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Overexpression of *Late Embryogenesis Abundant 14* enhances *Arabidopsis* salt stress tolerance

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ABSTRACT

Late embryogenesis abundant (LEA) proteins are implicated in various abiotic stresses in higher plants. In this study, we identified a LEA protein from *Arabidopsis thaliana*, AtLEA14, which was ubiquitously expressed in different tissues and remarkably induced with increased duration of salt treatment. Subcellular distribution analysis demonstrated that AtLEA14 was mainly localized in the cytoplasm. Transgenic *Arabidopsis* and yeast overexpressing *AtLEA14* all exhibited enhanced tolerance to high salinity. The transcripts of salt stress-responsive marker genes (*COR15a*, *KIN1*, *RD29B* and *ERD10*) were overactivated in *AtLEA14* overexpressing lines compared with those in wild type plants under normal or salt stress conditions. In vivo and in vitro analysis showed that AtLEA14 could effectively stabilize AtPP2-B11, an important E3 ligase. These results suggested that AtLEA14 had important protective functions under salt stress conditions in *Arabidopsis*.

1. Introduction

Soil salinity, one of the serious abiotic stresses, affects approximately 20% of the world's arable land and 40% of the irrigated land at different degrees [1]. To survive, plants have evolved diverse and elaborate mechanisms to protect themselves from salt stress through a series of physiological and morphological changes, such as the Salt Overly Sensitive (SOS) regulatory pathway, the mitogen-activated protein kinase (MAPK) cascade, the different categories of ion channels and the biosynthesis of products that can alleviate salt stress responses including antioxidants, chaperones, and late embryogenesis abundant (LEA) proteins [2–5].

Diverse groups of LEA proteins usually accumulate to high levels during the late stage of embryo development, which are also the universal products involved in the response to environmental stresses by stabilizing proteins, nucleic acids, cell membranes, and redox balance [6–8]. Consistent with their functions, the expression of most LEA genes can be apparently induced by various abiotic stresses, and the biological functions of plant LEA proteins have been well illustrated. Heterologous expression of *HVA1*, a LEA protein gene from barley, can enhance the drought and salt stress tolerance of transgenic wheat and rice [9,10]. Tomato *LE25* increases the salt and chilling stress tolerance when overexpressed in yeast [11]. Transgenic *Arabidopsis* plants overexpressing *NtLEA7-3* are much more resistant to cold, drought, and salt stresses [12]. In addition, other LEA proteins, such as PsLEAM from pea, IbLEA14 from sweet potato, D-19 from cotton and Em from wheat, also enhance abiotic stress tolerance [6,13–15].

In *Arabidopsis* genome, 51 LEA proteins have been identified and classified into nine distinct groups, namely, dehydrin, LEA_1, LEA_2, LEA_3, LEA_4, LEA_5, PvLEA18, SMP, and AtM [16]. Among the 51 genes, 22 members (43%) showed high expression levels in the non-seed organs, and most *LEAs* were induced under stress or hormone treatment, such as drought, high salinity, cold, and ABA [16]. The functions of different *Arabidopsis* LEA proteins have been studied. For example, it has been reported that Early Responsive to Dehydration (ERD) 10 and ERD14 of *Arabidopsis*, members of the dehydrin family, may function as chaperones under abiotic stresses [17]. *AtLEA4-5* is induced by various abiotic stresses, and its overexpressing lines show higher tolerance to severe drought compared with wild type plants [18,19]. The *Arabidopsis* LEA protein AtEM6, which is subgrouped to LEA_5, is required for normal seed development, and loss of *AtEM6* resulted in the premature

Abbreviations: LEA14, late embryogenesis abundant 14; GUS, β-glucuronidase; GFP, green fluorescent protein.

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dehydration of the distal end of siliques [20]. Constitutive expression of the cold-regulated gene *COR15a* enhances the freezing tolerance of chloroplast and protoplast [21]. However, the functional details of other *Arabidopsis LEA* members remain unclear.

