times t)]$$

where A_0 is absorbance of the control tube under light; A_s , absorbance of the sample; V_t , total volume of the extract liquid (mL); FW , weight of the fresh sample (g); V_s , volume of the enzyme extract used in the reaction mixture; and t , reaction time (min).

The GSH concentrations were also determined by using assay kits (Nanjing Jiancheng Biological Engineering Institute, China), based on the premise that 5, 5'-dithiobis (2-

nitrobenzoic acid) (DTNB) reacts with GSH to generate 2-nitro-5-thiobenzoic acid, a yellow chemical. The concentration in a particular sample solution was determined by measuring OD at 405 nm absorbance and was calculated from a standard formula and expressed as micromoles per liter (Li et al. 2008). All procedures were done in accordance with the manufacturer's instructions.

Results

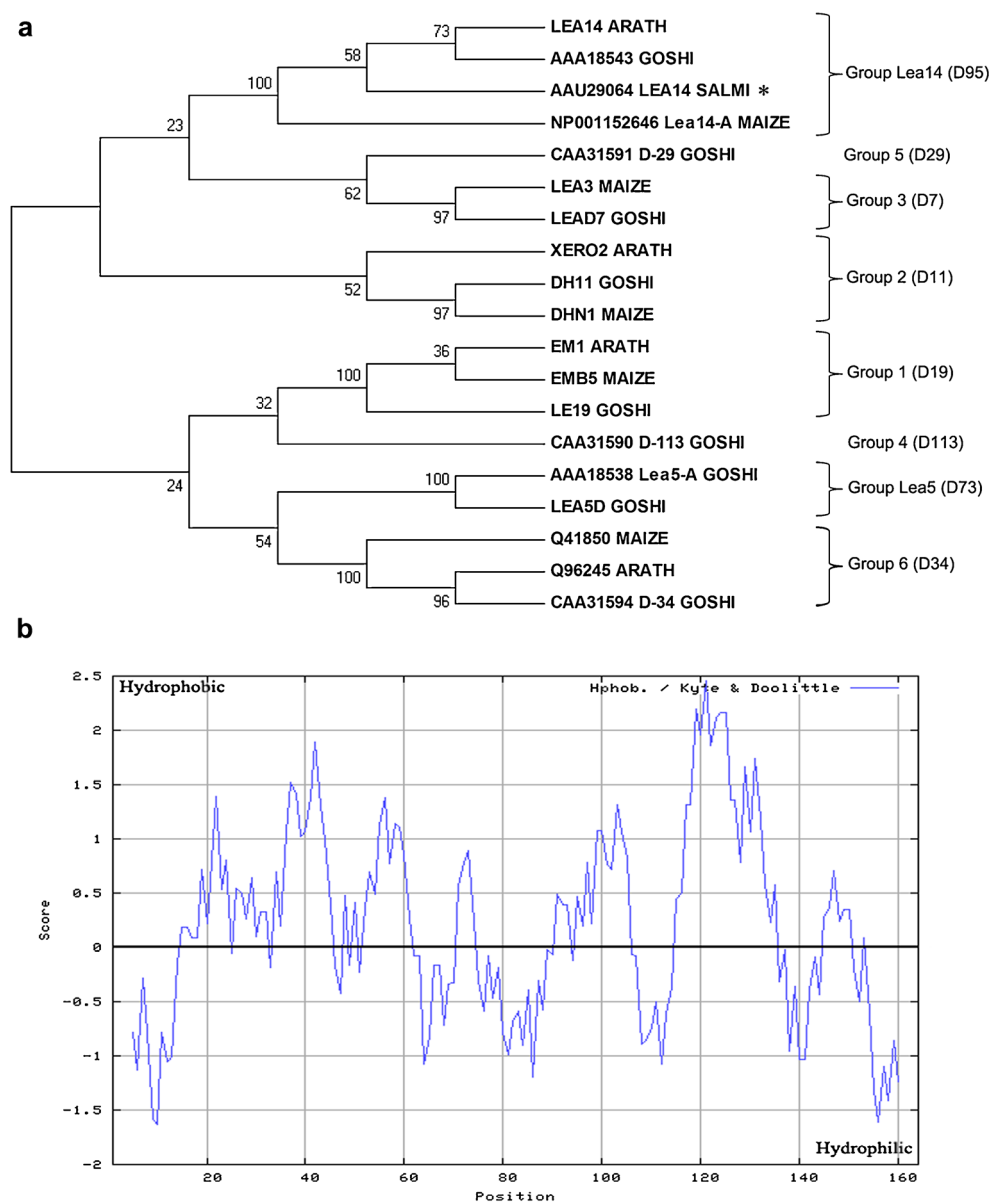
Phylogenetic characterization of *SmLEA*

