biologia plantarum
an international journal for experimental botany

BIOLOGIA PLANTARUM (2022) 66: 83-95 DOI: 10.32615/bp.2021.063

PgLEA, a gene for late embryogenesis abundant protein from *Panax ginseng*, enhances drought and salt tolerance in transgenic *Arabidopsis thaliana*

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Abstract

Late embryogenesis abundant (*LEA*) proteins are important for promoting the growth and stress tolerance of plants. They are widely involved in plant growth regulation and responses to hormones and environmental factors. However, knowledge of the functions of the *LEA* gene in ginseng species remains limited. In this study, a *Panax ginseng LEA* gene (*PgLEA*) expression vector was constructed, and stable transgenic *Arabidopsis* lines were established. The PgLEA protein was classified in the LEA-2 subgroup. Reverse-transcription quantitative PCR analysis showed that the expression of *PgLEA* increased under 300 mM NaCl or 10 % (m/v) polyethylene glycol treatments. Under salt and osmotic stresses, overexpression of *PgLEA* in transgenic *Arabidopsis* plants improved germination rate, root length, and survival rate compared to wild-type plants. In response to drought or salt stress, transgenic plants increased proline accumulation, decreased malonaldehyde content and ion leakage. Furthermore, the transgenic plants exhibited significantly increased activity of superoxide dismutase, peroxidase, and catalase, and reduced accumulation of hydrogen peroxide and superoxide. Moreover, overexpression of *PgLEA* affected the expression of genes related to salt/drought stress. Taken together, *PgLEA* is a positive regulator of drought and salinity stress, and positively functioned in pleiotropic effects through regulating osmotic balance, reactive oxygen species scavenging and inducing transcription of stress-related genes. *PgLEA* may enable ginseng plants to adapt to adverse environments. The data presented herein imply that *PgLEA* may be useful for breeding new stress-tolerant ginseng cultivars.

Keywords: CAT, drought stress, malondialdehyde, *Panax ginseng*, *PgLEA*, POD, proline, salt stress, SOD, transgenic *Arabidopsis thaliana.*

Introduction

Plants are usually sessile species and their growth and development are substantially influenced by the surrounding environment. Additionally, diverse environmental stressors, including drought and high salinity, severely restrict plant development, damage plant tissues, and under extreme conditions, can lead to death (Wang *et al*. 2003, Wu *et al*. 2014). Plants have various physiological and biochemical mechanisms to mitigate the harm caused by adverse conditions (Zhang *et al*. 2018). When plants are subjected to abiotic stress, they often synthesize a range of functional proteins that protect different tissues from damage. Among the plant cell-protective proteins induced by abiotic stress, there has been considerable interest in the late embryogenesis

Received 17 June 2021, *last revision* 18 October 2021, *accepted* 22 October 2021.
Abbreviations: CAT - catalase; COR47 - cold-regulated 47; DREB2A - dehydration-responsive element binding protein 2A; KIN1 - kinesin-1; P5CS1 - pyrroline-5-carboxylate synthase 1; PEG - polyethylene glycol; POD - peroxidase; RD19A - responsive to dehydration 19A; RD29A - responsive to desiccation 29A; ROS - reactive oxygen species; SOD - superoxide dismutase.
Acknowledgements: This work was supported by the Ministry of Science and Technology (2018YFC1706603, 2017YFC1702102) and the Jilin Province Science and Technology Development Plan (20200708060YY). We thank LetPub (www.letpub.com) for its linguistic assistance during the preparation of this manuscript.
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Conflict of interest: The authors declare that they have no conflict of interest.

abundant (LEA) proteins (Zeng *et al*. 2018).

LEA proteins were firstly discovered in seeds during the late stages of embryogenesis, but after that, they have been detected in the roots and many other tissues (Dure *et al*. 1981, Roberts *et al*. 1993). Moreover, LEA proteins are widely distributed not only in higher plants, but also in some animals, yeast, bacteria, and fungi. Based on amino acid compositions and phylogenetic relationships, the large LEA protein family has been divided into the following eight subgroups in the *Pfam* database: LEA1, LEA2, LEA3, LEA4, LEA5, LEA6, dehydrin, and seed maturation proteins (Jin *et al*. 2019b).

Most LEA proteins are relatively small (molecular mass of 10 - 30 kDa) and are rich in polar amino acid residues, including glycine, alanine, serine, and threonine, making them highly hydrophilic and thermostable (Liu *et al*. 2015). In higher plants, LEA proteins accumulate during the seed maturation stage as well as in vegetative organs in response to water deficiency. Accordingly, the expression of *LEA* genes in most plants may be important for responses to dehydration stress (Knox-Brown *et al*. 2020). Therefore, the functional analysis of LEA proteins has mainly focused on plant responses to abiotic stresses associated with dehydration. Specifically, studies have been conducted regarding the biological functions of LEA proteins related to salt, drought, and frost resistance (Jia *et al*. 2014, Liu *et al*. 2015).

Panax ginseng (C.A. Meyer), which has a long history as a medicinal plant, has been classified as a high-grade plant species in Shen Nong's Herbal Medicine resource (Chen *et al*. 2008). It has beneficial effects on the spleen and lungs and also boosts energy, soothes nerves, and protects against dementia (Hu *et al*. 2014, Jin *et al*. 2019a, Ogawa-Ochiai *et al*. 2019). However, ginseng cultivation usually requires 3 - 20 years (Wu *et al*. 2005). Additionally, its growth and development are highly affected by various environmental factors, especially drought and salt stress, which can lead to a series of damages and affect its yield and quality. At present, ginseng research has entered the genetic level. The function of ginseng stress-responsive genes is currently a topic of interest among researchers.

To date, there are no reports describing research regarding ginseng LEA proteins. Thus, the functions of ginseng LEA proteins remain unclear. Therefore, the roles of ginseng LEA proteins in response to abiotic stressors should be systematically investigated. In this study, to clarify the role of the *PgLEA* gene in response to abiotic stress, the *PgLEA* gene was inserted into *Arabidopsis thaliana via* genetic engineering to determine if transgenic plants with *PgLEA* overexpression could have enhanced tolerance to drought and salinity. The results of this study may be useful for improving the stress resistance of ginseng plants.

Materials and methods

Plants and growth conditions: Roots were collected from 5-year-old *Panax ginseng* (C.A. Meyer) plants grown at the Fusong ginseng planting base in the Jilin region of China. Five-year-old ginseng plants were grown in plastic pots (24 cm diameter, 16 cm height) containing forest soil and vermiculite (2:1). These plants were grown in a plastic greenhouse (16-h photoperiod, an irradiance of 100 µmol m^{-2} s^{-1}, day/night temperature of 26/18 °C, and 70 % relative humidity). Then ginseng plants at the leaf-expansion stage were subjected to 10 % polyethylene glycol (PEG) 6000 or 300 mM NaCl for 24 h to evaluate the effects of these stressors on *PgLEA* expression. Leaf, stem and root tissues were collected before the imposition of the stress. The leaves were collected at specific time points (0, 1, 3, 6, 12, and 24 h) after the imposition of the stress. The collected samples were quickly frozen in liquid nitrogen and stored at -80 °C for further analyses.

Arabidopsis thaliana L. Columbia (Col-0) ecotype seeds were surface-sterilized with 75 % (v/v) ethanol for 5 min and 2 % (m/v) NaClO for 7 min. The seeds were then thoroughly washed with sterile water. Thirty seeds were placed in Petri dishes containing half-strength Murashige and Skoog (MS) liquid medium and incubated in an artificial climate growth chamber at a temperature of 22 °C, a 16-h photoperiod, and an irradiance of 100 µmol m^{-2} s^{-1}.