In the current study, we explored *LEA14*, which belongs to the *Arabidopsis* LEA_2 subgroup. Compared with other dynamically disordered LEAs, this subgroup, particularly, AtLEA14, displays a stable three-dimensional structure [22]. Additionally, AtLEA14 contains significantly high numbers of hydrophobic residues, and it is likely to function differently from other LEA proteins that are highly hydrophilic [16]. However, the biological function of AtLEA14 remains unknown. Our results showed that *AtLEA14* could be dramatically induced by salt stress, and its overexpression enhanced the salt stress tolerance of transgenic *Arabidopsis* and yeast. Accumulation of AtLEA14 also upregulated the expression of abiotic stress-responsive genes, and stabilized the protein level of AtPP2-B11. These data suggested that *AtLEA14* was a valuable candidate for plant genetic improvement in the future.

2. Materials and methods

2.1. Plant materials and growth conditions

Arabidopsis seeds of each genotype from Columbia (Col-0) background were harvested at the same time from plants grown under the same condition. The sterile seeds were plated on 1/2 Murashige and Skoog (MS) medium containing 1% (w/v) sucrose. The seed-dotted plates were maintained in the dark at 4 °C for 3 d and then transferred to a growth chamber with 16-h-light/8-h-dark cycles at 22 °C.

2.2. Vector construction and plant transformation

To construct *35S::AtLEA14*, the *AtLEA14* coding sequence was amplified using Col-0 cDNA by PCR with gene specific primers (Table S1). The PCR products were cloned into pBI121 under the control of a cauliflower mosaic virus 35S promoter. A 1000 bp sequence upstream of ATG was amplified from genomic DNA to construct *pLEA14::GUS*. The PCR fragment was cloned into the *Hind*III and *Bam*HI sites of pBI121.

The transformation of *Arabidopsis* plants was performed by floral dip using *Agrobacterium tumefaciens* strain GV3101. Homozygous T3 lines were used for phenotypic analysis.

2.3. Histochemical GUS staining and GUS activity analysis

The *pLEA14::GUS* transgenic lines were incubated overnight at 37 °C in solution containing 1 mg/mL 5-bromo-4-chloro-3-indolyl-glucuronic acid, 5 mM potassium ferrocyanide, 0.03% Triton X-100 and 0.1 M sodium phosphate buffer (pH7.0). Then the plants were cleaned with 70% ethanol and pictures were taken by stereoscope. Plant protein extraction and analysis for GUS activity were performed as previously described [23]. GUS activity was obtained from at least five independent transformants, and each assay was repeated three times.

2.4. Seed germination assay

Plants of different genotypes were grown in the same conditions, and mature seeds were collected at the same time. Seeds were planted on the same plate containing 1/2 MS medium with different concentrations of NaCl (150 mM and 200 mM). Germination was defined as an obvious emergence of the radicle through the seed coat. Germination assays were carried out with three replicates of 100 seeds.

2.5. RNA extraction and real time RT-PCR analysis

Total RNA was isolated from *Arabidopsis thaliana* seedlings using a universal plant total RNA extraction kit (BioTeke, Beijing, China). cDNA was synthesized using PrimeScript reverse transcriptase with oligo dT primer using the PrimeScript RT master mix kit (Takara). A SYBR green real-time PCR master mix (Takara) and a Chromo 4 real-time PCR detector (Bio-Rad) were used. Real time

