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Overexpression of Arabidopsis *ZEP* enhances tolerance to osmotic stress

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ABSTRACT

Zeaxanthin epoxidase (ZEP) is an enzyme important in ABA biosynthesis and in the xanthophyll cycle. ABA, a plant hormone, is a key molecule that regulates plant responses to abiotic stress, such as drought and salinity, and is required for stress tolerance. To investigate the biological roles of the *Arabidopsis thaliana ZEP* gene (*AtZEP*) in stress response, we generated transgenic plants overexpressing the *AtZEP* gene and analyzed their responses to salt and drought stresses. *AtZEP*-overexpressing plants exhibited more vigorous growth under high salt and drought treatments than wild-type plants. In addition to enhanced *de novo* ABA biosynthesis, *AtZEP*-overexpressing plants also exhibited much higher expression of the endogenous stress-responsive genes *RD29A* and *Rab18* than wild-type plants under salt stress. Moreover, the stomatal aperture of the *AtZEP*-overexpressing plants was smaller than wild-type plants after exposure to light. Our results therefore indicated that *AtZEP* plays important roles in response to osmotic stress.

Most plants grow in an inconsistent environment that frequently imposes constraints on their growth and development. One of the most common adverse environmental factors encountered by land plants is osmotic stress, usually resulting from conditions of high salinity or periods of drought [1–3]. To overcome the serious problems caused by osmotic stress, known as water-deficit conditions, plants have developed unique systems to prevent water loss and retain water inside their cells [4].

When imposed by high salt or drought, osmotic stress signals are transmitted through at least two pathways; one is abscisic acid (ABA)-dependent, and the other is ABA-independent [5]. In the ABA-dependent pathway, osmotic stress increases the cellular ABA levels, which induce the expression of osmotic stress-responsive genes such as *Rab18*, *KIN1*, and *RD29B* [5–13]. In the ABA-independent pathway, osmotic stress-responsive genes are induced without ABA upregulation; instead, the C-repeat/dehydration-responsive elements (CRT/DRE) in the promoters of osmotic stress-responsive genes, along with the associated CRT/DRE-binding factor 1 (CBF1/DREB1B), mediate their upregulation [14–16]. Both signaling pathways eventually result in the activation of genes that help to maintain cellular homeostasis during osmotic stress [17].

Understanding of the mechanisms underlying the regulation of plant ABA levels is a crucial part of unraveling osmotic stress response [5,7,10–13]. ABA is most likely synthesized by two pathways, direct and indirect, and ABA in higher plants is synthesized from the indirect pathway [18]. Previous studies indicate that zeaxanthin epoxidase (*ZEP*), 9-*cis*-epoxycarotenoid dioxygenase (*NCED*), and aldehyde oxidase (*AAO*) are key regulation genes in the ABA biosynthesis pathway in *Arabidopsis* and other plant species [18].

Mutants with a functional defect in *ZEP* have been isolated from several species, including *Arabidopsis* [19–22], *N. plumbaginifolia* [23], rice [24,25], and tomato [26]. *Osaba1*, a mutant of rice *ZEP*, displayed low ABA levels and almost no ABA upregulation upon drought induction [25]. Antisense down-regulation of *LeZEP1* in tomato resulted in accumulation of zeaxanthin in the leaves [26]. In *Arabidopsis*, *AtZEP* is allelic to *LOS6*/*ABA1* [19]. *Arabidopsis los6*/*aba1* mutants, deficient in *AtZEP*, displayed reduced expression of the endogenous *RD29A* gene and of other stress-responsive genes under osmotic stress [19]. Moreover, under drought stress, the change in ABA levels in the *los6*/*aba1* mutants was minimal compared to wild-type plants [19]. Taken together, these results hint that ZEP might play a critical part in the ABA-mediated stress response, but stress response of *AtZEP*-overexpressing transgenic plant has not been extensively studied, yet.

In this study, therefore, we sought to further investigate the biological roles of *AtZEP* in stress response by overexpressing the gene in *Arabidopsis*. We generated transgenic plants ectopically expressing the *AtZEP* gene, and these plants had enhanced salt- and drought-tolerance phenotypes. The increased stress tolerance in *AtZEP*-overexpressing plants was probably due to higher levels of endogenous ABA and enhanced expression of stress-responsive genes. Our results suggest that *AtZEP* might play an important role in stress response.