Cloning and sequence analysis of PgLEA: Total RNA was extracted from ginseng roots according to an improved *TRIzol* method (Wolfe-Simon *et al*. 2006). Reverse transcription was performed using a reverse transcription kit (*Takara*, Dalian, China), and the first strand of cDNA was synthesized according to the manufacturer's protocol. The *PgLEA* sequence was obtained from the ginseng transcriptome database (https://www.ncbi.nlm.nih.gov/WebSub, PRJNA659400). Gene-specific PCR primers (LEA-F: TGCTCTAGAATGTCGTCATCTGATAAGCCAG; LEA-R: CGCGGATCCATCATCATCATCATCATCGCC C) were designed with *PRIMER 5.0* software. Reverse-transcription polymerase chain reaction (RT-PCR) was used to isolate the target *PgLEA* gene fragment. The process of PCR amplification was performed according to a previous report (Liu *et al*. 2017).

The homolog proteins of *PgLEA* were identified using protein *BLAST* in the *NCBI* database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The aligned multiple sequences were analyzed by *DNAMAN 6.0.* A phylogenetic analysis was performed using the *MEGA 7.0* program, with the neighbor-joining method. The strength of nodes in the tree was set by bootstrap analysis (1 000 replicates). The relative molecular mass and isoelectronic point were analyzed by *ExPASy* (http://www.expasy.org).

Construction of *PgLEA* transgenic *Arabidopsis* plants: The *PgLEA* gene was cloned into the pMDTM18-T vector (*Takara*). The recombinant vectors were transformed into *Escherichia coli* DH5α competent cells. The plasmid was extracted and double digested with BamHI and XbaI, and the target pMDTM18-T-*PgLEA* plasmid was sequenced by Beijing Genomics Institute (BGI, Beijing, China). The target gene fragment of *PgLEA* was incorporated into the pCAMBIA1303 binary plant expression vector (*Dingguo*

Changsheng Biotechnology Co., Beijing, China), which contained a CaMV 35S promoter and nopaline synthase (NOS) terminator. We extracted the recombinant plasmid pCAMBIA1303-*PgLEA* and sequenced it at the BGI.

The resulting plasmid pCAMBIA1303-*PgLEA* was inserted into *Agrobacterium tumefaciens* strain Agl0 and then transferred into *Arabidopsis* according to a floral dip method (Clough and Bent 1998). The potentially genetically modified seeds were harvested and positive plants were selected with 60 mg dm^{-3} hygromycin in a half-strength MS medium. The seeds were screened twice with hygromycin as previously described to obtain T3 transgenic *Arabidopsis* plants. The expression of *PgLEA* in the T3 transgenic *Arabidopsis* plants was determined by RT-qPCR. Three independent transgenic homozygous T3 lines (TG2, TG4, and TG6) and a control line were selected for subsequent experiments.

Identification of PgLEA transgenic plants: Total RNA was extracted from transgenic *Arabidopsis* plants according to the cetyltrimethylammonium bromide (CTAB) method (Hu *et al*. 2002), after which cDNA was synthesized by RT-PCR. cDNA was used as a template and *PgLEA* gene-specific primers were used for PCR to detect successful transformation of *PgLEA* in transgenic *Arabidopsis*.

Proteins were extracted from the wild-type (WT) and transgenic *Arabidopsis* plants and then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Western blot analysis was conducted with a polyclonal rabbit antiserum (dilution 1:1 000) raised against a partial *PgLEA* peptide as the primary antibody and AP-labeled goat anti-rabbit IgG (*GE*, Pittsburgh, USA) (dilution 1:1 000) as the secondary antibody. Protein bands were stained with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (*Sigma-Aldrich*, Darmstadt, Germany) and images were recorded.

Expression profile of PgLEA gene under stress treatment: Ginseng samples were collected at the leaf expansion stage. Total RNA was extracted from the roots, stems, and leaves according to the method of Hu *et al*. (2002). Total RNA was reversely transcribed into cDNA. The expressions of *PgLEA* in different tissues were measured by RT-qPCR instrument (*Mx3000p*, Agilent, Santa Clara, USA).

Total RNA was extracted from leaves at specific time points after stress treatment. Total RNA was reversely transcribed into cDNA. The expressions of *PgLEA* at different time points were measured by RT-qPCR.

Germination and root growth assays of *Arabidopsis* plants: *Arabidopsis thaliana* L. seeds were surface sterilized with 75 % (v/v) ethanol and 2 % (v/v) NaClO, and then rinsed. For the germination assay, the sterilized seeds were sown on Murashige and Skoog (MS) medium containing 300 mM NaCl or 10 % PEG 6000 (PEG), followed by vernalization for 3 d at 4 °C. Plates were incubated in an artificial climate growth chamber at a temperature of 22 °C, a 16 h-photoperiod, an irradiance of 100 µmol m^{-2} s^{-1}, and 70 % relative humidity. The number of seeds germinated was counted daily for 7 d, and then the germination rate was calculated.

For the root growth assay, sterilized seeds were sown on MS medium and vernalized at 4 °C for 3 d. Plates were then placed vertically in an artificial climate growth chamber under the above-mentioned culture conditions. Five-day-old seedlings were transferred and grown on MS medium supplemented with 300 mM NaCl or 10 % PEG 6000 (PEG) for 7 d. All the treatments were performed with three independent biological replicates. Taproot lengths were measured with a straightedge.

Drought and salt stress treatments of transgenic *Arabidopsis* plants: Four-week-old transgenic *Arabidopsis* seedlings (TG 2, TG 4, and TG 6) and the WT were exposed to multiple abiotic stress treatments. For the drought stress treatment, plants were not watered until more than 50 % of the plants showed varying degrees of wilting, and then they were rewatered. For the salt stress treatment, plants were irrigated with 300 mM NaCl every 4 d until they showed differences in salt stress tolerance. After drought and salt treatment, the plants were watered normally for 2 weeks. Then the phenotype was observed, and the survival rates were calculated. Samples were collected before and after the stress treatments and immediately ground to a powder in liquid nitrogen for further RT-qPCR analysis.

Measurement of proline content, ion leakage, and malonaldehyde content: The leaves of each plant were collected 2 weeks after drought or salt stress treatment. Proline content was determined by a method described by Bates (1973). The fresh leaf powder (0.5 g) was added to 10 cm^3 of 3 % (m/v) aqueous sulfosalicylic acid and boiled in a water bath for 15 min. Samples were centrifuged at 4000 *g* for 10 min, the supernatant was mixed with 2 cm^3 of glacial acetic acid and 3 cm^3 acid ninhydrin reagent and boiled in a water bath for 40 min. After cooling to room temperature, the absorbance was measured at 520 nm with a *UV-2550* spectrophotometer (*Shimadzu*, Tokyo, Japan). To analyze electrolyte leakage (EL), undamaged leaves were immersed in 20 cm^3 of dH_2O and then incubated on a gyratory shaker at 100 rpm at room temperature for 15 h. The electrical conductivity (C1) of the samples was measured. Samples were incubated in a high-pressure steam sterilization pot at 121 °C for 10 min and then cooled to room temperature. The electrical conductivity (C2) of the samples was measured again. Relative leaf EL was determined with the following formula: EL [%] = (C1/C2) × 100. The MDA content was measured with a commercial detection kit (*Beijing Solarbio Science & Technology Co.*, Beijing, China).