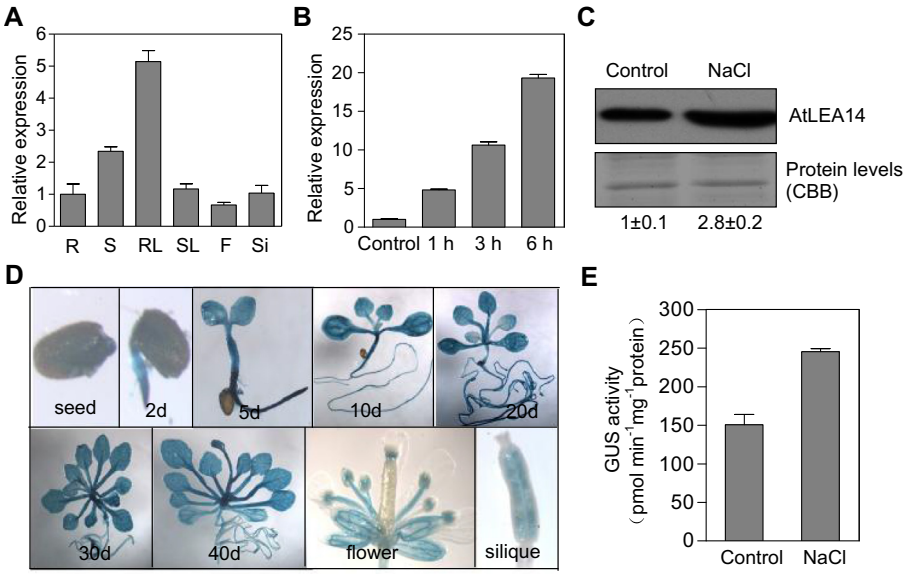

Fig. 1. The expression patterns of *AtLEA14*. (A) Transcript accumulation patterns of *AtLEA14* in different tissues. R: root; S: stem; RL: rosette leaf; SL: stem leaf; F: flower; Si: silique. AtLEA14 transcript (B) and protein (C) accumulation levels upon salt stress. Total RNA or protein from the 2-week-old plants were extracted and analyzed by real time RT-PCR (200 mM NaCl, 0 h, 1 h, 3 h and 6 h) and Western blot (200 mM NaCl, 3 h). Mean values of real time RT-PCR from three biological replicates were normalized to the levels of an internal control, *GAPDH*. Error bar indicates SD ($n = 3$). (D) GUS staining of the *pAtLEA14::GUS* transgenic lines at different developmental stages. (E) GUS activity of the leaves in 4-week-old *pAtLEA14::GUS* transgenic lines treated with or without 200 mM NaCl for 6 h.

RT-PCR experiments were performed three biological replicates under similar conditions with *GAPDH* as an internal control.

2.6. Subcellular localization analysis

The *35S::AtLEA14-GFP* and *35S::GFP* plasmids were constructed using pJIT163 transient expression vectors. The *AtLEA14* coding sequence without the stop codon was amplified using Col-0 cDNA by PCR with gene specific primers (Table S1). The *35S::AtLEA14-GFP* and *35S::GFP* plasmids were transformed into wild type *Arabidopsis* protoplasts by the PEG method [24]. The GFP signal was monitored 12 h after transformation using a laser confocal microscope (Zeiss, LSM 510).

2.7. Construction and generation of transgenic yeast

AtLEA14 was cloned into the pYES2 vector and transformed into the gold yeast cells according to the manufacturer's instruction (Clontech). The yeast transformed with pYES2 empty vector was used as a control. Transgenic yeast cells were planted on the YPD solid (2% galactose, 1% yeast extract, 2% peptone, 1.5% agar) or liquid medium containing different concentrations of NaCl (500 mM and 800 mM) with the gradient dilution. Photos were taken after 48 h at 30 °C. The absorbance of the stock cultures was determined at 600 nm after grown for an additional 48 h at 30 °C. Each assay was repeated three times.

2.8. Expression and purification of AtLEA14

AtLEA14 was cloned into pET30a and the recombinant plasmid was transformed into *E. coli* BL21 competent cells. When OD_{600} reached 0.4–0.6, isopropyl-β-D-thiogalactoside was added to LB culture to the final concentration of 1 mM. After incubation at 16 °C for 8 h, bacterial cells were harvested and recombinant AtLEA14 was purified using 6 × His-Tagged Protein Purification Kit (CWBIO).