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Materials and methods

Plant materials. All *Arabidopsis* plants were of the ecotype background Columbia. *Arabidopsis* seeds were surface-sterilized and water-imbibed in the dark for 3 d at 4 °C. The seeds were sown on 0.7% (w/v) agar plates containing one-half-strength Murashige and Skoog (MS) media [27] supplemented with B5 vitamins and 1.5% (w/v) sucrose. They were grown at 22 °C under 8-h light/16-h dark cycles. Two-week-old seedlings were transferred into soil and grown at 22 °C under 16-h light/8-h dark cycles.

Plasmid construction and plant transformation. To create an *AtZEP* (gene locus no. At5g67030) overexpression construct, *AtZEP* cDNA was cut from a full-length *Arabidopsis* clone (pda08179; RIKEN BioResource Center, Japan) and inserted into pPZP211 vector [28] with a modified *cauliflower mosaic virus* 35S promoter. The resulting plasmid (pPZP211-AtZEP) was introduced into *Agrobacterium tumefaciens* strain GV3101 (pMP90) by freeze-thaw method and then transformed into *Arabidopsis* ecotype Columbia by floral-dipping method [29]. Transgenic plants were selected on MS medium containing 25 mg/L kanamycin.

HPLC assay. Thirty-day-old wild-type plants and T_1, T_2 transgenic plants were frozen in liquid nitrogen and ground with a Mixer-Mill (Qiagen, USA). Plants were treated with 100% acetone, and the extract was gently mixed in the dark for 1 h at 4 °C. Cell debris was removed by centrifuging twice at 14,000 rpm, 4 °C, for 10 min (Micro 17R; Hanil, Korea). The extracts were then filtered through a 0.2-μM syringe filter. Pigment separation was performed in a high performance liquid chromatography (HPLC) system (HP 1100 series, Hewlett Packard, Waldbronn, Germany) on a Spherisorb ODS-1 column (Alltech, USA), with a solvent mixture of acetonitrile/methanol/0.1 M Tris–Cl (pH 8.0) (72:12:7, v/v) and with a 10-min linear gradient to methanol/hexane (4:1, v/v). All pigments were recovered from the column within about 25 min, at a flow rate of 2 mL/min. The eluted pigments were monitored at 445 nm. Concentrations of the pigments were estimated by using the conversion factors for peak area (in nmol) that had been calculated for this solvent mixture [30].

Semi-quantitative reverse transcription-PCR. Total RNAs were prepared using TRI reagent (Molecular Research Center, USA). RNA was treated with RNase-free DNase I (Promega, Madison, WI) following the manufacturer's instructions. RT using 5 μg total RNA was performed with moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) and a poly-T primer. PCR reactions employed the following primers (24–30 cycles): AtZEP-F (5′-ATGCCATCGATGCTTGACTG-3′), AtZEP-R (5′-ATCACAATGCGC ATTCCAGG-3′), RD29A-F (5′-GAAACAGAGTCTGCCGTGAC-3′), RD29A-R (5′-TGCTGCCTTCTCGGTAGAGA-3′), Rab18-F (5′-AATGCTTCACCGCT CCGGAT-3′), Rab18-R (5′-TTCTTCTCGTGGTGCTCACC-3′), GAPc-F (5′-CACTTGAAGGGTGGTGCCAAG-3′), and GAPc-R (5′-CCTGTTGTCG CCAACGAAGTC-3′). PCR amplification was performed with 24–30 cycles as follows: 94 °C for 45 s, 56 °C for 45 s, and 72 °C for 45 s, followed by 72 °C for 10 min.

Stresses treatment. T_2 and T_3 transgenic plants were used for stress treatments. For NaCl treatment, 7-day-old seedlings were transferred to MS medium supplemented with various concentrations of NaCl such as 0, 100, 140, 150, or 160 mM. For fluridone treatment, 1 μM fluridone (Wako) was added to MS medium containing 150 mM NaCl. For mannitol treatment, 7-day-old seedlings were transferred to MS medium supplemented with 0, 200, 300, or 400 mM mannitol. For drought treatment, 7-day-old seedlings were transferred to soil, and drought treatment was applied to 3-week-old plants by withholding watering for 21 d. Plants were subsequently re-watered and analyzed 3 d later. For water loss measurements, shoots of 4-week-old plants were detached from their roots and weighed immediately. After then, each shoot was placed in a plate on a laboratory bench and weighed at designated time intervals.

Measurements of stomatal aperture. Stomatal apertures of unstained epidermal strips were measured with a light microscope under bright-field illumination [31]. Briefly, rosette leaves from 4-week-old wild-type and T_2, T_3 transgenic plants were harvested in darkness at the end of the night period. To determine stomatal aperture, epidermal strips prepared from the underside of rosette leaves were placed in Petri dishes containing 5 mL of the incubation solution (10 mM Mes/KOH, 50 mM KCl, pH 6.0). To standardize the initial state, epidermal strips were kept in the incubation solution for 1 h in darkness at 22 °C. Leaves were then incubated at 22 °C for 3 h under illumination at 100 μmol m^{-2} S^{-1}. Leaves were then treated with 10 μM ABA for 3 h.