Determination of ROS and activities of antioxidant enzymes: The leaves were detached from WT and transgenic Arabidopsis plants 2 weeks after each treatment for ROS and measurement of antioxidant enzyme activities. Accumulations of $O_2^-$and H_2O_2 were examined by nitro blue tetrazolium (NBT) and diaminobenzidine (DAB) staining, respectively, according to the method described by Wu *et al*. (2017).

Briefly, the leaves were treated with DAB and NBT solution under vacuum infiltration. Samples were stained at room temperature in the dark for 16 h. Then the leaves were placed in 80 % (v/v) ethanol and boiled for 10 min to remove chlorophyll. They were then soaked in fresh acetonitrile for 4 h before they were examined and photographed. The activities of superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) were determined using commercial detection kits (*Beijing Solarbio Science & Technology Co.*). One unit of SOD was defined as the amount of enzyme that causes 50 % inhibition of absorption in comparison with a blank sample. One unit of POD was defined as the amount of enzyme that causes 0.005 absorbance increase at 470 nm in comparison with a blank sample. One unit of CAT was defined as the amount of enzyme needed to catalyze 1 µmol H_2O_2 per minute.

Expression analyses of stress-related genes: To further elucidate the effects of *PgLEA* on plant stress resistance, several genes associated with abiotic stress resistance [*RD19A* (responsive to dehydration 19A), *RD29A* (responsive to desiccation 29A), *COR15A* (cold-regulated 15 A), *COR47* (cold-regulated 47), *KIN1* (kinesin 1), *P5CS1* (pyrroline-5-carboxylate synthase 1), and *DREB2A* (dehydrationresponsive element binding protein 2A)] were selected based on the available literature (Yang *et al*. 2010, Guo *et al*. 2014, Qin *et al*. 2015). The transcription of these genes was analyzed by RT-qPCR using the $2^{-\Delta\Delta Ct}$ method, and *Arabidopsis thaliana* ubiquitin (*AtUBQIN*) was used as a reference gene. The calculation formula is as follows: ΔCT = CT(target, test) - CT(ref, test). Details regarding the PCR primers are provided in Table 1 Suppl.

Statistical analyses: For each experiment, at least three biological replicates were performed. The results were expressed as mean ± standard deviation (SD). Statistical analyses were performed by *SPSS 16.0* (*SPSS Inc.*, Chicago, IL, USA). The significance level was analyzed by Student's *t*-test, and differences between means were considered to be significant and extremely significant when the *P*-values were less than 0.05 and 0.01, respectively.

Results

The *PgLEA* gene was determined, and a three-dimensional structure of the *PgLEA* protein was predicted by the *SWISS-MODEL* server (Fig. 1 Suppl.). Details regarding the gene encoding the newly identified PgLEA protein are available in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank, accession number: QKN84106.1). The full-length *PgLEA* sequence comprises 942 bp and encodes a polypeptide with 314 amino acids. PgLEA has a predicted molecular mass of 34.76 kDa and a theoretical isoelectric point of 4.78. The *NCBI* database comparative analysis revealed that PgLEA contains two "LEA_2" motifs (PF03186), and according to the classification of LEA proteins (Hundertmark *et al*. 2008), the PgLEA protein is classified into subgroup LEA_2 (Fig. 2 Suppl.). *DNAMAN* was used for multiple sequence alignment (Fig. 1*A*); the results showed that PgLEA displays a high degree of similarity to other group LEA_2 proteins. To further identify the *PgLEA* gene in *P. ginseng*, a phylogenetic tree was constructed (Fig 1*B*). The phylogenetic analysis grouped PgLEA with 13 LEA_2 proteins from other species. Our results showed that PgLEA was most closely related to *Trema orientale* (PON50604.1), followed by *Ipomoea triloba* (XP_031129341.1).

To determine tissue-specific expression patterns of *PgLEA*, RT-qPCR was used to detect expression in the roots, leaves, and stems. *PgLEA* showed high expression in the leaves. The leaf expression was approximately 4- and 8-fold higher than root and stem expressions, respectively (Fig. 2*A*). Accordingly, under normal growth conditions, *PgLEA* is mainly expressed in the leaves at the leaf expansion stage.

In response to PEG treatment to simulate dehydration stress, the *PgLEA* expression gradually increased over time, peaking at 3 h before decreasing (Fig. 2*C*). Thus, the *PgLEA* gene was induced by osmotic treatment, and the expression varied with the duration of stress. The 300 mM NaCl treatment gradually upregulated *PgLEA* expression, with the highest expression detected at 6 h, after which the expression gradually decreased (Fig. 2*D*).

To determine the function of *PgLEA, PgLEA* was transformed and overexpressed in *Arabidopsis*. The RT-PCR results confirmed that the transgenic plants expressed the target gene (Fig. 3*A*). The same fragment was not amplified in the WT plants. Consistent with the RT-PCR results, a Western blot analysis involving an anti-*PgLEA* polyclonal antibody indicated that the transgenic *Arabidopsis* plants, but not the WT plants, produced a protein that was between 25 and 37 kDa (Fig. 3*B*). Three homozygous T3 transgenic lines were screened, and the expression of *PgLEA* was measured by RT-qPCR. The results suggested that three independent lines (TG2, TG4, and TG6) were highly expressing this gene (Fig. 2*B*). Therefore, these three transgenic plants were selected for further study.

To evaluate stress resistance of the wild-type and transgenic *Arabidopsis thaliana*, seed germination was measured. The T3 transgenic seeds were placed in a half-strength MS medium containing different concentrations of PEG or NaCl. When grown on a normal culture MS medium, no significant differences in germination rate were observed between the WT and transgenic seeds. As shown in Fig. 4, when the seeds were in the presence of PEG, the germination rates of the transgenic *Arabidopsis* lines were 52, 49, and 50.5 %, whereas only 28 % germination rate was observed in the WT line. In a growth medium with 300 mM NaCl, the germination rates of the transgenic seeds were 60.5 - 66 %, whereas only 36 % in WT seeds within 7 d.

Subsequently, the root length of transgenic *Arabidopsis* and WT plants was also investigated under osmotic or NaCl stress conditions. When grown on a medium without PEG or NaCl treatment, there was no significant difference in root lengths between the WT and transgenic seedlings. When grown on a medium containing PEG,

