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OVEREXPRESSION OF *AtLEA3-3* CONFERS RESISTANCE TO COLD STRESS IN *Escherichia coli* AND PROVIDES ENHANCED OSMOTIC STRESS TOLERANCE AND ABA SENSITIVITY IN *Arabidopsis thaliana*

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Previous studies have shown that the late embryogenesis abundant (LEA) group 3 proteins significantly respond to changes in environmental conditions. However, reports that demonstrate their biological role, especially in *Arabidopsis*, are notably limited. This study examines the functional roles of the *Arabidopsis* LEA group 3 proteins AtLEA3-3 and AtLEA3-4 in abiotic stress and ABA treatments. Expression of *AtLEA3-3* and *AtLEA3-4* is upregulated by ABA, high salinity, and osmotic stress. Results on the ectopic expression of *AtLEA3-3* and *AtLEA3-4* in *E. coli* suggest that both proteins play important roles in resistance to cold stress. Overexpression of *AtLEA3-3* in *Arabidopsis* (*AtLEA3-3-OE*) confers salt and osmotic stress tolerance that is characterized during germination and early seedling establishment. However, *AtLEA3-3-OE* lines show sensitivity to ABA treatment during early seedling development. These results suggest that accumulation of *AtLEA3-3* mRNA and/or proteins may help heterologous ABA re-initiate second dormancy during seedling establishment. Analysis of yellow fluorescent fusion proteins localization shows that *AtLEA3-3* and *AtLEA3-4* are mainly distributed in the ER and that *AtLEA3-3* also localizes in the nucleus, and in response to salt, mannitol, cold, or BFA treatments, the localization of *AtLEA3-3* and *AtLEA3-4* is altered and becomes more condensed. Protein translocalization may be a positive and effective strategy for responding to abiotic stresses. Taken together, these results suggest that *AtLEA3-3* has an important function during seed germination and seedling development of *Arabidopsis* under abiotic stress conditions.

***Keywords*: ABA, *AtLEA3-3*, osmotic stress, salt stress, seedling establishment, transgenic plant.**

INTRODUCTION

Abiotic stresses, such as drought, high salinity, and cold can considerably limit the growth and yield of crops. In response to such environmental stresses, plants have developed various physiological and biochemical strategies to minimize the possible damages caused by water deficits. These strategies generally include ion sequestration and accumulation of sugars and compatible, low-molecular-weight osmolytes, scavengers of reactive oxygen, small heat shock proteins, and putative osmoprotective proteins, such as the late embryogenesis abundant (LEA) proteins [1, 2].

LEA proteins were first identified 29 years ago, and shown to be accumulated to high levels during the maturation phase of cotton (*Gossypium hirsutum*) embryogenesis [3]. LEA and LEA-like proteins or their genes were subsequently found in the seeds, as well as in the vegetative organs, of many plants, especially when these plants were subjected to stressful conditions, such as cold, heat, salt, osmotic stress, or exogenous hormone applications [1, 2, 4]. LEA proteins have been classified into at least seven groups on the basis of their similarities in sequence or peptide compositions [4–7]. LEA group 3 members, for example, are characterized by an 11 amino acid motif, and are categorized into two subgroups, namely 3A (D-7 related proteins) and 3B (D-29 related proteins) [4, 6].

A unifying and outstanding feature of most of the LEA group 3 proteins of higher plants is their high hydrophilicity. Also, the 11-mer is able to form mainly amphipathic α-helices, which may dimerize into an unusual right-hand coiled-coil arrangement with a periodicity defined by the 11-mer motif [4, 5, 8]. Most group 3 LEA proteins are natively unfolded in solution. However, they adopt the α-helical conformation in the presence of sucrose and glycerol, or after fast drying [7–12]. After rehydration, the desiccated LEA proteins become unstructured, indicating that the

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structural transitions are fully reversible [12]. Hence, group 3 proteins possess properties that allow them to form an α-helical structure, and this structure is believed to be related to their cellular function during water limitation [13].

For the last two decades, the functions of group 3 LEA proteins have been extensively studied using different approaches. They were predicted to contribute to counteracting the damage brought about by water limitations. A variety of mechanisms has been proposed, including hydration buffering, ion sequestration, renaturation of unfolded proteins, or direct protection of other proteins or membranes [1, 4, 13–15]. Although the actual functional mechanism of LEA proteins remains elusive, *in vitro* studies have shown that LEA proteins help prevent aggregation, as well as protect the enzymes against inactivation during desiccation stress [16–21]. Other studies have suggested that LEA proteins act as a "molecular shield" to help prevent protein aggregation during water deficits [17] or help preserve membrane integrity [12]. Meanwhile, several groups have used organisms other than plants to test LEA protein functions. For example, expression of the wheat *TaLEA3* and *TaLEA2* in yeast improved hyperosmotic stress, salt, and freezing tolerance [22]. Overexpression of barley HVA1 proteins and soybean PM2 in yeast and bacteria, respectively, also conferred tolerance to salt stress [23, 24]. Recently, gain-of-function experiments in different plant species further reinforced the belief that group 3 LEA proteins are responsible for stress tolerance. Constitutive expression of barley *HVA1* in rice and wheat exhibited improved responses to water deficits [25, 26], and cold-acclimatized *Arabidopsis* plants expressing a wheat chloroplast LEA showed a significant increase in freezing tolerance [27].

All of the evidence obtained from gain-of-function experiments in different species indicates at the protection function of group 3 LEA proteins upon water deficit. However, few reports describing loss-of-function experiments addressing the function of group 3 proteins in plant and other organisms are available. In *Arabidopsis*, for example, more than 51 LEA or LEA-related genes can be found in the genome [6, 7]. Few reports uncover the function of group 3 LEA proteins based on genetic evidence, and most of the available information on the function of this group in stress conditions is obtained from transcriptomic projects and expression analysis. Thus, in *Arabidopsis*, more work needs to be done to elucidate the precise roles of LEA proteins under stress conditions.

In this study, two homologous genes, *At1g52690* and *At3g15670*, were selected to determine their roles during plant development. According to the nomenclature of Bies-Ethe've et al. [6], these genes are named *AtLEA3-3* and *AtLEA3-4*, respectively. Gene chip analysis revealed that transcripts expression was induced by dehydration, salinity, low temperatures, or ABA. The genes are regulated by transcriptional factors or other proteins, such as DREB 2A (Dehydration-Responsive Element Binding protein), HY5 (LONG HYPOCOTYL5), Zat12 (zinc-finger protein), and DELLA proteins (GA signaling repressors) [28–34]. *AtLEA3-3* and *AtLEA3-4* proteins belong to the LEA_4 cluster and are hydrophilic and stable in nature. The secondary structures of both proteins contain predominant helical blocks packed into compact globules. To further explore the functions of *AtLEA3-3* and *AtLEA3-4* in plants subjected to abiotic stresses, the survival of *E. coli* expressing *AtLEA3-3* and *AtLEA3-4* under high salt, osmotic or cold stress, as well as seed germination and early seedling development of *AtLEA3-3-OE* lines under high salinity conditions, osmotic stress or ABA treatments, were analyzed. It is evident that *AtLEA3-3* plays a positive role during seed germination and early seedling establishment under salt or osmotic stress conditions. Also, the overexpression of *AtLEA3-3* may intensify the process of ABA-induced secondary dormancy during early seedling development.