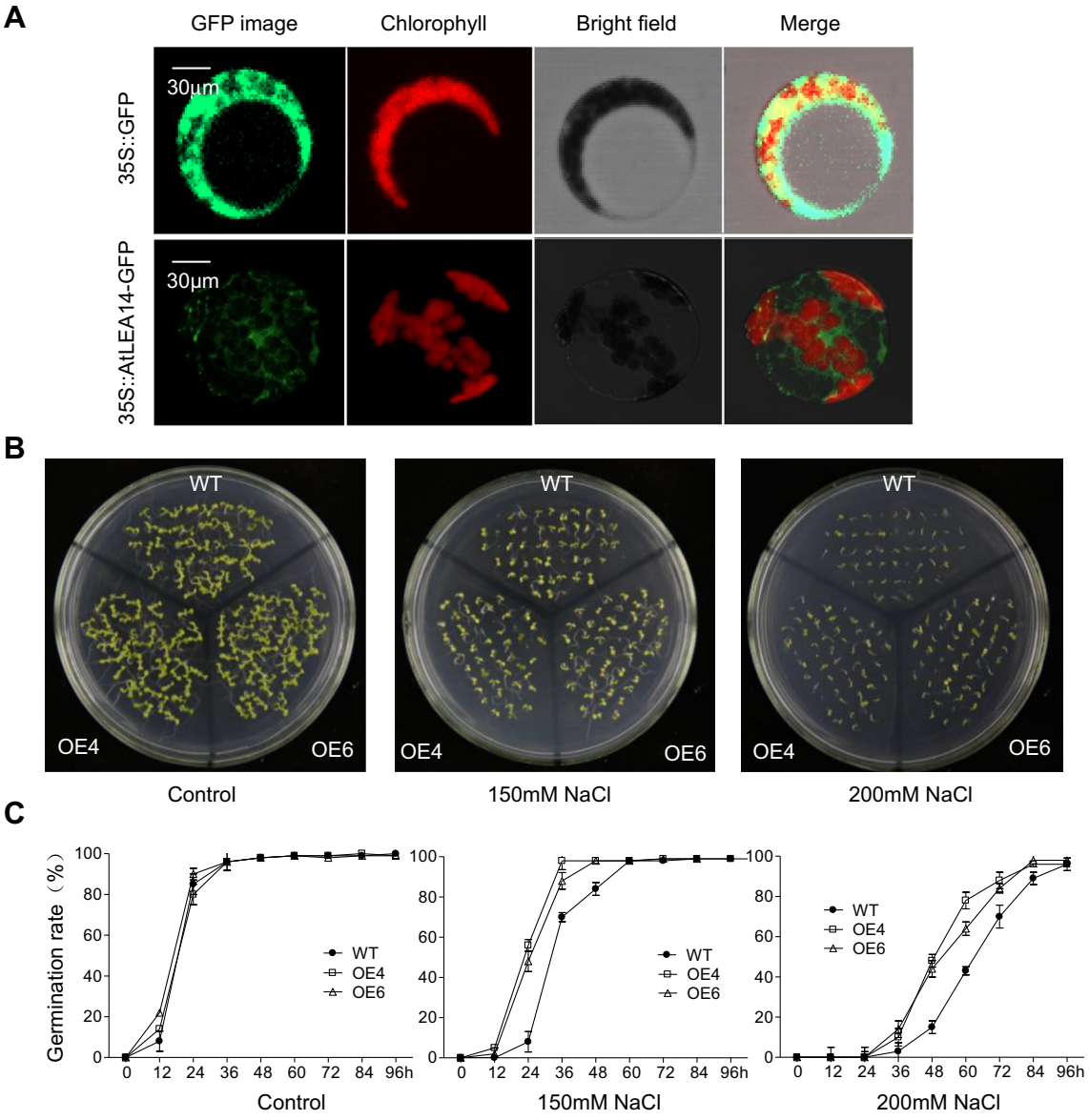

Fig. 2. Subcellular localization of AtLEA14, and phenotypic studies of wild type and *AtLEA14* overexpressing lines at germination stage. (A). Subcellular localization of *35S::GFP* and *35S::AtLEA14-GFP* constructs in *Arabidopsis* leaf protoplasts. (B) Salt response of wild type and *35S::AtLEA14* transgenic lines at germination stage. (C) Seed germination rates of wild type and transgenic plants grown on 1/2 MS medium containing 150 mM and 200 mM NaCl. Data showed the mean ± SD of at least three biological replicates. 100 seeds per genotype were counted in each replicate.

2.9. Immunoblot assays

The immunoblot assays were performed as described by Li et al. [25]. Coomassie Brilliant Blue R250 staining was used to show protein loading levels, and the protein levels were quantified using ImageJ software.