We constructed a phylogenetic tree to compare *SmLEA* with other LEA proteins from *Arabidopsis thaliana*, *Zea mays*, and *Gossypium hirsutum*. Eight different groups were identified; six of the most distinct are highlighted in Fig. 1a. *SmLEA* belongs to Group LEA14, along with LEA14 from *A. thaliana* and *G. hirsutum*. Group LEA14 proteins have hydrophobic residues, which are different from other groups of hydrophilic proteins. According to the Kyte and Doolittle (1982) hydropathy plot, *SmLEA* is predicted to have a highly hydrophobic structure (Fig. 1b). Among the LEAs shown in Fig. 1a, the hydrophobic LEA14 proteins show similar features. The grand average of hydropathy (GRAVY) index of *SmLEA* was 0.207, a value comparable to *Arabidopsis* LEA14 protein (0.019 for LEA14_ARATH), whereas EM1_ARATH (Group 1) was -1.489.

Recombinant *SmLEA* protein expression

To study the physicochemical characteristics and function of *SmLEA*, we introduced plasmid pET-*SmLEA* and an empty pET28a vector into *E. coli* BL21 cells grown in an LB medium. Affinity tag sequences were removed from the pET28a expression vector to express the native protein. From total cell lysates, we observed the induction of *SmLEA* product with an apparent molecular mass of approximately 19 kD (Fig. 2a). This mass was almost 2 kD larger than the predicted value of 17.34 kD (Close et al. 1989; Ceccardi et al. 1994). The maximum accumulation of *SmLEA* occurred at 6 h after induction with IPTG (Fig. 2a). After IPTG induction, the cultures were diluted and transferred to an LB medium. Growth curves of *E. coli* cells expressing *SmLEA* and control cells (harboring pET28a) almost overlap (Supplementary Fig. 1). For salt-stress treatment, the transformed strain of BL/*SmLEA* went into the logarithmic phase after 20 h, which was 10 h earlier than the control (Fig. 2b). *E. coli* cells expressing *SmLEA* were more tolerant to salt stress than control cells. This indicated that *SmLEA* can enhance the cellular tolerance to salt stress of *E. coli* cells.

Fig. 1 Phylogenetic tree and hydrophathy plot for SmLEA and related plant proteins. **a** Six distinct subgroups out of eight are highlighted. Proteins were examined from *Salvia miltiorrhiza* (AAU29064 LEA14 SALMI, marked with *), *Arabidopsis thaliana* (EM1_ARATH, XERO2_ARATH, Q96245_ARATH, LEA14_ARATH), *Zea mays* (EMB5_MAIZE, DHN1_MAIZE, LEA3_MAIZE, Q41850_MAIZE, NP001152646 LeA14-A MAIZE), and *Gossypium hirsutum* (LE19_GOSHI, DH11_GOSHI, LEAD7_GOSHI, CAA31590 D-113 GOSHI, CAA31591 D-29 GOSHI, CAA31594 D-34 GOSHI, AAA18543 GOSHI, AAA18538 LeA5-A GOSHI, LEA5D_GOSHI). Codes are those used in forming SwissProt protein identifiers, where names generally comprise the first three letters of genus and first two letters of species. **b** Hydrophathy plot of deduced SmLEA amino acid sequence analyzed via Kyte-Doolittle algorithm (Kyte and Doolittle 1982)



Stress tolerance by SmLEA-transgenic *S. miltiorrhiza*

The overexpressing vector and empty vector control were introduced into *S. miltiorrhiza* via *Agrobacterium*-mediated transformation, and transgenic plants were identified by genomic PCR analysis and quantitative RT-PCR (Supplementary Fig. 2). Therefore, two overexpressing transgenic lines 11 and 41 with the highest transcription levels and empty vector control plants were selected for further analysis.

To address whether *SmLEA* can improve stress tolerance as well as other LEA proteins do, we evaluated relative root lengths and MDA concentrations as indicators of the extent to which salinity or drought treatment damaged our plants.