EXPERIMENTAL

Plant materials and treatments. Columbian ecotype of *Arabidopsis thaliana* was used as the wild type (WT) plant in all of the experiments. Seeds were placed on Murashige and Skoog (MS) medium and were synchronized for 3 days at 4°C before being placed under LD (16 h light/8 h dark) conditions for 7 days. The seedlings were then treated with H_2O, 10 μM ABA, 100 μM ABA, 150 mM NaCl, 200 mM NaCl, or 400 mM Mannitol. The seedlings were harvested after 3 h and stored at –80°C for subsequent isolation of RNA. All of the treatments were repeated in three independent experiments.

RNA extraction and RT-PCR analysis. Total RNA was extracted from the frozen tissues using an RNeasy kit (RNAiso™-mate for Plant Tissue, "TaKaRa") and treated with RNase-free DNase I ("TaKaRa") according to the manufacturer's instructions. Using the RevertAid-™ First Strand cDNA Synthesis Kit ("Fermentas"), 0.5–1 μg of total RNA was reverse-transcribed. Semi-qRT-PCR was performed according to Bies-Ethe've et al. [6]. For quantitative RT-PCR, 1 μL of 3–5 times diluted cDNA was used in a total reaction volume of 20 μL. Quantitative RT-PCR was also performed with Rotor-gene 3000 with SYBR® Premix Ex Taq™ II (Perfect Real Time, "TaKaRa"). The final primer concentration was 0.4 μM. All primers used are listed in the Supplemental Table S1 (http://www.molecbio.com/downloads/2011/5/supp_pengshanzhao_en.pdf) which may be found online. The reaction protocol was as follows: Stage 1 – pre-denaturalization at 95°C for 30 s, 1 cycle; Stage 2 – PCR reaction at 95°C for 10 s, 56°C for 20 s, 72°C for 20 s, 35–40 cycles; Stage 3 – melting curve analysis, ramp from 72 to 95°C, raising by 1°C at each step while

Rosette leaf characteristics of the wild type, vector line and *AtLEA3-3-OE* line plants grown in LD at 32DAS

Measurement[a]	5th Rosette Leaf			6th Rosette Leaf		
	WT	*vector*	*AtLEA3-3-OE*	*WT*	*vector*	*AtLEA3-3-OE*
Length of leaf petiole, cm	1.08 ± 0.25	1.11 ± 0.21	1.27 ± 0.19**	1.11 ± 0.26	1.08 ± 0.22	1.26 ± 0.17**
Length of leaf blade, cm	1.40 ± 0.22	1.47 ± 0.29	1.53 ± 0.25*	1.34 ± 0.27	1.47 ± 0.21	1.58 ± 0.24**
Width of leaf blade, cm	0.73 ± 0.11	0.78 ± 0.12	0.80 ± 0.08**	0.7 ± 0.10	0.76 ± 0.14	0.81 ± 0.10**
Length/width ratio	1.92 ± 0.27	1.89 ± 0.31	1.90 ± 0.22	1.94 ± 0.37	1.99 ± 0.39	1.96 ± 0.27

[a] Measurements taken from the fifth and sixth rosette leaf. Results shown as the average ±SE. $n \geq 22$.

* And ** indicate statistical significance of the difference in comparison with the wild-type plants at $P < 0.05$ and $P < 0.01$, respectively.

waiting for 30 s after the first step, followed by waiting for 5 s after each subsequent step. The efficiency of amplification of various cDNA fragmnets was assessed relative to the amplification of transcripts from *actin*2 (*At3g18780*). Each RNA sample was assayed in triplicates. The results were analyzed by relative quantitative analysis using Rotor-Gene Real-Time Analysis Software 6.1.

Plasmid construction. The full-length open reading frames (ORFs) U12613 and U10209, corresponding to *AtLEA3-3* and *AtLEA3-4* respectively, were ordered from the Arabidopsis Biological Resource Center (ABRC). The cDNA of *AtLEA3-3* and *AtLEA3-4* was amplified were high fidelity polymerase PrimeSTAR™ ("TaKaRa") using gene specific primers (Table S1). The sequences of the resulting amplification products were then verified. After digestion by SalI/SmaI for *AtLEA3-3* and SalI/SacI for *AtLEA3-4*, the fragments were introduced into the same sites of the transient expression vector pA7-CFP/YFP and the fusion of CFP-AtLEA3-3/AtLEA3-4 or AtLEA3-3/AtLEA3-4-YFP was controlled by the cauliflower mosaic virus (CaMV) 35S promoter. To determine prokaryotic expression, the amplicons of *AtLEA3-3* and *AtLEA3-4* were restricted with SalI/SmaI, and then cloned into pGEX-4T-1 to obtain recombinant pGEX-4T-1-*AtLEA3-3/AtLEA3-4*. These were then introduced into the *E. coli* strain BL-21. To construct the binary vectors, the amplicons of *AtLEA3-3* and *AtLEA3-4* were digested by BamHI and HindIII, and were cloned into the modified binary vector p1307-2 × *35S*:GFP. The recombinant vectors containing *GFP-AtLEA3-3/AtLEA3-4* were subsequently transformed into *Agrobacterium* GV3101 by the freeze-thaw method. The plasmids of pVKHEn6-GFP-HDEL were provided by Ian Moore from the University of Oxford [35].

Protein expression and stress tolerance assays in *E. coli*. Prokaryotic expression in *E. coli* BL-21 cells was carried out according to Dalal et al. [36], except that two inducing times (1 and 4 h) were used. For the stress tolerance assays, aliquots from IPTG-induced cultures were diluted consecutively and plated with LB medium containing 500 and 800 mM NaCl and 7% sorbitol at 37°C. All assays were performed in triplicates. After incubating for 12–18 h at 37°C, the number of colonies was counted, and the amount of colony-forming units (cfu) was calculated. For cold treatments, aliquots from IPTG-induced cultures were exposed to cold stress (4°C for 24 h and –20°C for 1 h). In addition, cell viability was measured as described earlier. For all treatments, the mean values of the three experiments were determined from three parallel transformants with an empty vector and pGEX-4T-1-*AtLEA3-3/AtLEA3-4* constructs.

Constitutive expression of *AtLEA3-3* in *Arabidopsis*. *Arabidopsis* transformation was carried out using the floral dip method. Transgenic plants were obtained by screening successive generations in terms of hygromycin resistance and green fluorescence.

To evaluate the stress tolerance of *AtLEA3-3-OE* lines at the germination stage, about 50 surface-sterilized seeds from WT and T4 generation transgenic plants each were planted in triplicates on MS medium. The medium contained 1% (w/v) sucrose and 1% agar, pH 5.8, supplemented with or without different concentrations of NaCl, mannitol or ABA as indicated. The seeds were incubated at 4°C for 3 days before being placed at 22°C under 16 h light/8 h dark (LD) conditions. The germination rates (emergence of radicals) and seedling establishment (opened green cotyledons) were then scored everyday for 7 days.