3. Results

3.1. Expression pattern analysis of AtLEA14

To reveal the expression patterns of *AtLEA14*, total RNA was extracted from root, stem, rosette leaf, stem leaf, flower, and silique, and then analyzed by real time RT-PCR. The results showed that *AtLEA14* was present in different organs, and relatively higher levels were observed in stem and rosette leaf (Fig. 1A). Hundertmark and Hincha have demonstrated that *AtLEA14* could be induced by cold, drought, high light, salt, heat, and mildew [16]. Since our research interests are mainly focused on salt stress, we then examined the effect of salt stress on the transcript accumulation of *AtLEA14*. As shown in Fig. 1B, *AtLEA14* was obviously induced by salt stress. Consistent with this finding, an increase of AtLEA14 protein was also observed after NaCl treatment (Fig. 1C).

Histochemical GUS staining revealed that *AtLEA14* was expressed in all tissues (Fig. 1D). Quantitative GUS activity assay of *pAtLEA14::GUS* transgenic lines also displayed a nearly 1.7-fold increase under salt stress compared with the control, which was consistent with the results of real time RT-PCR and Western blot results (Fig. 1E). Overall, these results suggested that AtLEA14 was ubiquitous and involved in the salt stress responses.

3.2. Subcellular localization of AtLEA14

The protein distribution pattern is closely correlated with its biological functions. To examine the subcellular localization, GFP was fused in frame to the C-terminus of AtLEA14. Plasmids containing *35S::AtLEA14-GFP* were transformed into *Arabidopsis* leaf protoplasts using a polyethylene glycol-mediated method [24] and then examined. As shown in Fig. 2A, confocal microscopy revealed a strong fluorescence signal in the cytoplasm attributed to the AtLEA14-GFP fusion protein compared with the localization pattern of the control (free GFP), indicating that AtLEA14 mainly functioned in the cytoplasm.

3.3. AtLEA14 enhances the salt tolerance of transgenic Arabidopsis

To investigate the biological functions of *AtLEA14* under salt stress, two independent transgenic lines, namely, OE4 and OE6, in which *AtLEA14* was highly expressed, were selected for further analysis. When the seed germination rate was assayed, no significant differences were found between the wild type and the *AtLEA14* overexpressing lines under normal growth conditions. However, when the transgenic and wild type seeds were assayed on NaCl containing medium, the *AtLEA14* overexpressing lines germinated faster than wild type (Fig. 2B). After 2 days of salt treatment with 200 mM NaCl, ~50% of the OE4 and OE6 seeds germinated, whereas only ~20% of wild type seeds germinated (Fig. 2C). We then examined the root length of wild type and transgenic lines under normal and salt stress conditions, respectively. After treatment for two weeks with 150 mM NaCl, the root length of wild type plants were significantly shorter than that of *AtLEA14* overexpressing plants, whereas no differences were observed