Quantification of ABA levels. To determine the ABA levels in wild-type and T_2, T_3 transgenic plants, 2-week-old seedlings were treated with 300 mM NaCl for 5 h. ABA extraction and determination were performed as previously described [32]. In brief, 30 to 1000 mg of plant material was frozen in liquid nitrogen and ground with a Mixer-Mill (Qiagen, USA). Plant material was treated with 1 to 2 mL of ABA extraction buffer (10 mM HCl and 1% [w/v] polyvinylpolypyrrolidone in methanol). The extract was mixed overnight at 4 °C. After centrifugation, the supernatant was neutralized with 1 M NaOH, and ABA levels were quantified using the Phytodetek-ABA kit (AGDIA Inc.), following the manufacturer's protocol. Raw values for ABA levels were standardized by plant mass and extraction volume.

Results and discussion

Generation of transgenic plants overexpressing the AtZEP gene

To investigate the functions of *AtZEP* in stress response, we generated transgenic plants overexpressing *AtZEP* under the *cauliflower mosaic virus* 35S promoter (Fig. 1A). We analyzed five individual T_1 plants to select lines overexpressing *AtZEP*, and investigated the expression levels of *AtZEP* using semi-quantitative RT-PCR and HPLC. Our RT-PCR results revealed that two plants among the five plants showed much higher expression of *AtZEP* than the wild-type and the other transgenic plants (Fig. 1B). The two plants had no detectable zeaxanthin, while wild-type plants showed a small amount of zeaxanthin that was discernable by HPLC (Table 1). Furthermore, we detected no zeaxanthin in the T_2 generation of the two T_1 plants, confirming that *AtZEP* overexpression was heritable and highly penetrant (data not shown). Based on these experiments, we selected these two T_1 lines, T-4 and T-5, for our subsequent studies.

Transgenic plants overexpressing AtZEP show enhanced tolerance to salt stress

Several studies have demonstrated that ABA plays an important role in response to osmotic stress [5], we therefore hypothesized

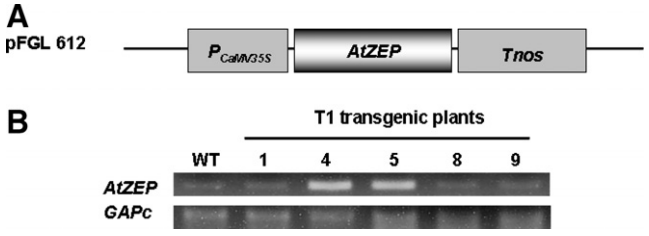

Fig. 1. *Arabidopsis* transgenic plants with constitutive overexpression of *AtZEP* gene. (A) Schematic map of the *CaMV35S::AtZEP* binary vector. (B) Expression analysis of *AtZEP* transcript was determined in wild-type and *AtZEP*-overexpressing *Arabidopsis* transgenic plants by semi-quantitative RT-PCR. *GAPc* expression level was analyzed as an internal control.

Table 1
Pigment contents in *AtZEP*-overexpressing plants, as determined by HPLC

Pigment (%)	WT	T_1 transgenic plants	
		T-4	T-5
Zeaxanthin	4.9 ± 0.14	0 ± 0.00	0 ± 0.00
Antheraxanthin	10.5 ± 0.70	3.9 ± 0.14	3.85 ± 0.21
Violaxanthin	84.6 ± 0.56	96.1 ± 0.14	96.15 ± 0.21

The plants were grown at 22 °C under 16-h light/8-h dark cycles (100–200 μmol photons·m^{-}2 s^{-}1 light intensity). Thirty-day-old wild-type plants and T_1 transgenic plants were pretreated with darkness for 1 h. The errors represent standard deviations from duplicate measurements.

that AtZEP might play an important role in response to osmotic stress. To test this idea, we studied the response of our *AtZEP*-overexpressing transgenic plants to salt stress. In these experiments, 7-day-old seedlings of T_2 and T_3 plants of the T-5 line were transferred to MS medium containing various concentrations of NaCl. Compared to wild-type seedlings, the *AtZEP*-overexpressing seedlings showed more developed rosette leaves and roots in the presence of 150 mM NaCl (Fig. 2A). Primary root length of the *AtZEP*-overexpressing seedlings under NaCl treatment was longer than those of wild-type seedlings (Fig. 2B). Also, the fresh weight (FW) of *AtZEP*-overexpressing seedlings was higher than that of wild-type seedlings after salt treatments (Fig. 2C). Similarly, enhanced levels of salt tolerance in T-4 transgenic plants were also observed (data not shown). Taken together, these results indicate that the *AtZEP*-overexpressing plants were more tolerant to salt stress than the wild-type plants.