For root elongation measurements, 4-day old seedlings were transferred from the germination medium and placed with root tips pointing upward or downward on agar plates (placed vertically) supplemented with different concentrations of NaCl or mannitol. After 7 days of growth in the treated media, the root lengths and numbers of lateral roots of the seedlings were determined for the upward and downward oriented seedlings respectively. Each plate contained six *AtLEA3-3-OE* and six WT seedlings. Three replicate plates were used for each treatment.

For the water-loss analysis, five rosette leaves from 35 days-after-sowing (DAS) WT and *AtLEA3-3-OE* plants were detached and weighed at different times to determine the rate of water loss. This particular experiment was carried out at least three times.

Isolation of *Arabidopsis* mesophyll protoplasts and confocal imaging. *Arabidopsis* mesophyll protoplasts were isolated from the rosette leaves of 4-week-old plants as described by Yoo et al. [37]. Plasmids were purified using Qiagen columns according to the manufacturer's protocol. The fusion constructs were introduced into *Arabidopsis* protoplasts by polyethylene glycol (PEG)-mediated transformation and then protein expression was monitored with a Zeiss LSM 510 META laser-scanning fluorescence microscope.

To detect different fluorescence in *Arabidopsis* protoplasts, a Plan-Neofluor 40x/0.75 lens was used at a 2.0 scan zoom. The detector gain was set to avoid saturated pixels in images of fluorescence proteins expressing protoplasts and the data was acquired from the approximate center of the cell. For simultaneous imaging of YFP and chlorophyll autofluorescence in *Arabidopsis* protoplasts, a line-sequential multitrack configuration with 514 nm excitation and a BP 535–590 IR filter for YFP, and a 488 nm excitation from a He-Ne laser and an LP 560 filter for chlorophyll autofluorescence was used. To detect CFP together with chlorophyll autofluorescence, 458 nm excitation and a BP 480–520 IR filter for CFP and a LP 560 filter for chlorophyll autofluorescence were used. To detect GFP together with chlorophyll autofluorescence, 488 nm excitation and a BP 500–550 IR filter for GFP and a LP 560 filter for chlorophyll autofluorescence were used. For GFP and YFP colocalization experiments, GFP and YFP fluorescence was analyzed using the 458 and 514 nm excitation lines from a 25 mW argon laser with the line-sequential multitrack scanning mode of the Zeiss LSM510 microscope. Fluorescence was detected using a 458/514 nm primary dichroic, a 490 nm secondary dichroic, a BP 500-530 IR filter for GFP, and a BP 535–590 IR filter for YFP.

Statistical analysis. Each experiment was repeated at least three times. All values are expressed as mean ± SE. All comparisons were done using the Student's t-test for independent samples.

RESULTS

Expression of AtLEA3-3 and AtLEA3-4 is induced by ABA, high salinity, and osmotic stress

To investigate the regulation of *AtLEA3-3* and *AtLEA3-4* expression under different types of stress, 7-day old seedlings were treated with H_2O, 10 μM ABA, 100 μM ABA, 150 mM NaCl, 200 mM NaCl, and/or 400 mM Mannitol for 3 h. The transcript levels of *AtLEA3-3* and *AtLEA3-4* were analyzed by real-time PCR assay (fig. 1*a*,*b*). Compared to the control, the mRNA of *AtLEA3-3* and *AtLEA3-4* were accumulated dramatically under treatments. The maximal accumulations of *AtLEA3-3* and *AtLEA3-4* mRNA were observed after treatment with 200 mM NaCl.

Ectopic expression of AtLEA3-3 and AtLEA3-4 enhances the viability of E. coli under cold conditions

To evaluate the possible function of *AtLEA3-3* and *AtLEA3-4 in vivo* and under stressful conditions, the *AtLEA3-3* and *AtLEA3-4* coding sequences were overexpressed using pGEX-4T-1 and confirmed by SDS-PAGE (fig. S1; Supplemental – http://www.molecbio.com/downloads/2011/5/supp_pengshanzhao_en.pdf). The molecular mass of each of the expressed proteins was about 40 and 50 kDa, respectively, which was consistent with the predicted molecular masses of 18.1 and 24.19 kDa. Empty vectors were introduced into *E. coli* cells as a control. Similar growth rates were observed for test and control recombinant *E. coli* under normal conditions. Since there was no significant difference in protein quantity between 1 and 4 h inducement time, 1 h IPTG inducing time was used in the other stress tolerance assays.

The effects of high salt concentrations, osmotic, and cold stress on the survival of recombinant *E. coli* cells were measured by counting the colony-forming units (cfu). As shown in fig. 1*c*,*d*, there was no significant difference between the control cells and the recombinant cells under 300, 500 mM NaCl, and 7% sorbitol stress conditions. Fig. 1*d* shows that *E. coli* cells (harboring *AtLEA3-3* and *AtLEA3-4*) exhibited significantly higher survival rates than the control cells under cold conditions (4°C for 24 h), but not under freezing ones (–20°C for 1 h). These results show that *AtLEA3-3* and *AtLEA3-4* can enhance the cellular tolerance of *E. coli* cells to low temperatures.

Overexpression of AtLEA3-3 in Arabidopsis prompts vegetative growth and AtLEA3-3-OE plants acquire strong water retention ability

Transgenic *Arabidopsis* plants overexpressing *AtLEA3-3* under the control of a CaMV 35S promoter were generated. Among the 3 : 1 line segregation for hygromycin resistance (presumed one T-DNA insertion) in T2 plants, three lines were randomly selected and their expression levels of *AtLEA3-3* were examined by semi-qRT-PCR (fig. 2*a*). Homozygotous lines (named *AtLEA3-3-OE*), which showed the highest expression level of *AtLEA3-3*, were used for further analysis. Transgenic plants with the *GFP-AtLEA3-4* construct were also generated. All the transgenic lines unexpectedly showed weak GFP fluorescence (data not shown).

Compared to the WT plants, transgenic plants exhibited obvious phenotypic alterations, such as leaf expansion. The leaves of *AtLEA3-3-OE* plants grown under long day (LD) conditions were larger than those of the WT plants. Table 1 describes the 5th and 6th rosette leaf characteristics of WT plants, transgenic plants with an empty vector (*vector* lines), and *AtLEA3-3* plants grown in LD conditions. The length of leaf petioles in *AtLEA3-3-OE* plants was significant-