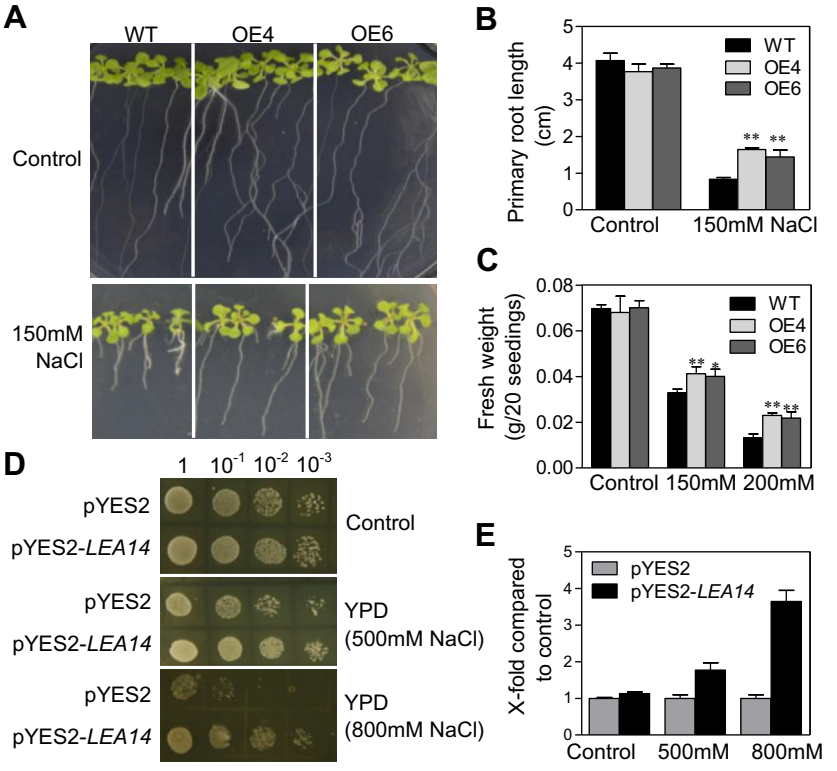

Fig. 3. Responses of *AtLEA14* overexpressing plants to salt stress at seedling stage, and roles of *AtLEA14* in yeast. (A) Photographs of 2-week-old wild type and transgenic plants grown on 1/2 MS agar plates with or without 150 mM NaCl. (B) The primary root length of 2-week-old plants on 1/2 MS medium containing different concentrations of NaCl. (C) The fresh weight of wild type and transgenic plants grown on 1/2 MS medium containing different concentrations of NaCl. *P*-value was determined by *t*-test ($^{*}P < 0.05$, $^{**}P < 0.01$). (D) Photographs of the yeast containing pYES2 empty vector or *AtLEA14* on YPD solid medium with different concentrations of NaCl. (E) Absorbance of the different stock cultures at 600 nm in YPD liquid medium containing different concentrations of NaCl.

when grown on 1/2 MS agar medium (Fig. 3A and B). In addition, the *AtLEA14* overexpressing lines yielded higher fresh weight than wild type plants under stress conditions (Fig. 3C). These findings suggested that transgenic lines overexpressing *AtLEA14* were more tolerant to salt stress than wild type plants.

3.4. Overexpression of ALEA14 enhances the salt tolerance in yeast

To further verify the effect of *AtLEA14* in response to salt stress, the pYES2-*AtLEA14* and pYES2 empty vector were transformed into yeast (Gold Yeast, Clontech). The positive and control transformants were spotted on the YPD solid medium with different concentrations of NaCl (500 mM and 800 mM), and grown for an additional 48 h at 30 °C. When salt stressed, the positive and control transformants all exhibited growth inhibition. However, the transgenic yeast harboring *AtLEA14* exhibited stronger tolerance to NaCl, especially to 800 mM NaCl (Fig. 3D). In order to confirm these results, the transgenic strains were inoculated to the fluid medium containing different concentrations of NaCl, and the absorbance of the stock cultures was determined 48 h later. We found the absorbance of yeast strain containing *AtLEA14* was much higher than that of the empty vector control (Fig. 3E). In summary, AtLEA14 could enhance the salt stress tolerance in yeast.

3.5. Overexpression of AtLEA14 increases the expression of salt-stress induced genes

Considering that *AtLEA14* overexpression lines altered the tolerance to high salinity, we determined whether other salt-responsive genes are also affected. The expression levels of *COR15a*, *KIN1*, *RD29B* and *ERD10* were investigated via real time RT-PCR analysis. As shown in Fig. 4A, even under normal growth conditions, a relatively higher expression level was observed for each examined gene in transgenic plants. These genes were all sharply induced after NaCl treatment, but the overexpressing line obviously accumulated more transcripts (Fig. 4A). Thus, these results suggest that AtLEA14 functioned in the salt stress response by indirectly regulating transcript accumulation of salt-inducible genes.