No growth difference was observed between the pAK and pAK-SmLEA transgenic plants when cultured on MS media (Supplementary Fig. 3). When grown on 60 mM NaCl or

150 mM mannitol, the roots from pAK lines were 27 or 20 % shorter, respectively, than those from plants on the standard MS medium. By comparison, roots of pAK-SmLEA lines on 60 mM NaCl or 150 mM mannitol were 1 and 3 % (line 11) or 13 and 5 % (line 41) shorter, respectively, than those of plants on the MS medium (Fig. 3a, Supplementary Table 2). Under stress conditions, plants of line 11, which expressed *SmLEA* at a higher level, also exhibited greater rootability. When we applied either salt or drought stress to the shoots of pAK and pAK-SmLEA transgenic plants at the four-leaf stage, both types showed some distinct phenotypical alterations, including stunted growth, reduced leaf expansion, and aberrant root development. The growth inhibition due to salt or drought stress of plants was much more pronounced on pAK lines than pAK-SmLEA transgenic plants (Supplementary Fig. 3).

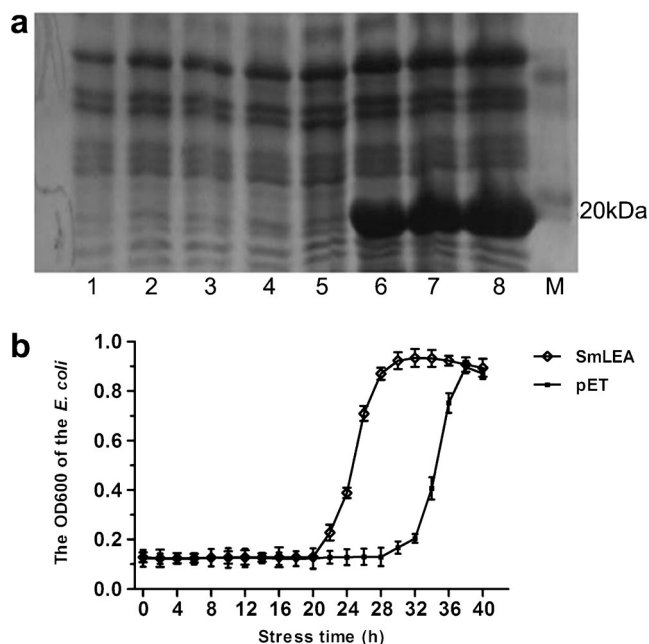


Fig. 2 Overexpression of *SmLEA* and its effect on salt stress response in *E. coli*. **a** SDS-PAGE analysis of *SmLEA* overexpression (*M*, low molecular weight protein marker; *lane 1*, before induction of BL21 with empty pET-28a vector; *lanes 2, 3, and 4*, 1 mM IPTG induction for 2, 4, and 6 h, respectively, of BL21 with empty pET-28a; *lane 5*, before induction of BL21 with pET-*SmLEA* vector; *lanes 6, 7, and 8*, 1 mM IPTG induction for 2, 4, and 6 h of BL21 with pET-*SmLEA* vector). **b** Growth curves of IPTG-induced *E. coli* harboring pET28a and pET-*SmLEA* in LB medium supplemented with 800 mM NaCl

MDA concentrations were measured in leaves of pAK and pAK-*SmLEA* transgenic plants (Fig. 3b). When the plants were grown on 60 mM NaCl or 150 mM mannitol for 15 days, MDA levels rose significantly to varying degrees in all lines. However, MDA concentrations were lower in pAK-*SmLEA* lines than in pAK lines (Fig. 3b). Analyses of lipid peroxidation also demonstrated that our overexpressing transgenic lines were more tolerant to NaCl or mannitol exposure when compared with pAK lines. This was manifested by their lower accumulations of MDA as well as less damage done to their cell membrane systems under those conditions.

Rates of water loss, superoxide dismutase activity, and glutathione concentrations in transgenic and control plants

After 1 h of dehydration, the pAK lines retained 51.43 % of their initial moisture content compared with the control, while levels in the two pAK-*SmLEA* lines were only 30.37 to 37.33 % of that for the control (Fig. 4). Thus, water loss was less rapid in *SmLEA*-overexpressing transgenics.