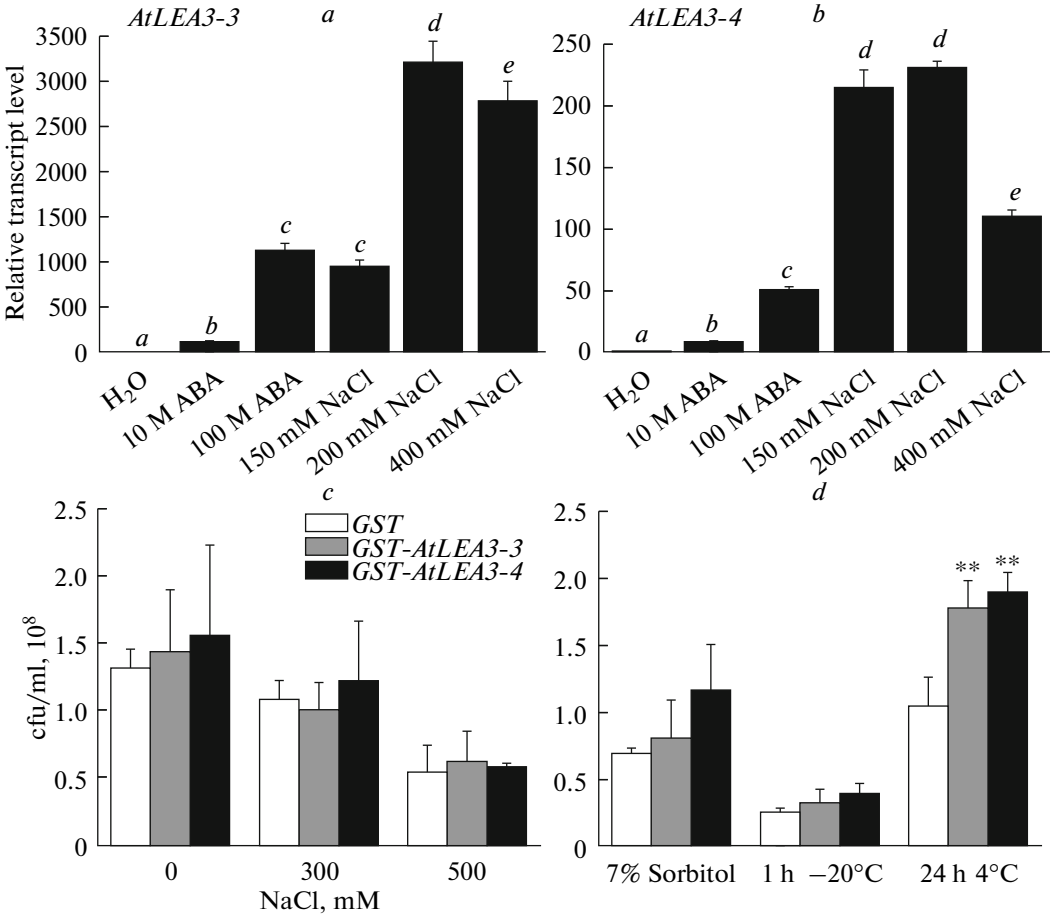

Fig. 1. Expression patterns of *AtLEA3-3* and *AtLEA3-4* under abiotic stress and ABA treatment, and the heterogeneous expression of *AtLEA3-3* and *AtLEA3-4* in *E. coli* BL21 confers the host's resistance to cold stress. Real-time PCR assay of the accumulation of *AtLEA3-3* (*a*) and *AtLEA3-4* (*b*) gene transcripts in *Arabidopsis* 7-day old seedlings in response to ABA, high salinity, and osmotic stress. The expression levels were normalized relative to that of *Actin 2*, and the levels of *AtLEA3-3* and *AtLEA3-4* transcripts in the controls were set to 1.0. Error bars represent SE for three independent experiments. Bars with different letters were significantly different at the 0.01 level. *c* – Survival of IPTG-induced *E. coli* harboring pGEX-4T-1 and pGEX-*AtLEA3-3*/*AtLEA3-4* on LB medium containing 300 and 500 mM NaCl. (*d*) Survival of IPTG-induced *E. coli* harboring pGEX-4T-1 and pGEX-*AtLEA3-3*/*AtLEA3-4* on LB medium containing 7% sorbitol or exposed to cold stress at 4°C for 24 h and −20°C for 1 h before plating. An aliquot of *E. coli* cells was plated on the LB medium, allowing them to form colonies for 16–18 h. Finally, the colony forming units (cfu) were calculated. Each column represents an average of three replicates, and bars indicate SEs. For (*c*), ** indicate significant differences from the corresponding control under the same treatments at $P < 0.01$.

ly longer than that in the WT plants and the *vector* lines. Both the length and the width of the rosette leaves were also increased in the *AtLEA3-3-OE* plants compared to those in WT plants and the *vector* lines. However, the length-width ratio of the rosette leaves did not differ significantly among these plants. Furthermore, at 35 DAS, the aboveground fresh weight (FW) of the *AtLEA3-3-OE* plants was higher than that of the WT plants (fig. 2*b*). Another striking phenotype was that most of *AtLEA3-3-OE* plants displayed reduced fertility of the first-appeared flowers on the main stem, which was rare in WT plants (fig. 2*c*). However, fertility in later flowers of the *AtLEA3-3-OE* plants was restored under normal conditions. The total number of siliques per plant was even higher in the *AtLEA3-3-OE* plants (data not shown).

To assess the water retention ability of transgenic *Arabidopsis*, *AtLEA3-3-OE* lines and WT plants were selected for a detached rosette water loss rate assay. Seven time points were selected for measurement of the FW changes in the detached rosette leaves. The *AtLEA3-3-OE* plants showed lower water loss rates at each time period and the final relative water contents of the *AtLEA3-3-OE* plants rosettes were significantly higher than those of WT plants (fig. 2*d*).

Overexpression of AtLEA3-3 improves salt and osmotic stress tolerance during germination

To characterize the ability of *AtLEA3-3* to respond to abiotic stresses, its behavior under salt or osmotic stress conditions was successively examined. In the ab-