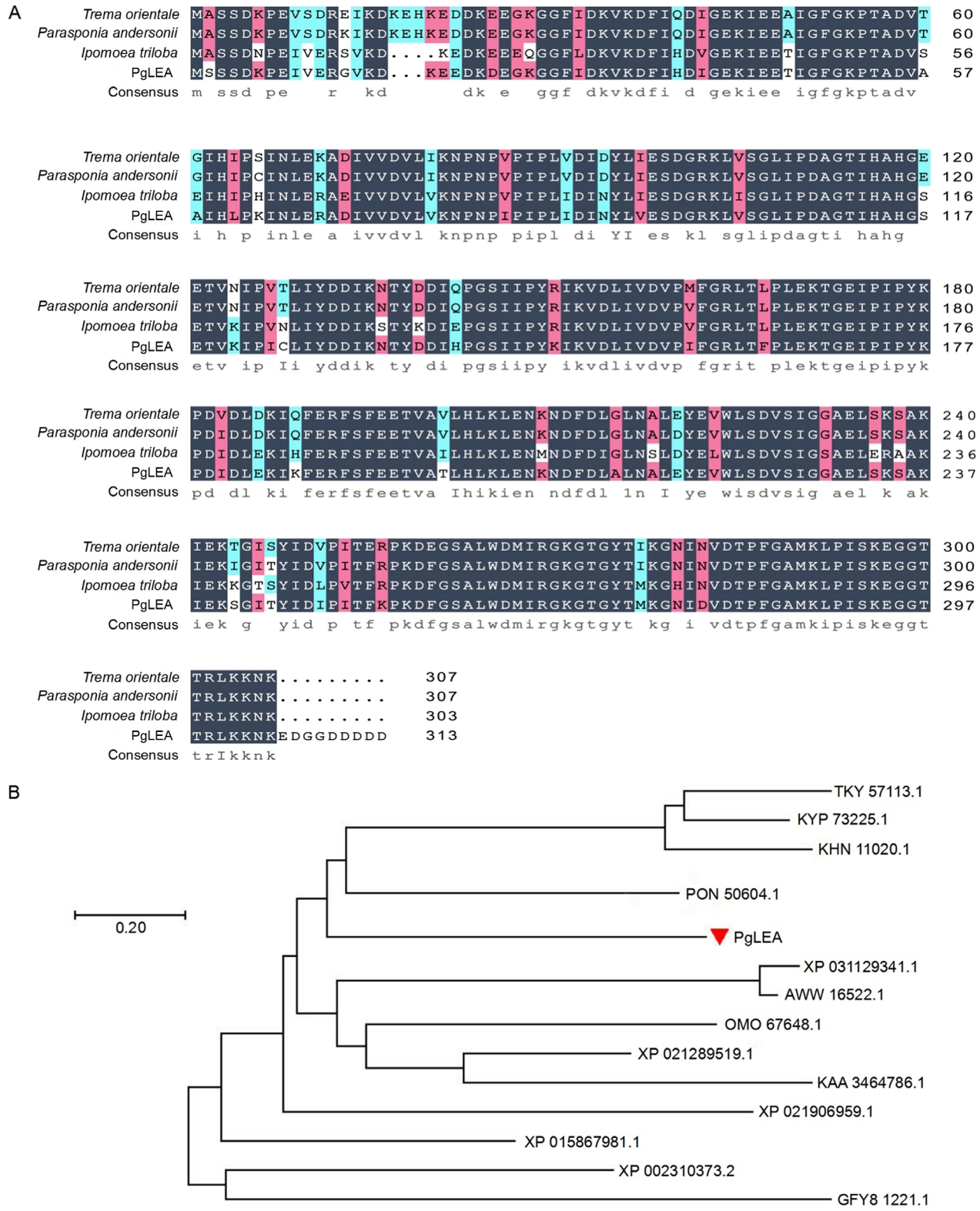

Fig. 1. Sequence analysis of PgLEA from *Panax ginseng*. *A* - Sequence alignment of PgLEA with other homolog proteins, including those from *Trema orientale* (PON50604.1) and *Ipomoea triloba* (XP_031129341.1). *B* - Phylogenetic tree of PgLEA. The analyzed sequences were from *Spatholobus suberectus* (TKY57113.1), *Cajanus cajan* (KYP73225.1), *Glycine soja* (KHN11020.1), *Trema orientale* (PON50604.1), *Ipomoea triloba* (XP_031129341.1), *Ipomoea pes-caprae* (AWW16522.1), *Corchorus capsularis* (OMO67648.1), *Herrania umbratica* (XP_021289519.1), *Gossypium australe* (KAA3464786.1), *Carica papaya* (XP_021906959.1), *Ziziphus jujuba* (XP_015867981.1), *Populus trichocarpa* (XP_002310373.2), and *Actinidia rufa* (GFY81221.1).

the root lengths of the transgenic *Arabidopsis* plants were 1.4 - 1.5 greater than those of the WT plants. When *Arabidopsis* seedlings were grown on a medium containing 100 - 200 mM NaCl, the root lengths of transgenic plants were longer than those of the WT plants, and they had more substantial differences as the concentration of NaCl increased. When grown on MS medium in the presence of 300 mM NaCl, the average root length of transgenic *Arabidopsis* was 2.6 - 3.0 cm long, which was 1.2- to 1.4- times longer than in WT plants.

To study the performance of *PgLEA* transgenic plants under drought and salt stress in the soil, the WT and transgenic *Arabidopsis* plants were tested at the seedling stage. Under normal growth conditions, the transgenic *Arabidopsis* plants showed no obvious phenotypic differences compared with WT plants. After 4-week-old seedlings underwent drought treatment for a continuous 14 d, WT *Arabidopsis* plants became yellowish and wilted, and some plants died. However, transgenic *Arabidopsis* plants only showed yellow leaves and exhibited less severe dehydration stress symptoms (Fig. 3 Suppl.). Furthermore, the survival rate of the *PgLEA*-overexpressing plants was 3- to 3.3-times higher than that of the WT plants after 14 d of drought stress. After 2 weeks of exposure to 300 mM NaCl, the plants showed different degrees of yellowing leaves, yet the transgenic *Arabidopsis* plants grew better

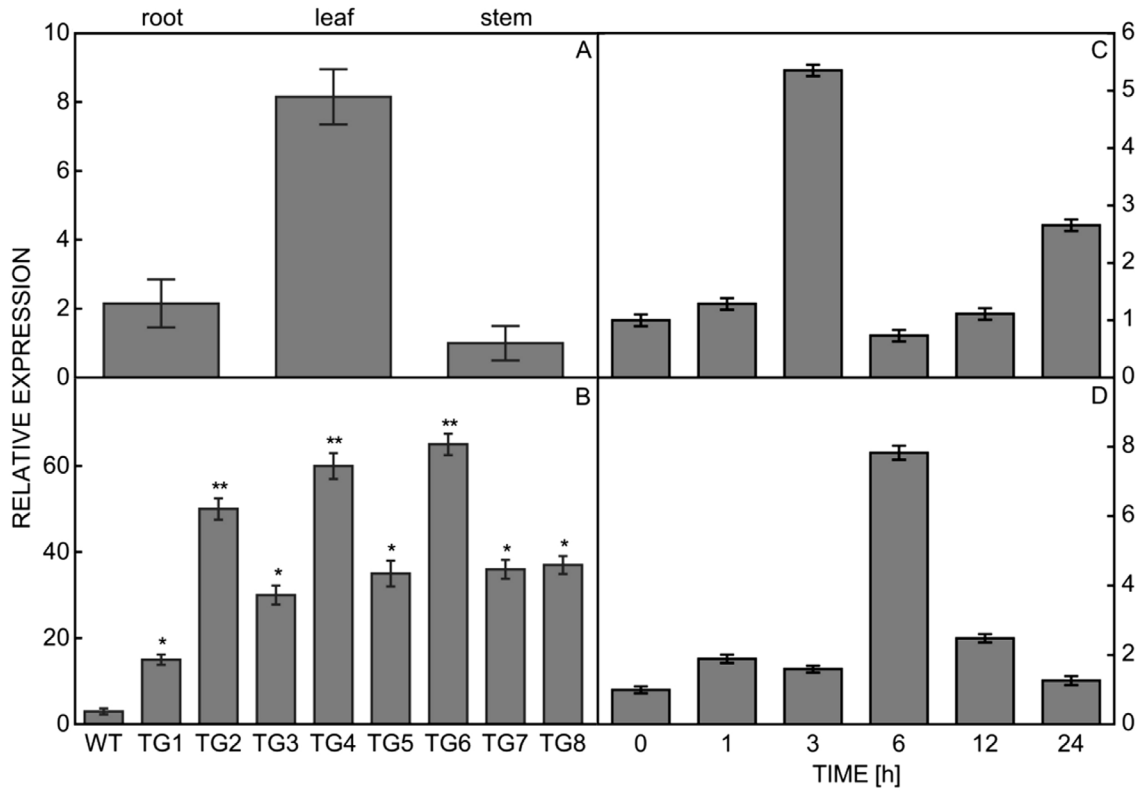

Fig. 2. Expression patterns of *PgLEA*. *A* - Expression of *PgLEA* in different ginseng tissues. *B* - The expression of *Panax ginseng* LEA (*PgLEA*) in WT and transgenic *Arabidopsis* lines. *C* - Time-course expression of *PgLEA* in ginseng under drought treatment. *D* - Time-course expression of PgLEA in ginseng under salt treatment. WT - wild-type *Arabidopsis*, TG1 to TG8 - transgenic *Arabidopsis* lines.