The pAK and pAK-*SmLEA* transgenic plants with similar vitality under normal growth conditions were used for treatment (Fig. 5a, c). When plants grown in 1/2 Hoagland's solution were subjected to salt stress (100 mM) or drought (15 % PEG)

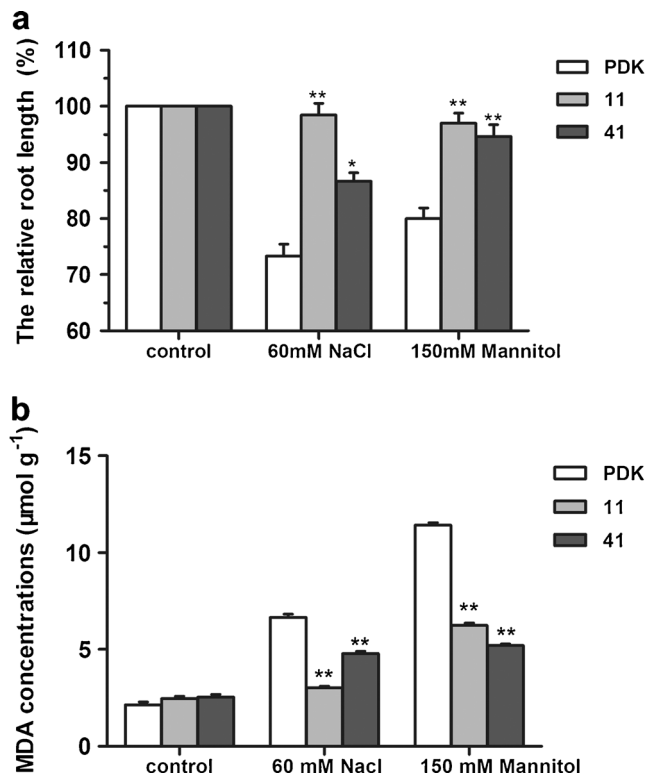


Fig. 3 Effects of *SmLEA* overexpression on stress tolerance. **a** Relative root lengths for pAK lines (PDK) and pAK-*SmLEA* lines (lines 11 and 41) after salt and drought treatments. Length for untreated plants taken as 100 %. **b** MDA concentrations in pAK lines (PDK) and pAK-*SmLEA* lines (lines 11 and 41) after stress treatment. Error bars indicate statistically significant differences at * $P < 0.05$ and ** $P < 0.01$

for 4 days, lines 11 and 41 continued to grow, whereas pAK lines exhibited leaf wilting and nearly died (Fig. 5b, d).

These stress responses were further analyzed by monitoring SOD activity and glutathione concentrations. SOD is a key enzyme which can scavenge harmful reactive oxygen species that accumulated during stresses. Lines 11 and 41 had greater SOD activity than pAK lines after treated with either 100 mM NaCl or 15 % PEG (Fig. 5e). GSH is one of the low molecular weight antioxidants that play an important role in intracellular defense against ROS-induced oxidative damage. Meanwhile, GSH can help recycle other antioxidants. When challenged with high salt or drought, GSH concentrations were lower in pAK lines than in pAK-*SmLEA* lines (Fig. 5f). These findings suggested that the enzyme activity and the antioxidant concentrations were enhanced in *SmLEA* transformants in response to salt and drought stress.

Discussion

When plants are subjected to abiotic and biotic stresses, various key responsive genes are induced (Munns 2005; Mahajan and Tuteja 2005). *LEA* genes, encoding water stress-related

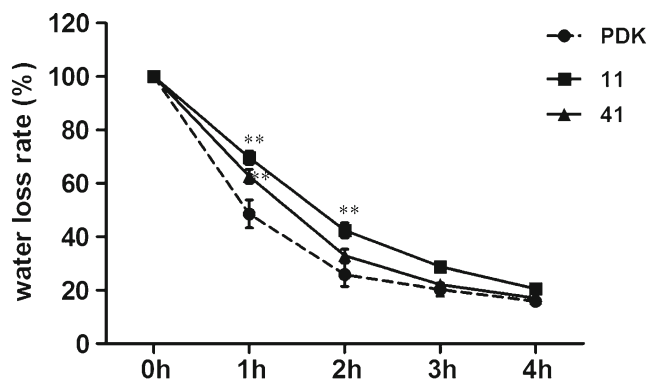


Fig. 4 Comparison of water loss rate among pAK lines (PDK) and pAK-*SmLEA* lines (lines 11 and 41) under dehydration. Error bars show significant differences at $**P < 0.01$

proteins, are regulated by developmental stage, ABA, and dehydration signals. These proteins are often involved in adaptive responses to salt and dehydration (Tunnacliffe and Wise 2007). We isolated *SmLEA* from an EST library generated from drought-stressed *S. miltiorrhiza*. Its overexpression in both bacterium and plants provided a tool for examining its functional role in conferring tolerance to abiotic stresses.