Transgenic plants overexpressing AtZEP show tolerance to drought stress

We next sought to investigate how *AtZEP* overexpression affected tolerance to another osmotic stress, drought. For these experiments, we utilized two types of drought stress to determine whether *AtZEP* overexpression protected the *Arabidopsis* seedlings: We grew wild-type and *AtZEP*-overexpressing seedlings on medium containing mannitol, or we withheld water from seedlings transplanted into soil.

On MS medium containing 400 mM mannitol, *AtZEP*-overexpressing seedlings of the T-5 line showed more developed rosette leaves and lateral roots than wild-type plants (Fig. 3A). In addition, the *AtZEP*-overexpressing seedlings under mannitol treatments displayed longer primary root length and higher FW than the wild-type seedlings (Figs. 3B and C). In the parallel experiments, we did not water 3-week-old *AtZEP*-overexpressing plants and wild-type plants for 3 weeks. The *AtZEP*-overexpressing plants showed enhanced drought tolerance than the wild-type plants. After water was withheld for 3 weeks, 100% of the *AtZEP*-overexpressing plants were still alive, while none of the wild-type plants survived (Fig. 3D).

Reduced water loss is a major factor contributing to drought tolerance [33]. The rate of water loss from *AtZEP*-overexpressing plants of the T-5 line was lower than that from wild-type plants, as measured by the FW loss of detached shoots (Fig. 3E). After dehydration for 3 h, the FW of the *AtZEP*-overexpressing plants was reduced to approximately 52%, whereas wild-type plants retained 44% of their initial weight. T-4 transgenic plants also showed similar results (data not shown). Taken together, these results indicate that *AtZEP*-overexpressing plants are more tolerant to drought stress than wild-type plants. Because both high salinity and drought contribute to osmotic stress on plants, our results further support that *AtZEP*-overexpressing plants are tolerant to osmotic stress, and that *AtZEP* is involved in the response to osmotic stress.

Overexpression of AtZEP increased ABA levels and induced expression of ABA-responsive genes

Because ZEP is involved in the first step of ABA biosynthesis, we hypothesized that the stress-tolerant phenotype of *AtZEP*-overexpressing plants may be due to an increase in ABA levels, which would lead to enhanced expression of ABA-regulated stress-