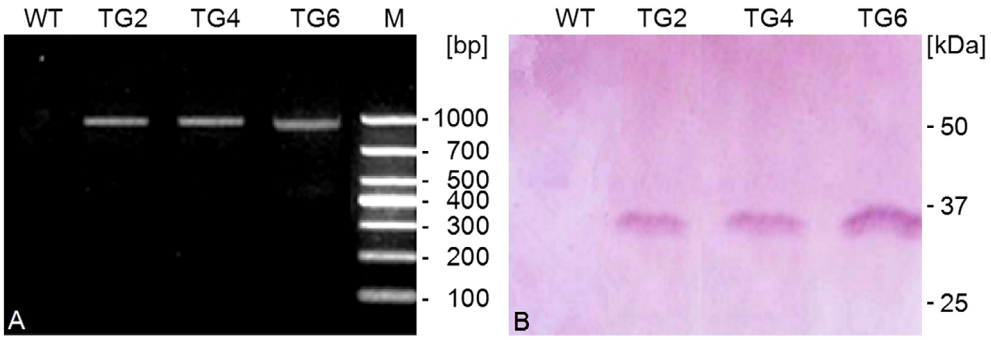

Fig. 3. RT-PCR analysis (*A*) and Western-blot detection (*B*) of transgenic *Arabidopsis* lines. WT - wild-type *Arabidopsis*, TG2, TG4 and TG6 - transgenic *Arabidopsis* lines, M - DL 1000TM DNA marker.

than the WT plants. Specifically, the yellowing of leaves was more extensive in the WT plants, some of which died (Fig. 3 Suppl.). After re-watering for 1 week, the survival rate of the WT *Arabidopsis* was only 30 %, which was in contrast to the 72 to 78 % survival rates of the transgenic *Arabidopsis* (about 2.6-times higher) (Fig. 4*E*). Phenotypic characterization suggested that overexpression of *PgLEA* apparently improved the drought and salt resistance of transgenic *Arabidopsis*.

To investigate the physiological differences between the WT and transgenic *Arabidopsis* lines under drought and salt stress conditions, some important physiological

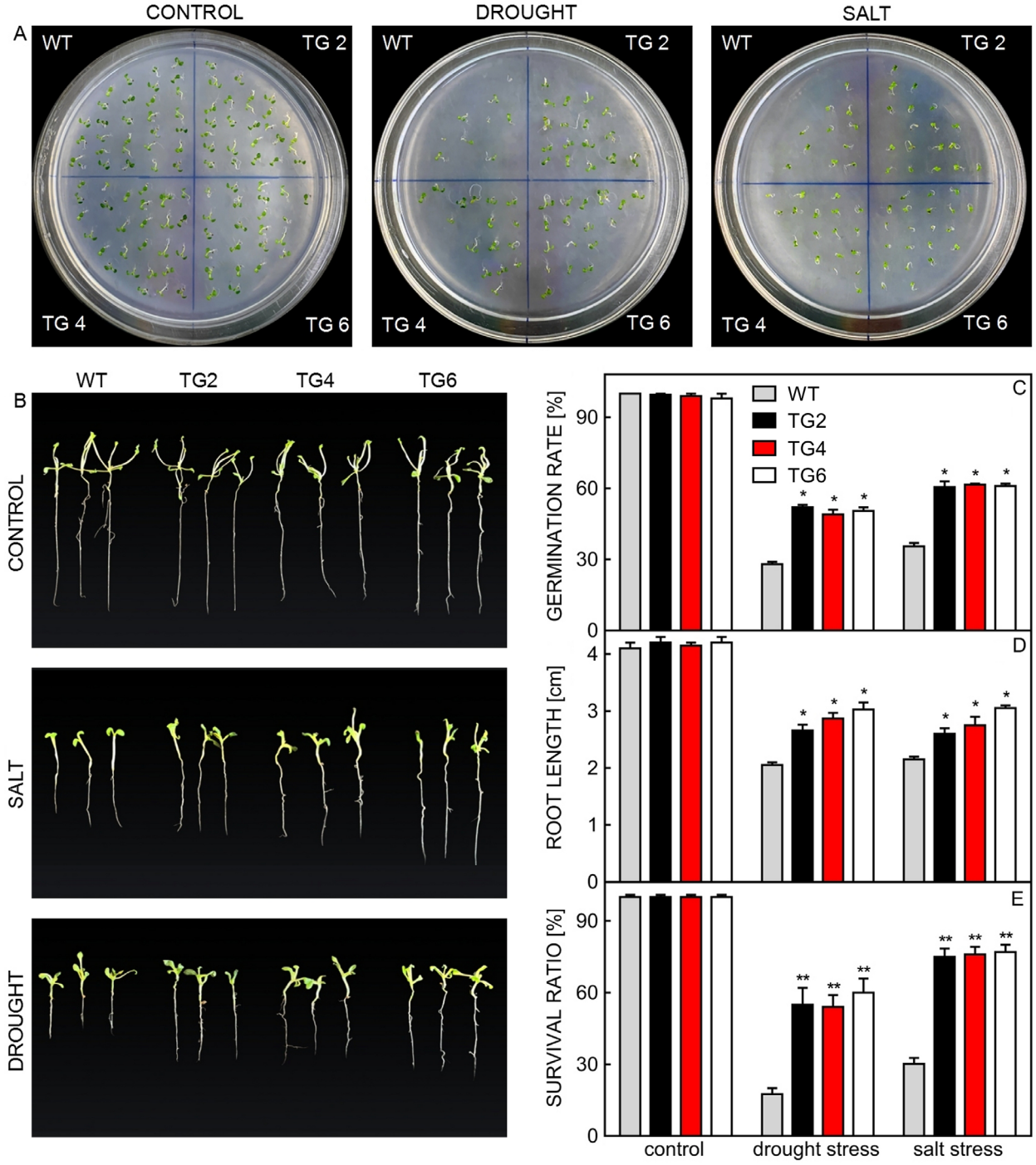

Fig. 4. Overexpression of *PgLEA* enhanced the seed germination rate and root length in *Arabidopsis* lines. *A* - Photographs of *Arabidopsis* seeds germinated on MS medium alone or on MS medium with PEG 6000 or NaCl for 7 d. *B* - Photographs of *Arabidopsis* seedlings on MS medium or MS medium with PEG 6000 or NaCl for 7 d. *C, D, E* - Analysis of the germination rate, root length, and survival of the WT and transgenic lines under drought and salt stress. WT - wild-type *Arabidopsis*, TG2, TG4 and TG6 - transgenic *Arabidopsis* lines. Means ± SDs, $n = 3$; ** and * indicate statistically significant differences compared to the control at $P < 0.01$ and $P < 0.05$, respectively.

parameters were determined. Under normal conditions, there was no significant difference in proline content, MDA content, or EL between transgenic *Arabidopsis* and WT lines. However, under drought and salt treatments, the proline content was higher in the transgenic plants than that in the WT. The MDA content and EL were lower in transgenic plants than WT plants (Fig. 5). These physiological indices suggested that the transgenic plants had stronger resistance to drought/salt stress.