3.6. AtLEA14 affects the stability of its interacting protein AtPP2-B11

Given the protective nature of LEA proteins, we speculated that the increased salt stress tolerance of *AtLEA14* overexpressing lines was mostly attributed to the stabilization of important factor(s) via AtLEA14 binding. To validate our hypothesis, we examined the protein level of a well characterized AtLEA14 interacting protein AtPP2-B11, an important E3 ligase involved in *Arabidopsis* abiotic stress tolerance [25]. Western blot assay showed that OE4 and OE6 lines accumulated more AtPP2-B11 proteins than the wild type plants regardless of salt stress treatment (Fig. 4B). In order to exclude the possibility that the increased protein content was caused by higher mRNA levels, the expression of *AtPP2-B11* was detected in wild type and AtLEA14 overexpressing lines. As shown in Fig. 4C, the expression level of *AtPP2-B11* was indistinguishable between the wild type and transgenic plants under the same growth conditions. These results suggested that AtLEA14 could stabilize the protein level of AtPP2-B11 in vivo. To further confirm this result, a stricter in vitro test was carried out. AtLEA14 was

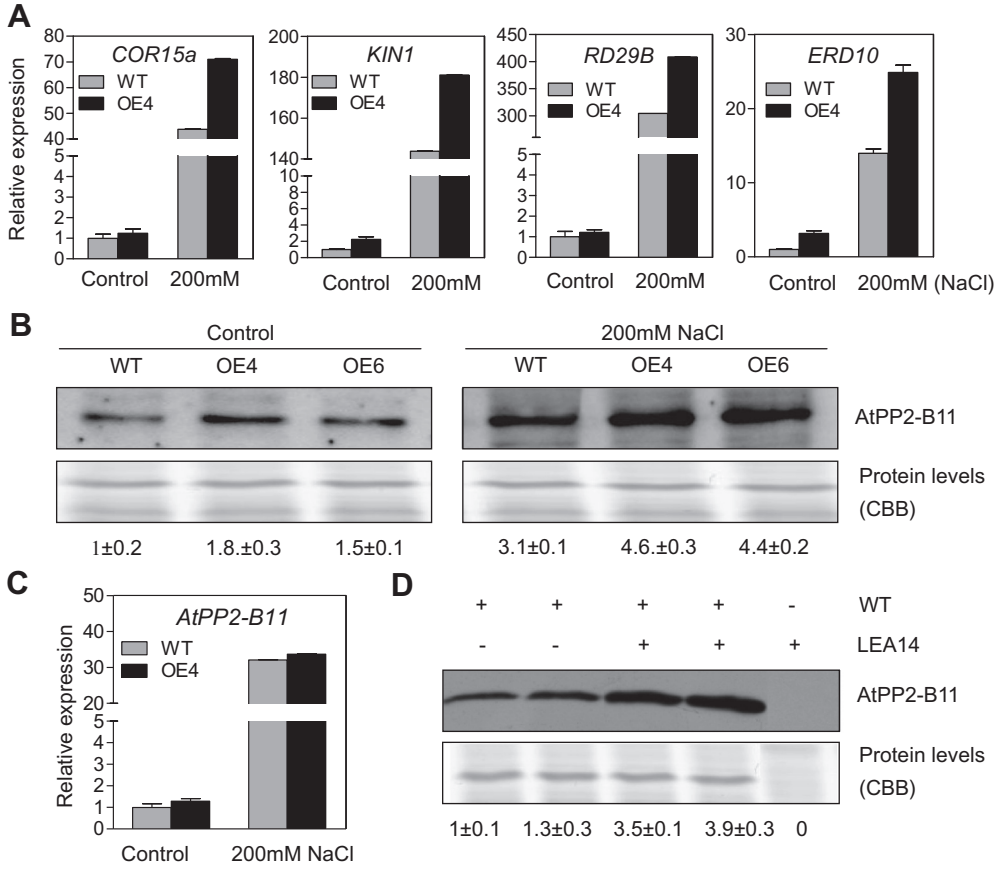

Fig. 4. Real time RT-PCR analysis of the salt inducible genes, and AtLEA14 stabilized its interaction protein AtPP2-B11. (A) Total RNA from 2-week-old plants was extracted and analyzed by real time RT-PCR. The graphs indicated the induction fold of the *COR15a*, *KIN1*, *RD29B* and *ERD10* in response to salt stress (200 mM NaCl for 6 h) as compared with control. (B) The protein levels of AtPP2-B11 in 2-week-old wild type and *AtLEA14* transgenic plants under normal and salt stress conditions (200 mM NaCl for 6 h). (C) The expression levels of *AtPP2-B11* in 2-week-old wild type and *AtLEA14* transgenic plants under normal and salt stress conditions (200 mM NaCl for 6 h). (D) AtLEA14 affected the protein stability of AtPP2-B11 in vitro. Error bar indicates SD ($n = 3$).