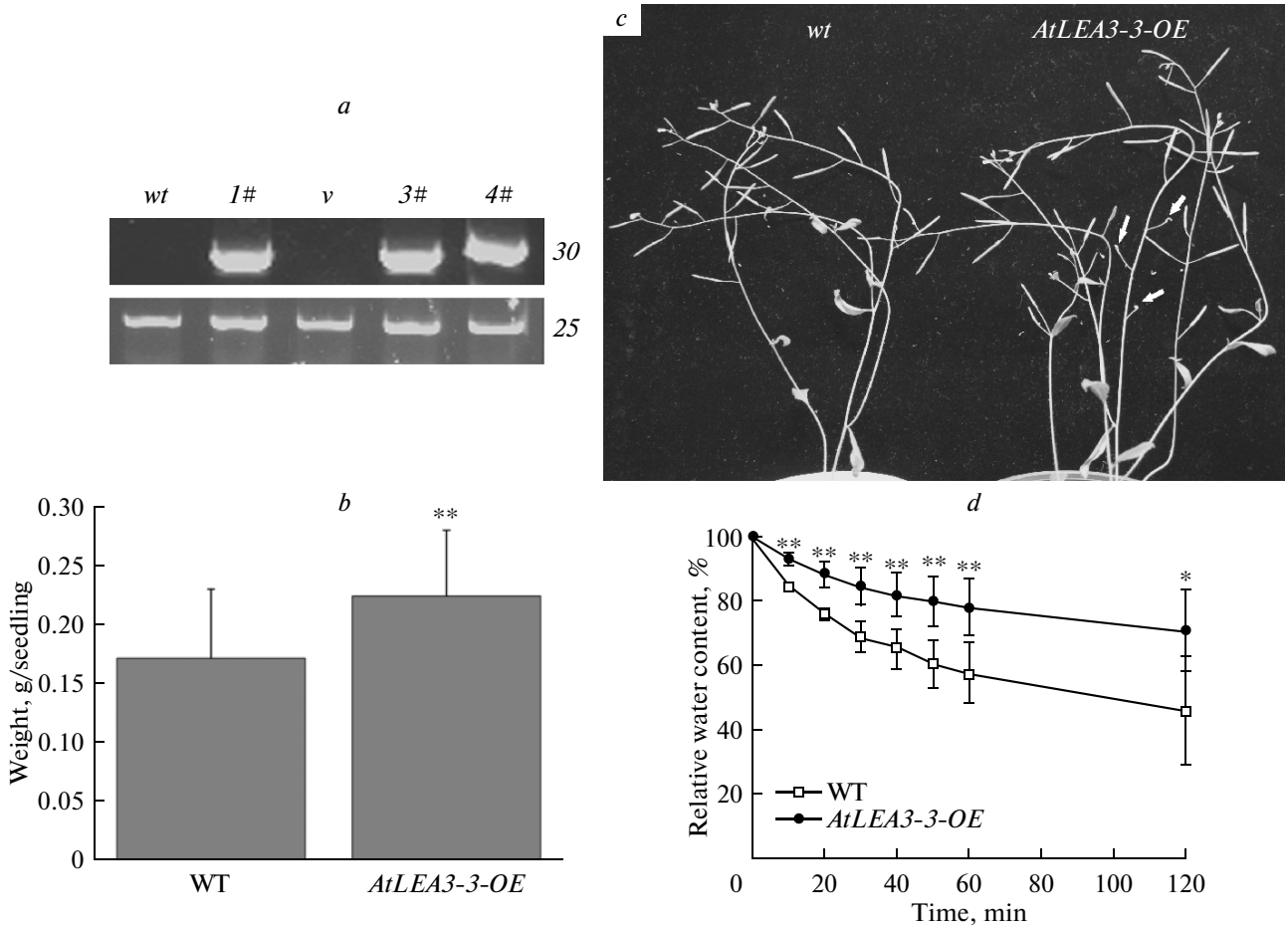

Fig. 2. Molecular characterization and phenotypes of *AtLEA3-3-OE* plants. *a* – Expression of independent transgenic lines by semi-RT-PCR with total RNA isolated from 7-day-old plants. *Actin2* was used as a control. For *AtLEA3-3-OE*, 30 cycles and for *Actin2*, 25 cycles were used. V – vector. *b* – The aboveground FW of WT and AtLEA3-3-OE plants at 35 DAS ($n = 22$). *c* – Phenotype of reduced fertility. White arrows show the abnormal siliques. *d* – Water loss from detached leaves of WT and *AtLEA3-3-OE* lines**.** Water loss is expressed as the percentage of initial FW. Values are means from five leaves for each of the three independent experiments. * and ** indicate significant differences from the corresponding control under the same treatments at $P < 0.05$ and $P < 0.01$, respectively.

sence of stress, seed germination was exceptionally similar between the WT and *AtLEA3-3-OE* plants (data not shown). The seeds of WT and *AtLEA3-3-OE* plants on MS basal medium with various additional concentrations of NaCl or mannitol were then germinated to investigate the effect of salt or osmotic stress. The germination of *AtLEA3-3-OE* seeds was found to be tolerant to both salt and osmotic stresses (fig. 3*a*,*b*). When the NaCl concentration was as high as 200 mM, a drastic decrease in the germination rate was observed on the seeds of WT plants (almost 63%), whereas the transgenic plants lost only about 10% of their germination capacity (fig. 3*a*; $P < 0.01$). Likewise, on the medium containing mannitol, the *AtLEA3-3-OE* seeds showed similar kinetics in germination. As the concentration of mannitol increased, the positive effect of the overexpression of the *AtLEA3-3* gene on seed germination became more evident, especially in the 300 and 400 mM mannitol media, but to a lesser extent (fig. 3*b*; $P < 0.05$). These results indicate that the *AtLEA3-3* gene may play a positive role in plant seed germination and enhance the germination rate under salt and osmotic stress.

Overexpression of AtLEA3-3 results in tolerance to salt and osmotic stress during early seedling development

To further examine the effects of *AtLEA3-3* overexpression on plant stress response, the stress responses of the WT plants were compared to those of *AtLEA3-3-OE* during post-germination development in the presence of NaCl or mannitol. The *AtLEA3-3-OE* seedling growth, including cotyledon greening and expansion, was found to be more tolerant to salt and osmotic stress treatments (fig. 3*d*,*e*). Severe inhibition in WT seedling growth was observed when NaCl and manni-