Abiotic stress results in an accumulation of ROS, which can cause damage to membrane systems. Therefore, the amount of ROS was studied in *PgLEA* overexpressing and WT plants under drought/salt stress conditions. The accumulation of H_2O_2 and O_2^- in WT and transgenic lines were determined by DAB and NBT staining (Fig. 6*A,B*). Under normal conditions, there were no remarkable differences in staining between WT and *PgLEA*-overexpressing lines, and both WT and transgenic leaves accumulated low amounts of H_2O_2 and O_2^-. After exposure to drought/salt stress, both *PgLEA*-overexpressing lines and the WT showed increased staining, indicating ROS

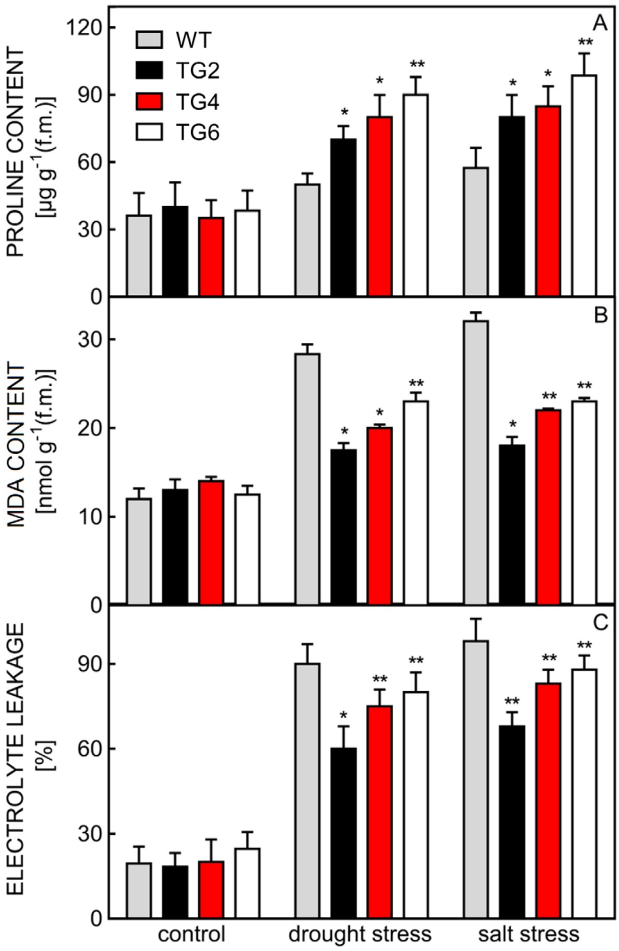

Fig. 5. Analyses of proline content (*A*), MDA content (*B*), and electrolyte leakage (*C*) in WT and transgenic plants under normal, drought, and salt stress conditions. WT - wild-type *Arabidopsis*, TG2, TG4 and TG6 - transgenic *Arabidopsis* lines. Means ± SDs, $n = 3$; ** and * indicate statistically significant differences compared to the control at $P < 0.01$ and $P < 0.05$, respectively.

accumulation under drought or salt stress. Nevertheless, compared with WT lines, the *PgLEA* transgenic lines accumulated a much lower amount of H_2O_2 and O_2^-. The staining results suggested that overexpression of *PgLEA* reduced the accumulation of ROS under drought and salt stresses. In addition, antioxidant enzymes play critical roles in ROS scavenging and can help maintain the cellular ROS balance. In the present study, the activities of SOD, POD, and CAT in leaves of WT and transgenic *Arabidopsis* plants were evaluated under normal and stress conditions (Fig. 6*C-E*). Under normal conditions, the activity of antioxidant enzymes, SOD, POD, and CAT revealed no remarkable differences between the WT and transgenic plants. Conversely, under drought and salt stresses, the activities of SOD, POD, and CAT increased more significantly in the leaves of transgenic *Arabidopsis* than in WT plants. These results revealed that *PgLEA* transgenic plants might enhance drought and salt resistance by scavenging excessive ROS.

Finally, the altered expression of abiotic stress-related genes was investigated in *PgLEA* transgenic *Arabidopsis* plants (Fig. 7). The results showed no obvious differences in expressions between the WT and transgenic *Arabidopsis* plants under normal conditions. Most of these genes were induced by salt and drought to varying degrees, and they were more highly expressed in the transgenic *Arabidopsis* compared with that in WT. For instance, in response to drought stress, the expressions of *COR47*, *RD29A*, *KIN1*, and *RD19A* were higher in transgenic *Arabidopsis* than in WT lines. Upon exposure to salinity stress, the expressions of *RD29A*, *RD19A*, *DREB2A* (dehydration-responsive element-binding protein 2A) and *P5CS1* (pyrroline-5-carboxylate synthase 1) were 20-, 10- , 45- and 50-fold higher, respectively, in the transgenic *Arabidopsis* than in WT lines.

Discussion

As some of the most important stress-related proteins in the plant kingdom, LEA proteins are growth regulators that can enhance adaptive responses to salt and dehydration stresses (Wu *et al.* 2014). However, to the best of our knowledge, *P. ginseng* LEA proteins have not been characterized. We explored the transcriptome database for ginseng and obtained the *PgLEA* gene sequence. *Arabidopsis* has the advantages of a small genome, a small plant size, a short growth cycle, and a simple genetic operation. Therefore, *Arabidopsis* was selected as a transgenic plant in this study. To further investigate functional characteristics and elucidate the underlying molecular mechanism of *PgLEA*, a *PgLEA* expression vector was constructed to generate transgenic *Arabidopsis* plants *via A. tumefaciens*-mediated transformation.

NCBI database comparative analysis showed that *PgLEA* belongs to the LEA-2 subgroup. Multiple alignments revealed that PgLEA displays a high degree of similarity to other group LEA-2 proteins. The expression of LEA-2 proteins can be induced by some abiotic stresses such as PEG, NaCl, and low temperature. Previously,

SiLEA14 was reported to be dramatically induced by osmotic and NaCl stresses (Wang *et al*. 2014). *AtLEA14* was reported to be induced by cold, drought, salt, and heat stress (Hundertmark *et al*. 2008). *AdLEA* expression was induced by high temperature, dehydration, salinity, and oxidative stress (Sharma *et al*. 2016). Consistent with our research, the expression of the *PgLEA* gene was induced by drought and salt treatments.