Researchers have shown that the expression system of *E. coli* is a simple, convenient, and effective model for determining the function of a heterogeneous protein in those cells under stress

treatments (Liu and Zheng 2005). For example, high salt can cause intracellular dehydration and damage to both proteins and cellular membranes (Liu and Zheng 2005). Here, our findings that salt tolerance was enhanced in recombinant bacterial cells indicated that expression of *SmLEA* in the host cells can protect them against such damage (Fig. 2b). This role by *SmLEA* proteins in *E. coli* is similar to that of other LEA proteins, e.g., soybean (*Glycine max*) PM2, *Brassica napus* (Chinese cabbage) LEA4-1, and tomato *le4*, in bacterial cells (Liu and Zheng 2005; Dalal et al. 2009; Zhang et al. 2000). Their expression directly increases stress tolerance by the host, implying that some protective mechanisms might be common to both prokaryotes and eukaryotes under adverse conditions.

Various hydrophilic LEA proteins presumably act as broad protectants against numerous sources of dehydration. Transgenic studies with yeast and rice have confirmed that several of those proteins, induced under drought or salinity, help to maintain minimum cellular water content, thereby stabilizing the cellular architecture (Xu et al. 1996; Kim et al. 2005; Zhang et al. 2000). However, hydrophobic LEA proteins may have a somewhat different function, despite their strong response to dehydration (Kim et al. 2005). The deduced amino acid sequence of *SmLEA* shares homology with that of tomato “Lemmi 9” and resembles others in the hydrophobic LEA14 group (Liu and Wang 2009) (Fig. 1).