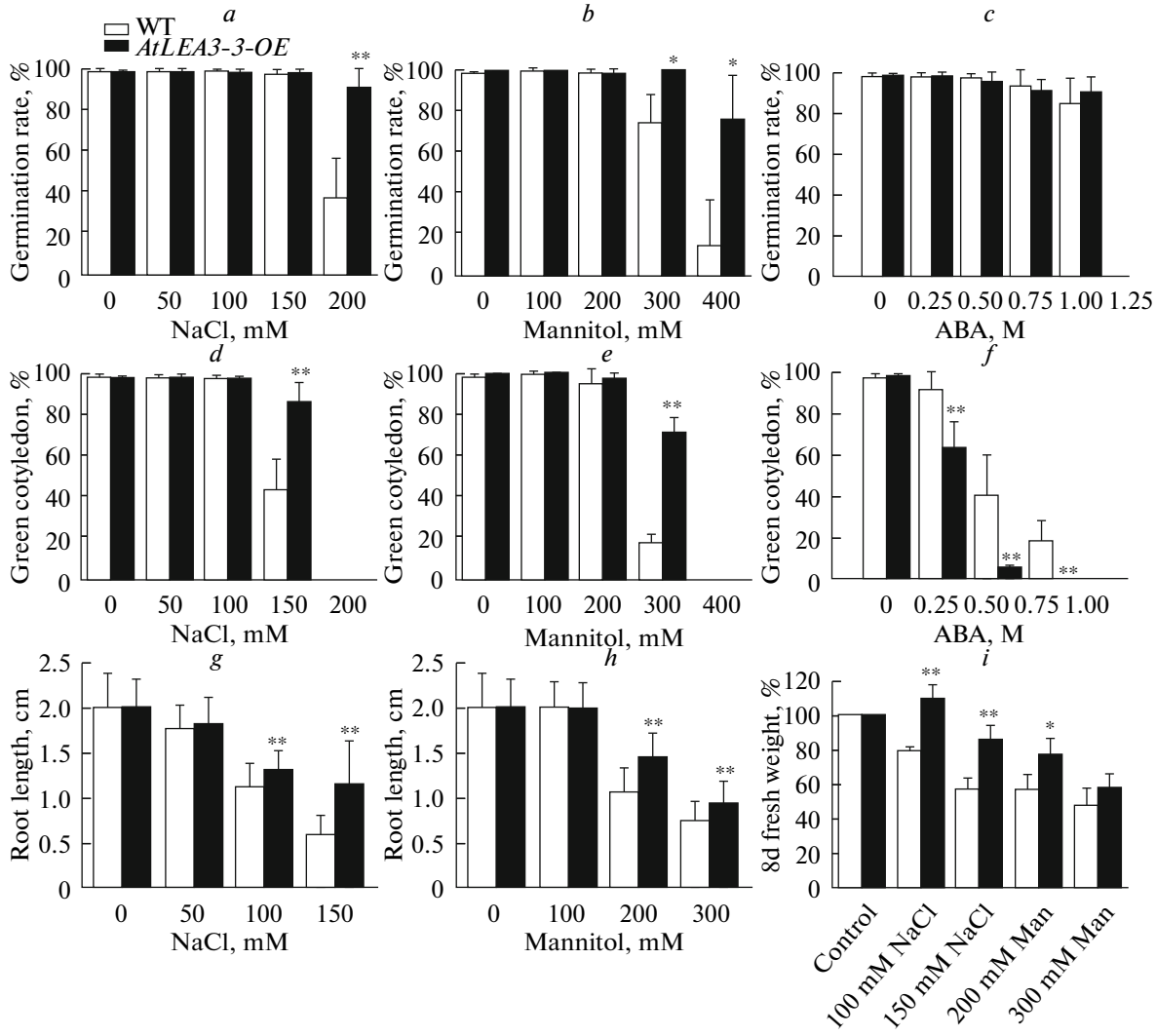

Fig. 3. Germination and early seedling development in response to salt, osmotic stress and ABA treatment in *AtLEA3-3-OE* lines. *a–c* – Germination rate of WT and *AtLEA3-3-OE* seeds 7 days after stratification on the media containing NaCl (*a*), mannitol (*b*) and ABA (*c*). *d–f* – Percentage of germinating seedlings turning green for the WT and *AtLEA3-3-OE* lines 7 days after stratification on the media containing NaCl (*d*), mannitol (*e*), and ABA (*f*). *g–h* – Root growth in response to salt (*g*) and osmotic stress treatments (*h*) in WT and *AtLEA3-3-OE* during postgermination development. (*i*) The FW of 8-d WT and *AtLEA3-3-OE* seedlings under the stress treatments. Mean values and standard errors were obtained from three independent experiments ($n = 50$). * And ** indicate significant differences from the corresponding controls under the same treatments at $P < 0.05$ and $P < 0.01$ respectively.

tol concentrations were 150 and 300 mM respectively. Compared to WT (43%), about 87% of the *AtLEA3-3-OE* seedlings showed open green cotyledon in the presence of 150 mM NaCl, 7 days after stratification under LD conditions (fig. 3*d*; $P < 0.01$). In the 300 mM mannitol medium, WT plants hardly had any ability to establish expanded green seedlings. In contrast, 70% of the seedlings of the *AtLEA3-3-OE* plants grew normally (fig. 3*e*; $P < 0.01$). The average root length of *AtLEA3-3-OE* plants was similar with the WT plants under normal conditions. However, the root of *AtLEA3-3-OE* was noticeably longer compared to WT plants under osmotic or salt stress conditions (fig. 3*g*,*h*; $P < 0.01$). Furthermore, quantification of FW at 8 days after stratification demonstrated that the *AtLEA3-3-OE* plants seedlings exhibited significantly greater FW than the WT plants grown at 100 and 150 mM NaCl or 200 mM mannitol (fig. 3*i*; NaCl, $P < 0.01$; mannitol, $P < 0.05$). These results indicated that the overexpression of the *AtLEA3-3* gene enhances early seedling establishment during salt and osmotic stress treatment in transgenic *Arabidopsis*.

Overexpression of AtLEA3-3 causes increased ABA sensitivity during seedling establishment

Real-time PCR assay demonstrated that the expression of *AtLEA3-3* in seedlings was significantly in-

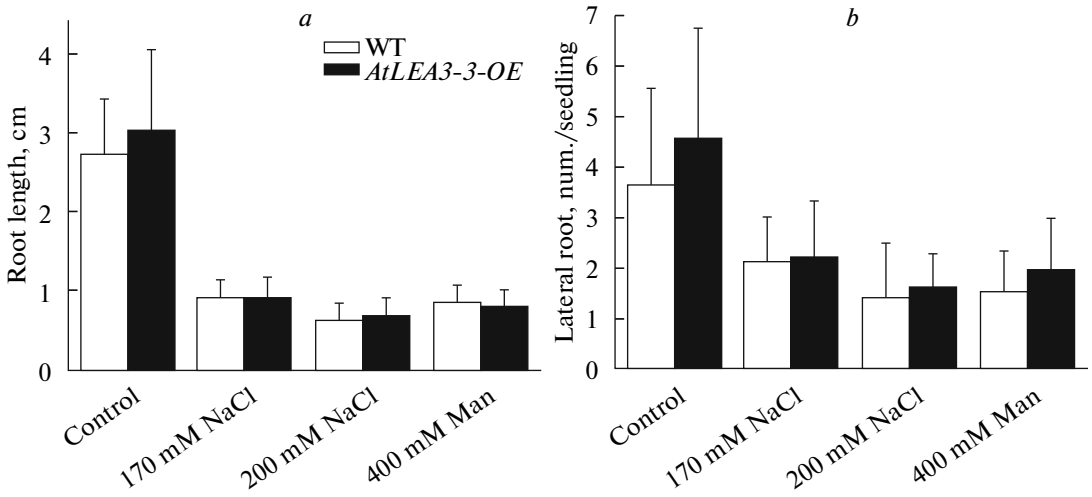

Fig. 4. Vegetative growth of the wild type and *AtLEA3-3-OE* plants under stress. Four-day old seedlings germinated on MS medium were transferred to MS medium supplement with 170 and 200 mM NaCl and 400 mM Mannitol. Root lengths (*a*) and the lateral root numbers per seedling were recorded 7 days after the transfer (*b*). All values are means (±SE) of three independent experiments ($n = 18$).

duced by ABA. It was hypothesized that overexpression of the *AtLEA3-3* gene could regulate germination and vegetative growth through ABA response. To investigate this, the seeds of WT and *AtLEA3-3-OE* line plants were germinated on the medium containing various concentrations of ABA and their germination and seedling establishment were evaluated. As shown in fig. 3*c*, the germination rate of *AtLEA3-3-OE* seeds was similar to that of WT seeds under ABA treatment. However, with increasing concentrations of ABA, cotyledon greening of *AtLEA3-3-OE* seedlings was inhibited dramatically (fig. 3*f*). The percentage of cotyledons turning green of *AtLEA3-3-OE* seedlings was reduced by more than 28% at as low as 0.25 μM ABA. At 0.5 μM ABA, only about 5% of the *AtLEA3-3-OE* germinated seedlings turned green while 40% of WT seedlings were green at 7 days under LD conditions. These results suggest that *AtLEA3-3* plays an important role in mediating stress response via the ABA signal transduction pathway.

AtLEA3-3 gene dose not mediate stress responses during vegetative growth

To investigate whether or not the *AtLEA3-3* gene also mediates stress responses during vegetative growth, a root growth assay was conducted to measure the physiological changes between WT and *AtLEA3-3-OE* plant seedlings. As shown in fig. 4*a*,*b*, the root elongation and the lateral root number of *AtLEA3-3-OE* plants were not significantly different from those of WT plants. All of these results suggest that overexpression of the unique *AtLEA3-3* gene cannot confer to salt and osmotic tolerance during vegetative development. However, we cannot rule out the possibility that the *AtLEA3-3* gene also mediates stress signaling and response pathways during vegetative development.