Transgenic *A. thaliana* plants overexpressing *PgLEA* were generated to examine the role of *PgLEA* in salt and drought resistance. Seed germination determines whether plants can grow and develop normally (Wu *et al*. 2018). Drought and salt stresses inhibit the normal germination of seeds. *AtLEA14*-overexpressing *Arabidopsis* was reported to exhibit enhanced tolerance to high salinity during germination (Hundertmark *et al*. 2008). *IpLEA* significantly influences the salt/drought stress tolerance during seed germination (Zheng *et al*. 2019). In this study, the germination rates of *PgLEA*-overexpressing *Arabidopsis* plants under osmotic or salt stresses were higher than of WT plants, indicating that *PgLEA* was involved in plant germination. When plants suffer drought stress, damage can be reduced by root elongation (Parent *et al*. 2010). The root length of *JcLEA*-overexpressing *Arabidopsis* plants is higher than that of WT plants under drought conditions (Liang *et al*. 2013). Here, *PgLEA* overexpression in *Arabidopsis* led to an increase in root length under osmotic or salt stress compared with WT plants. Drought and salt stress can also lead to adverse changes in plant growth and can even cause plant death. *CmLEA-S* overexpressing transgenic tobacco lines showed fewer signs of wilting and chlorosis under salt and drought stress (Poku *et al*. 2020). Consistent with our research, the survival rate of *PgLEA*-overexpressing *Arabidopsis* plants subjected to drought and salt treatments was higher than that of WT plants. According to the change rule of multiple

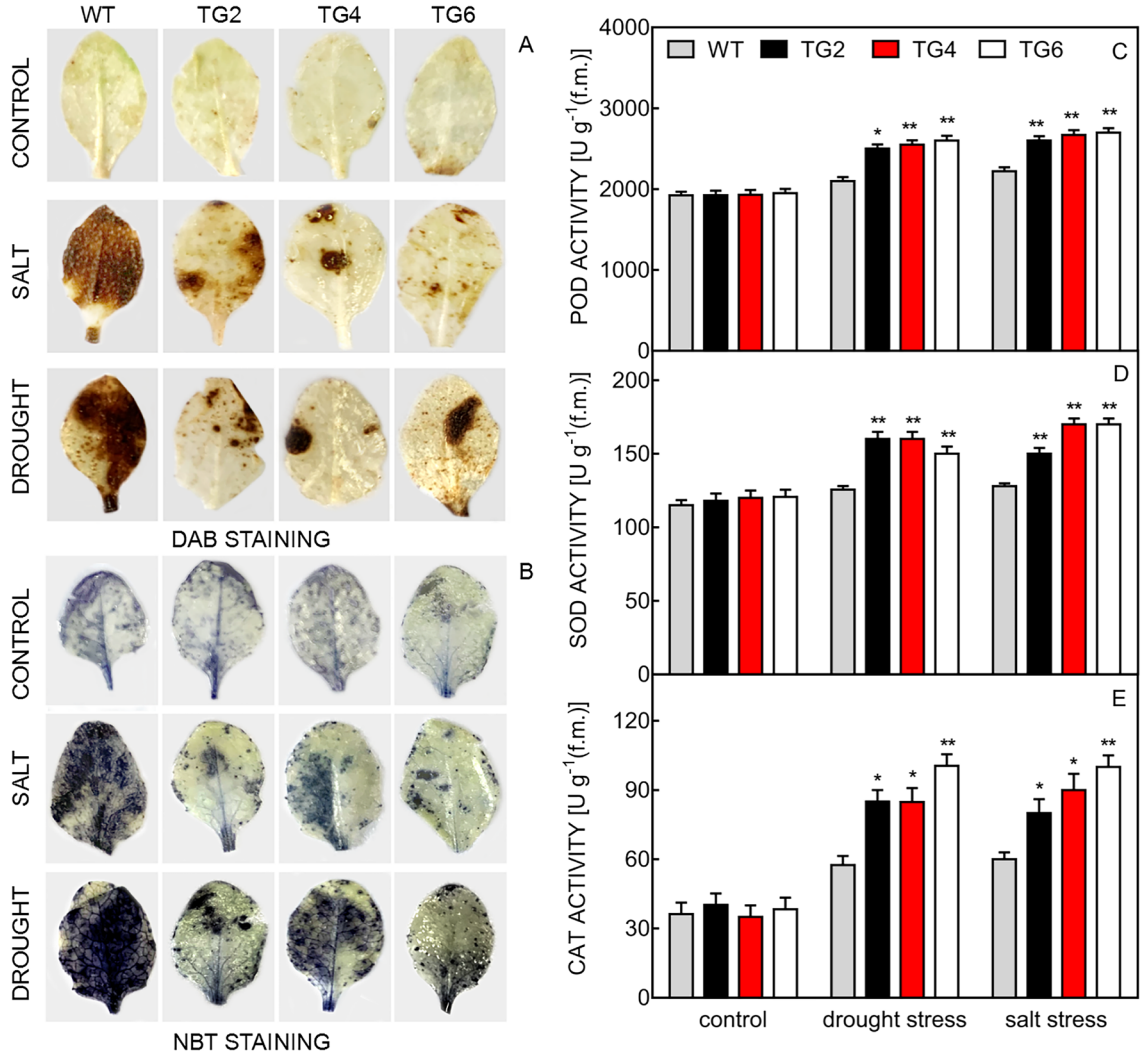

Fig. 6. Oxidative stress and ROS scavenging analyses of the *PgLEA* transgenic lines and WT plants 2 weeks after drought or salt stress treatment. *A, B* - Histochemical staining with DAB and NBT was used to detect H_2O_2 and O_2^-, *C* - Peroxidase (POD) activity, superoxide dismutase (SOD) activity, and catalase (CAT) activity. WT - wild-type *Arabidopsis*, TG2, TG4 and TG6 - transgenic *Arabidopsis* lines. Means ± SDs, $n = 3$; ** and * indicate statistically significant differences compared to the control at $P < 0.01$ and $P < 0.05$, respectively.

growth indices, it was concluded that *PgLEA* was involved in *Arabidopsis* growth regulation under drought and salt stress.

Proline is considered to be a broadly distributed and powerful osmolyte, and can also function as a radical scavenger (Szabados and Savouré 2010, Kong *et al*. 2015). Plants tend to accumulate more proline in response to various abiotic stressors (Dobra *et al*. 2010, Ami *et al*. 2020). Thus, the proline content may be a useful physiological index for evaluating plant salt and drought tolerance. Abiotic stress can damage the plant cell membrane and cause the outflow of cell fluid, eventually leading to an increase in relative conductivity. MDA is the most important membrane lipid peroxidation product of plants under abiotic stress conditions, and changes in MDA content can represent the extent of damage to the ultrastructure (Wang *et al*. 2010). Therefore, both the relative conductivity and content of MDA can reflect