expressed and purified from *Escherichia coli*, and then added to the total protein extracted from wild type plants. The expressed AtLEA14 protein adding to buffer only (no plant extract added) served as a negative control. After incubation at 22 °C for 2 h, the protein level of AtPP2-B11 was determined. AtPP2-B11 became more stable when AtLEA14 was added, and less AtPP2-B11 protein was degraded during the course of incubation (Fig. 4D). Combined with the in vivo and in vitro results, we concluded that AtLEA14 could exert its roles by stabilizing its interacting protein(s) under normal or salt stress conditions.

4. Discussion

Higher plants have developed various mechanisms to adapt to everchanging environments at different levels, such as the expression and regulation of LEA proteins. *Arabidopsis LEA14* belongs to the LEA_2 group, which contains three homologues [16]. Intriguingly, these three proteins are unique as they are predicted to be relatively hydrophobic, except in the C-terminal region [16]. The LEA_2 group genes were modulated by adverse environmental conditions, and could modulate the stress tolerance under various environmental conditions [14,26,27]. Given the important function of LEA_2 proteins in abiotic stress response, we selected this function unknown *LEA14* from *Arabidopsis* for further investigation.

The expression patterns and subcellular localization analysis revealed that *AtLEA14* was ubiquitous in different organs and functioned mainly in the cytoplasm, suggesting unknown housekeeping roles of AtLEA14 in *Arabidopsis* development. Moreover, *AtLEA14* was strongly induced by salt stress, and its overexpression in *Arabidopsis* and yeast led to considerable increased tolerance to salt stress. These results clearly illustrated that LEA14 proteins were intimately involved in salt stress responses. As we mainly focused on salt stress, the biological functions of *AtLEA14* under other stress conditions, such as drought and temperature extremes, should be examined further. Next, we also aim to characterize the biological functions of the other two genes in *Arabidopsis* LEA_2 group, as well as the functional redundancy among these three members.

Previous studies reported that some LEAs could function as protectants and chaperones to stabilize the dehydration-sensitive proteins by forming specific and transient complexes with their client proteins [3,13,28]. We deduced that AtLEA14 conferred higher enzyme activity and/or longer lifespan of upstream transcription activator(s), which was responsible for the higher expression of salt-inducible genes and subsequent stress tolerance. Our previous study has demonstrated that AtLEA14 could interact with AtPP2-B11 and overexpression of *AtPP2-B11* suppressed AtLEA14 at both transcript and protein level when under drought stress conditions [25]. However, the regulation mechanism between *AtLEA14* and *AtPP2-B11* remained unknown under salt stress conditions. Based on the in vivo and in vitro analysis, we found that AtLEA14 could affect the protein stability of its interacting protein AtPP2-B11 under normal or salt stress conditions. To our knowledge, this is the first report on LEA protein, which exhibits protective activity to an E3 ligase. Compared with other LEAs that were natively unfolded, AtLEA14 was demonstrated to have defined secondary and tertiary structures in solution, implying a narrower spectrum of function. Therefore, it will be interesting to discover other unknown AtLEA14 interacting partners. As E3 ligases are important components in protein selective degradation, and salt stress inducible genes are overactivated in AtLEA14 overexpressing plants, we speculate that the stabilization of AtPP2-B11 proteins by AtLEA14 reinforces the elimination of negative regulators in salt stress responses. In agreement with this hypothesis, the *AtPP2-B11* overexpressing lines also exhibited higher salt tolerance. By identifying the interacting proteins of AtLEA14 and substrates of AtPP2-B11, we will see a more comprehensive picture of *AtLEA14* in modulating salt stress responses.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.10.136.

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