Intracellular localization of the AtLEA3-3 and AtLEA3-4 proteins

To determine the intracellular localization of the AtLEA3-3 and AtLEA3-4 proteins, plasmids containing YFP, *AtLEA3-3-YFP*, and *AtLEA3-4-YFP* were independently introduced into *Arabidopsis* protoplasts using PEG-mediated transformation (fig. 5*a*). In cells where the empty YFP vector was introduced alone, fluorescence was evenly distributed in the cytoplasm, as well at the nucleus. However, in the cells expressing the *AtLEA3-3-YFP* and *AtLEA3-4-YFP* constructions, fluorescence was clearly found to be localized only in the cytoplasm. The YFP signals of fusion proteins presented as knotted mesh-like patterns distributed in the region overlapped with the vacuolar space. The YFP signals of *AtLEA3-3-YFP* were also shown as a compact structure similar to the nucleus. Transient expression of *CFP-AtLEA3-3* and *GFP-AtLEA3-3* in *Arabidopsis* protoplasts showed that *AtLEA3-3* localized in the cytosol in a reticular or tubular shape. A compact CFP fluorescence structure was also shown in cells with *CFP-AtLEA3-3* (fig. S2*a*,*b* – see Supplemental). Transient expression of *CFP-AtLEA3-4* showed that *AtLEA3-4* was also distributed in the cytosol. A few luminous CFP spots were shown in the cytosol of cells expressing *CFP-AtLEA3-4* (fig. S2*a*).

In order to determine whether or not the knotted mesh-like pattern appeared in the ER structure, the transgenic plants with green fluorescent protein fused with an ER retrieval signal (GFP-HDEL) were used to prepare the protoplasts, and plasmids containing *AtLEA3-3-YFP* and *AtLEA3-4-YFP* were then inde-

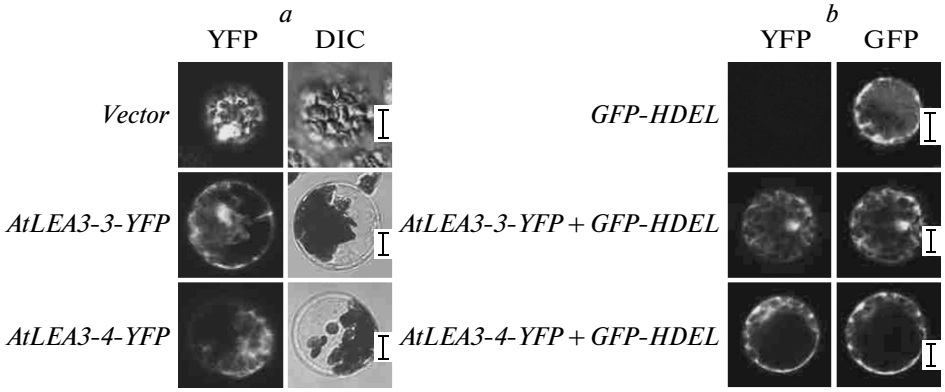

Fig. 5. The transient expression patterns of proteins AtLEA3-3 and AtLEA3-4 in *Arabidopsis* mesophyll protoplasts. (*a*) *Arabidopsis* protoplasts were independently transformed with plasmids containing *YFP*, *AtLEA3-3-YFP*, and *AtLEA3-4-YFP*. (*b*) Transgenic plants with GFP-HDEL were used to prepare the protoplasts. Plasmids containing *AtLEA3-3-YFP* (middle panel) and *AtLEA3-4-YFP* (bottom panel) were independently transformed. Protoplasts without transformation were used as a negative control (upper panel). YFP/GFP signals were visualized by fluorescence microscopy using different channels. YFP/GFP: the YFP/GFP signal. Here and in fig. 6 DIC – Differential interference images.

pendently transformed. As shown in fig. 5*b*, the YFP signals of the *AtLEA3-3-YFP* and *AtLEA3-4-YFP* were colocalized with the GFP signals of the GFP-HDEL. With these combinations of fluors, bleed-through into the GFP channel may be an issue as YFP is excited to about 30% of its maximum by the 488 nm line [38]. To rule out this possibility, GFP-HDEL protoplasts without transformation were used as a negative control. It was found that no YFP signals were detected and GFP signals were normal with sequential excitations at 458 and 514 nm. These *in vivo* localization results indicate that the *AtLEA3-3* and *AtLEA3-4* proteins are localized primarily in the ER, suggesting that the function of these two LEA proteins is associated with the ER.

Changes in localization of AtLEA3-3 and AtLEA3-4 proteins in response to abiotic stresses and BFA treatment

Since *AtLEA3-3* and *AtLEA3-4* were increased by abiotic stress and ABA treatments, an attempt to test whether or not the abiotic stress and BFA treatment could change the localization of AtLEA3-3 and AtLEA3-4 proteins was initiated. After treating with 50 mM NaCl for 2 h, 4°C for 6 h, and BFA for 1 h (fig. 6*a*,*c*,*d*), the fluorescence of fusion proteins appeared condensed to the areas where the chloroplasts were gathered, emitting red autofluorescence. A structure with dense yellow signals, which was similar to the nucleus, was also visible in both cell lines. Likewise, when treated with 500 mM Mannitol for 2 h (fig. 6*b*), the fluorescence was also compact but to a lesser extent. In contrast, the HDEL-GFP protoplast without transformation revealed that the localization of the green fluorescence was invariable during treatment with abiotic stresses and BFA (fig. S3 – see Supplemental), suggesting that the integrity of the ER structure remained intact and excluding the nonspecific effect of the treatments. Although the localization patterns of these two LEA proteins changed and condensed during the treatment, their final location in the cytosol or other organelles could not be determined from the present experiment. Thus, further studies should be done to find out their exact distribution.

DISCUSSION

Bioinformatic analysis revealed that *AtLEA3-3* and *AtLEA3-4* are stable proteins and are highly hydrophilic with over 90% residues falling in the hydrophilic regions with negative scores, containing 50 and 63 charged residues, respectively. To investigate the function of the *AtLEA3-3* and *AtLEA3-4* protein in detail, PSIpred was used to predict the secondary structure of these proteins [39]. Both proteins contain predominantly helical blocks with small regions of random coils at the N- and C-terminal (data not shown). Furthermore, phylogenetic analysis demonstrated that *AtLEA3-3* and *AtLEA3-4* grouped with barley HVA1 in the same cluster (fig. S4). Barley HVA1 was the best characterized protein as determined through the application of transgenic tools to study the function of LEA protein. Overexpression of *HVA1* in transgenic plants displayed enhanced tolerance to water deficit and salt stress [25, 26]. The expression of *AtLEA3-3* and *AtLEA3-4* was regulated by the transcriptional factors DREB2A and AtMYB118 [32, 33, 40]. Previous statements led us to hypothesize the possible involvement of AtLEA3-3 and AtLEA3-4 in plant responses to changing environmental conditions.

Several previous works on plant LEA function introduced their coding sequences in yeast and bacteria to investigate their possible functions *in vivo* [22–24].