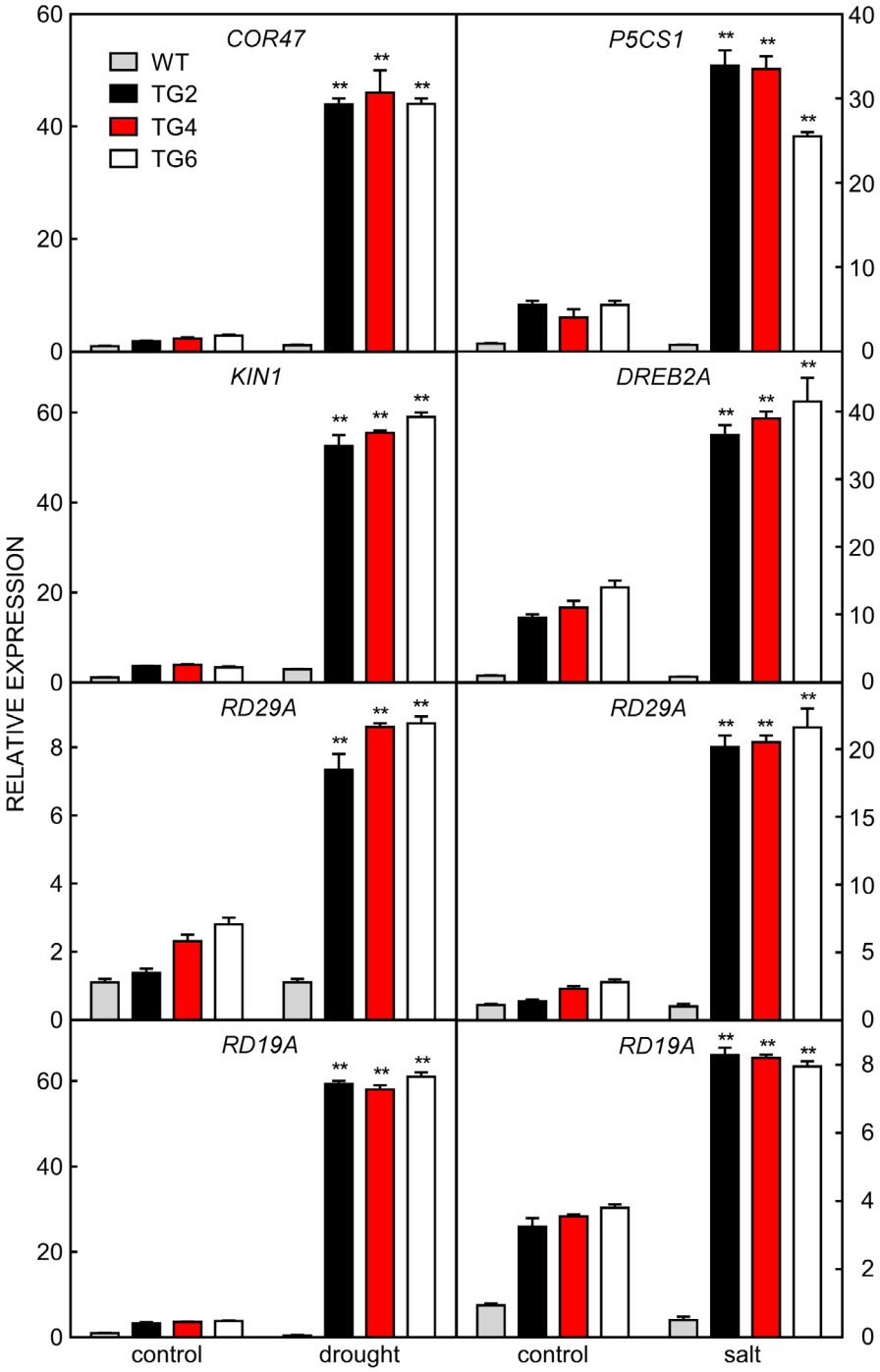

Fig. 7. The expressions of stress-related genes in *Arabidopsis* under drought and salt stress. WT - wild-type *Arabidopsis*, TG2, TG4 and TG6 - transgenic *Arabidopsis* lines. Means ± SDs, $n = 3$; ** indicates statistical significance compared to the control at $P < 0.01$.

the degree of damage to the plant cell membrane system under stress conditions. The amount of electrolyte leakage in *SiLEA14* transgenic foxtail millet plants is apparently lower than that of the WT lines under drought treatment. Furthermore, under salt and drought treatments, the proline content is enhanced in *SiLEA14* transgenic foxtail millet plants when compared to WT (Wang *et al*. 2014). Additionally, *ZmLEA3* overexpression causes lower MDA and relative electrolyte leakage in transgenic tobacco compared with WT plants under osmotic stress (Liu *et al*. 2013). In the present study, overexpressing *PgLEA* in *Arabidopsis* plants significantly stimulated the accumulation of proline under drought and salt stress. Furthermore, *PgLEA*-transgenic plants had significantly less MDA, and lower electrolyte leakage rates compared to the WT under drought and salt stress. The results suggested that *PgLEA* overexpression can minimize cell membrane damage in transgenic *Arabidopsis* plants.

Abiotic stress can cause an excessive accumulation of ROS, which can damage biological molecules and even result in cell death (Zhang *et al*. 2016). To protect membranes against damage, plants have evolved an antioxidant enzyme defence system, involving SOD, POD, and CAT (Miller *et al*. 2010, Wang *et al*. 2017). SOD converts superoxide radicals to H_2O_2. POD and CAT eliminate H_2O_2 by breaking it down to H_2O and O_2. They can effectively scavenge the increased amounts of toxic ROS that plants produce under abiotic stress (Asada *et al*. 2000, Tattini *et al*. 2015, Wang *et al*. 2018). In the current study, under drought and salt stress, *PgLEA*-overexpressing *Arabidopsis* plants accumulated a much lower amount of ROS, and showed higher SOD, POD, and CAT activity, compared to WT plants. These results suggested that the protective effect conferred by *PgLEA* might be mediated by enhanced antioxidant enzyme activities and ROS scavenging capacities to protect *Arabidopsis* plants from oxidative damage under drought/salt stress.

Drought and salt conditions induced the expression of genes related to stress resistance to improve plant resistance. Previous studies revealed that *RD19A*, *RD29A*, *COR47*, *KIN*, *DREB*, and *P5CS1* are inducible by salt or drought stress (Guo *et al*. 2014, Li *et al*. 2011). *DREB1A* acts as a positive regulator of drought tolerance (Li *et al*. 2020). Plants can respond to the external salinity stress *via* the expression of *P5CS* (Yamaguchi-Shinozaki 2001). In this study, under drought and salinity stress conditions, *RD19A, RD29A, KIN1,* and *COR47,* were significantly upregulated in *PgLEA*-overexpressing transgenic *Arabidopsis* plants compared with WT plants, indicating that *PgLEA* may mediate the activation of these stress-responsive genes. The *COR47, RD29A*, *KIN1*, and *RD19A* genes were significantly more highly expressed in transgenic *Arabidopsis* plants than in WT plants under drought conditions, suggesting that *PgLEA* regulates the expression o*f COR47, RD29A*, *KIN1*, and *RD19A* after exposure to drought stress. In response to salt stress, *RD19A*, *DREB2A*, *RD29A*, and *P5CS1* expressions increased in transgenic plants under saline conditions, suggesting that *PgLEA* is a regulatory factor affecting *RD19A, DREB2A, RD29A*, and *P5CS1* expression in response to salt stress. To our knowledge, *P5CS* is a regulatory enzyme that plays a crucial role in proline biosynthesis in plants (Kishor *et al*. 2014). The increased expression of *P5CS1* in transgenic plants in response to salt stress led to the accumulation of proline. Accordingly, *PgLEA* upregulated the expression of multiple stress-responsive genes and improved the adaptation of transgenic *Arabidopsis* plants to abiotic stress.

Conclusions

There is considerable interest in stress-responsive genes among ginseng researchers. A late embryogenesis abundant protein, *PgLEA*, was isolated from *Panax ginseng* herein. *PgLEA* revealed inducible expression patterns in response to drought and salt stresses. Overexpression of *PgLEA* increased tolerance to salt and drought stress in *Arabidopsis thaliana.* The *PgLEA* transgenic plants accumulated more proline and suffered less membrane damage compared to the WT plants under drought and salt stresses. *PgLEA* overexpression in transgenic *Arabidopsis* plants resulted in higher activities of ROS-scavenging enzymes (SOD, POD, and CAT). Moreover, *PgLEA* overexpression might regulate the expression of stress-responsive genes in plants. Our findings suggest that *PgLEA* possesses pleiotropic effects on transgenic plants and serves as a ROS scavenger, resulting in improved plant tolerance to drought and salinity stress. Our results provided a theoretical and experimental basis for *PgLEA* function in improving the tolerance ability of plants under multiple abiotic stressors, making it a promising candidate gene for increasing ginseng stress resistance through breeding.

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