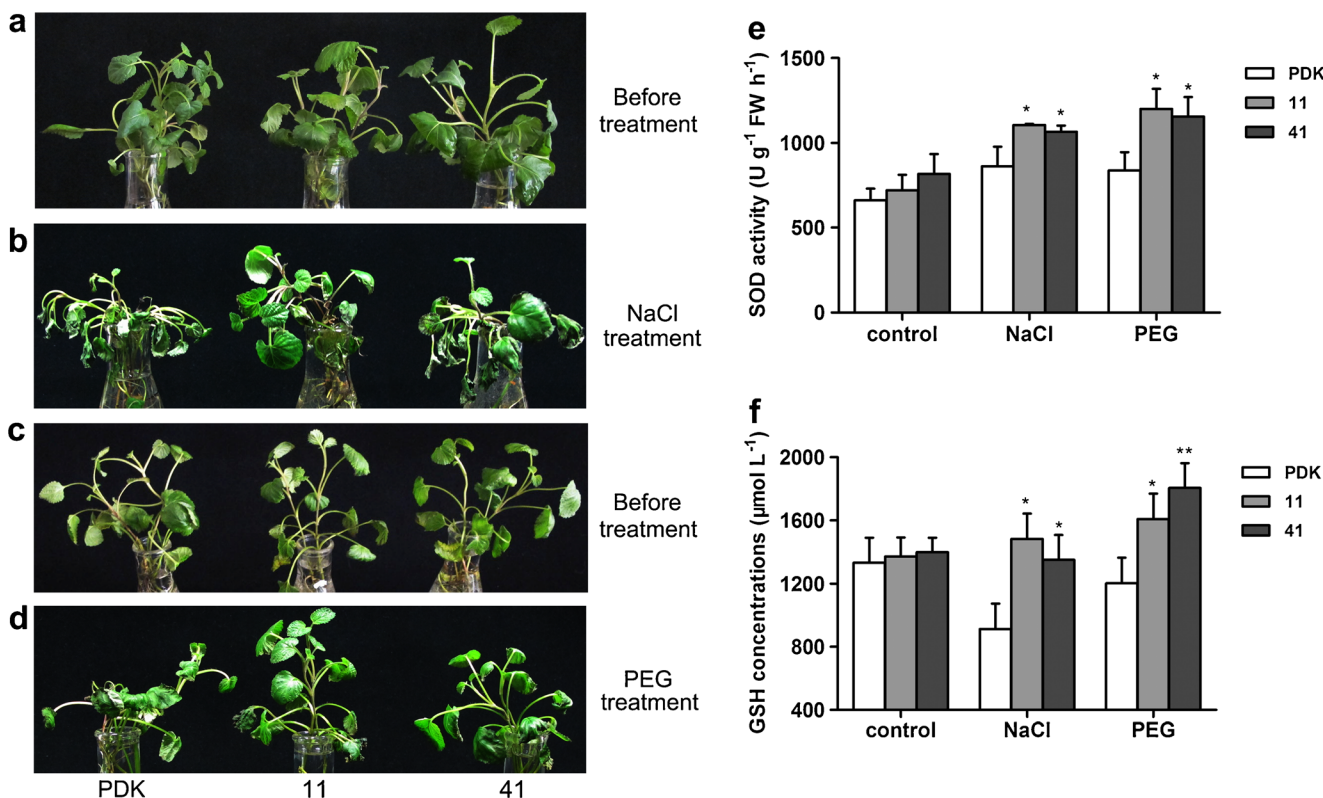


Fig. 5 Changes in SOD activity (e) and GSH concentrations (f) in stressed pAK lines (PDK) and pAK-*SmLEA* transgenic lines (lines 11 and 41) before/after incubation with 100 mM NaCl (a/b) or 15 % PEG (c/

d). Average and standard deviation of three independent experiments are plotted. Values are means \pm SD. Error bars show significant differences at $*P < 0.05$ and $**P < 0.01$

To verify the role of SmLEA in abiotic stress tolerance, we transformed *S. miltiorrhiza* with vectors that overexpress SmLEA under the control of a constitutive CaMV35S promoter. The presence of either NaCl or mannitol in the growing media can reduce plant's ability to take up water, leading to slower growth. When exposed to either of those components, our overexpressing plants showed faster root elongation compared with the pAK transgenic plants (Fig. 3a), thereby indicating that stress tolerance was improved in the pAK-SmLEA transgenic plants. Changes in MDA levels in response to stress are important markers for evaluating tolerance because they illustrate the extent of damage to the ultrastructure (Wang et al. 2010). Here, MDA concentrations were lower in transgenic lines 11 and 41 than in pAK lines following NaCl or mannitol exposure (Fig. 3b). Their enhanced tolerance was also evidenced by their slower rates of water loss (Fig. 4). Many research projects have focused on superoxide dismutases and glutathione because they are essential components of an organism's protective mechanism. Within the detoxifying process, SOD is the first line of defense, converting superoxide radicals to H₂O₂ (Asada 2000). Glutathione then participates in the removal of H₂O₂ through the ascorbate-glutathione cycle (Noctor and Foyer 1998). Transgenic lines 11 and 41 had high SOD activity and GSH concentrations, which effectively prevented or minimized oxidative damage to the biological macromolecules (Fig. 5e, f).

Because high levels of SmLEA expression are associated with greater drought and salt tolerance in transgenic plants, we might suggest a working hypothesis whereby SmLEA participates in a subset of development involved in stress responses by *S. miltiorrhiza*. In fact, our results are consistent with those reported for other genes in the LEA14 group (Kim et al. 2005; Park et al. 2003, 2011). Therefore, based on all of these different expression profiles from various plant species, we suggest that each hydrophobic LEA protein assumes a distinctive role against dehydration stress. Within this context, our findings provide further support that hydrophobic LEA proteins primarily confer protection under water deficits that arise from drought or high salinity.

The correlation found between SmLEA gene expression or SmLEA protein accumulation and stress tolerance is additional evidence of a role for LEA proteins in stress tolerance. Although the molecular basis for this improvement has not been completely determined for SmLEA-transformed *S. miltiorrhiza*, our data clearly suggest that this gene is involved in the plant response. Characterization is still needed to identify specific molecular and physiological functions. Further investigation of how SmLEA participates in multiple abiotic-stress responses will contribute to a deeper understanding of the cross-reactions in plants. We expect that our examinations of SmLEA-overexpression in transgenic plants will provide valuable information for the development of crops with enhanced tolerance.

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Conflict of interest The authors declare that they have no conflict of interest.

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