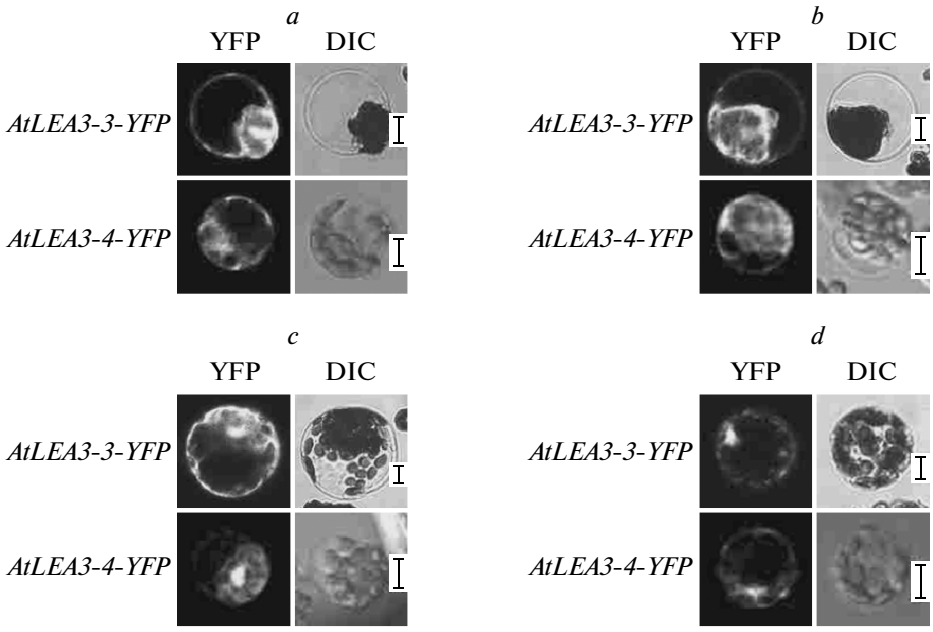

Fig. 6. Localization changes in response to abiotic stresses and BFA treatment. The effect of salt (*a*), osmotic (*b*) and cold stress (*c*) and BFA treatment (*d*) on the subcellular distribution changes of *AtLEA3-3-YFP* and *AtLEA3-4-YFP*. Protoplasts were treated with 50 mM NaCl for 2 h, 500 mM Mannitol for 2 h, 4°C for 6 h, or BFA for 1 h at 16–18 h after the transformation.

In this study, the coding sequences of *AtLEA3-3* and *AtLEA3-4* were introduced into *E. coli* stain BL21. It was found that the heterologous expression of *AtLEA3-3* and *AtLEA3-4* enables cold tolerance in *E. coli*. These results were consistent with previous reports that one and three low temperature response elements were represented in the *AtLEA3-3* and *AtLEA3-4* promoter sequences, and that the expression of *AtLEA3-3* was cold-inducible [7, 29]. The viability of *E. coli* with or without these two LEA proteins unexpectedly showed insignificant differences under high salinity, osmotic, and freezing stresses.

In this study, the overexpression of *AtLEA3-3* in *Arabidopsis* and the transgenic lines lead to some alterative phenotypes. During the vegetative growth stage under LD conditions, the leaf size of *AtLEA3-3-OE* lines was obviously larger than those of the WT plants. We propose that overexpression of *AtLEA3-3* in *Arabidopsis* may promote the growth rate and confer higher tolerance to environmental stress in transgenic lines. Interestingly, 3-week-old WT and *AtLEA3-3-OE* lines were subjected to drought stress by withholding water until seedlings display wilting where, after rewatering, the transgenic lines showed more significant early flowering compared to WT (data not shown). Another interesting observation was the reduced fertility of the first-appeared flowers. Microarray analysis demonstrated that the transcript of *AtLEA3-3* was DELLAs down-regulated in *Arabidopsis* unopened flower buds [34]. Overexpression of *AtLEA3-3* was proposed to have affected normal GA signal transduction during early flower development. More experiments are needed to test this hypothesis in the future.

To survive drought and high-salinity conditions, plants respond and adapt to stresses by inducing the expression of a number of genes. The results of this study implicate a function for AtLEA3-3 in the abiotic stress tolerance of *Arabidopsis*. Overexpression of *AtLEA3-3* caused tolerance to salt and osmotic stress manifested during seed germination and early seedling establishment of *Arabidopsis* plants (fig. 3*a*,*b*,*d*,*e*). The strong water retention ability in *AtLEA3-3-OE* lines infers that *AtLEA3-3* also counteracts dehydration stress.

Recent studies have found that *ABI3* and *ABI5* play roles in the ABA-induced post-germination developmental arrest checkpoint in *Arabidopsis* [41, 42]. This research revealed that the *AtLEA3-3* gene mainly modulates growth and plant adaptation through an ABA-dependent signal transduction pathway, and we provided several lines of evidence to support this hypothesis. First, the expression of *AtLEA3-3* and *AtLEA3-4* was strongly induced by ABA treatments (fig. 1*a*,*b*). Second, overexpression of *AtLEA3-3* in WT conferred supersensitivity to ABA application. Although the germination rate was similar between WT and *AtLEA3-3-OE* lines, the cotyledon expansion and greening of *AtLEA3-3-OE* lines was significantly inhibited by the ABA treatment (fig. 3*c*,*f*). Lopez-Molina et al. [42] showed that during the post-germination developmental arrest, transcript levels of late embryogenesis genes, such as *AtEm1* and *AtEm6*, were also re-induced. This re-induction of seed transcriptional reg-

ulators (*ABI3* and *ABI5*) and *LEA* gene expression suggest that a late embryogenesis program was recruited following stratification on ABA [42]. There have been extensive studies that showed that ABI5 can bind to ABA-responsive DNA elements (ABREs) and is capable of activating reporter genes containing ABREs [43]. There were also reports that four and one ABRE core motifs were presented in the *AtLEA3-3* and *AtLEA3-4* promoters, respectively [7]. Therefore, the expression of *AtLEA3-3* and *AtLEA3-4* may be regulated by ABI5 during seed development. As such, the overexpression of *AtLEA3-3* in WT is proposed to promote the re-induction of this late embryogenesis pathway with other LEA proteins in early seedling establishment during ABA treatment.

Previous work using bioinformatics methods demonstrated that *AtLEA3-3* and *AtLEA3-4* localized in the cytosol [7]. The transient expression results in this study showed that *AtLEA3-3* and *AtLEA3-4* proteins were distributed in the ER and AtLEA3-3 was also localized in the nucleus. Moreover, salt, osmotic, cold stress, and BFA treatments altered the distribution of these two proteins. With these treatments, AtLEA3-3 and AtLEA3-4 proteins are hypothesized to adopt an α-helical conformation, stabilizing the cellular structures or preserving the membrane integrity. These results suggest a new functional role of the *AtLEA3-3* and *AtLEA3-4* proteins in abiotic stresses, and demonstrate that protein translocalization is a positive and effective strategy of response to abiotic stresses.

To the best of our knowledge, few works demonstrating the functions of the LEA group 3 proteins using transgenic methods in *Arabidopsis*, are available. Based on the results of this study and of several others, *AtLEA3-3* is proposed to play a positive role during the germination and subsequent growth of *Arabidopsis* plants under high salinity and osmotic stress conditions. Accumulation of the mRNA and/or protein of *AtLEA3-3* may help heterologous ABA re-initiate the second dormancy. Nevertheless, more *in vivo* works need to be carried out to better understand the response mechanism of *AtLEA3-3* and *AtLEA3-4* during the abiotic stress adaptation processes in plants.

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