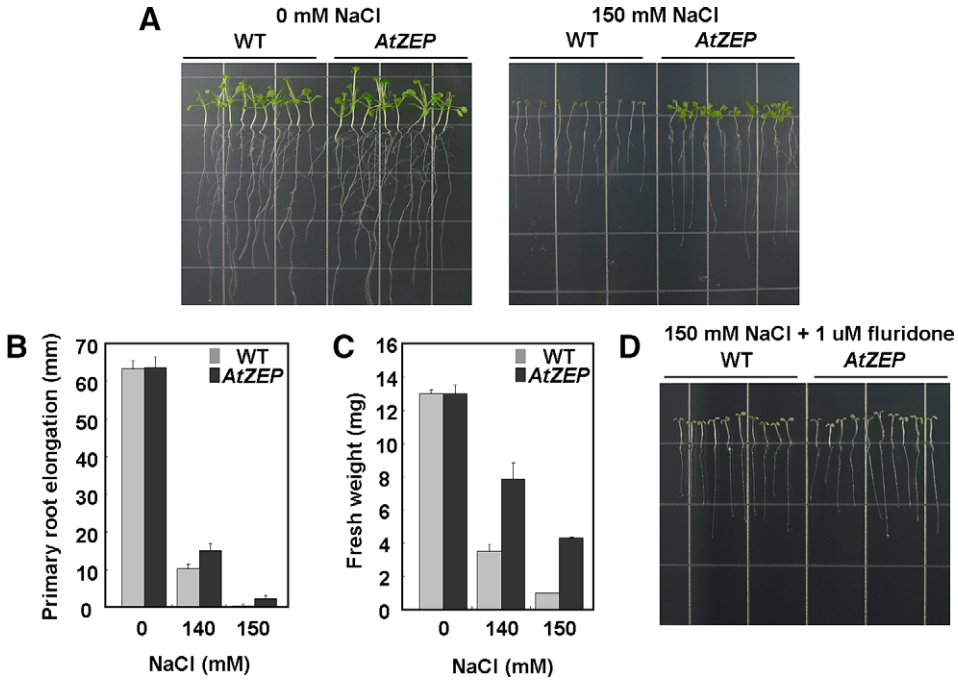

Fig. 2. NaCl tolerance of *AtZEP*-overexpressing *Arabidopsis* transgenic plants (T-5 line). (A) Wild-type and *AtZEP*-overexpressing seedlings were treated with or without 150 mM NaCl for 10 d. (B) Primary root growth of wild-type and *AtZEP*-overexpressing seedlings were measured after treatment with or without NaCl for 10 d. Error bars represent standard deviation (n = 10 plants). (C) Plant FW was measured after 10 d of growth on indicated medium. Error bars represent standard deviation (n = 10 plants). (D) Wild-type and *AtZEP*-overexpressing seedlings were treated with 150 mM NaCl and 1 μM fluridone for 10 d.

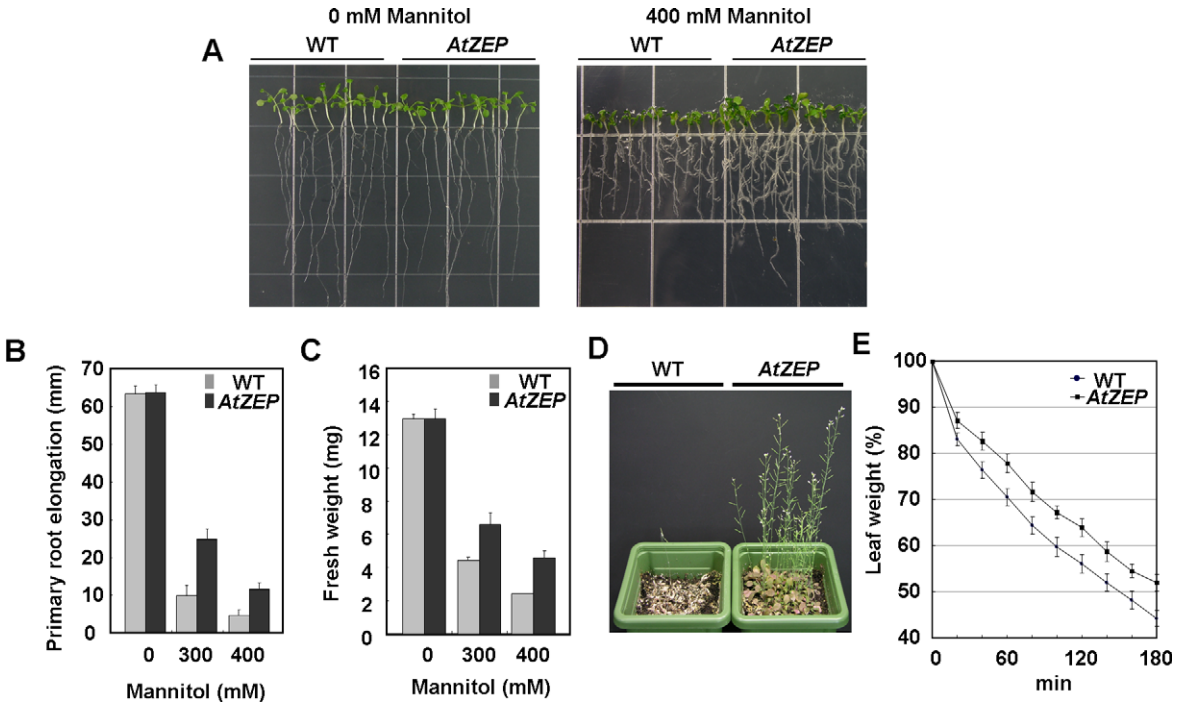

Fig. 3. Drought tolerance of *AtZEP*-overexpressing *Arabidopsis* transgenic plants (T-5 line). (A) Seven-day-old wild-type and *AtZEP*-overexpressing seedlings were treated with or without 400 mM mannitol for 10 d. (B) Primary root growth of wild-type and *AtZEP*-overexpressing seedlings were measured after treatment with or without mannitol for 10 d. Error bars represent standard deviation (n = 10 plants). (C) Plant FW was measured after 10 d of growth on indicated medium. Error bars represent standard deviation (n = 10 plants). (D) Three-week-old wild-type and *AtZEP*-overexpressing plants were treated with drought stress for 21 d. (E) For water-loss measurements, the aerial part of 4-week-old plants was separated from the roots, placed on weighing dishes, and allowed to dry slowly on the laboratory bench. Error bars represent standard deviation (n = 10 plants).

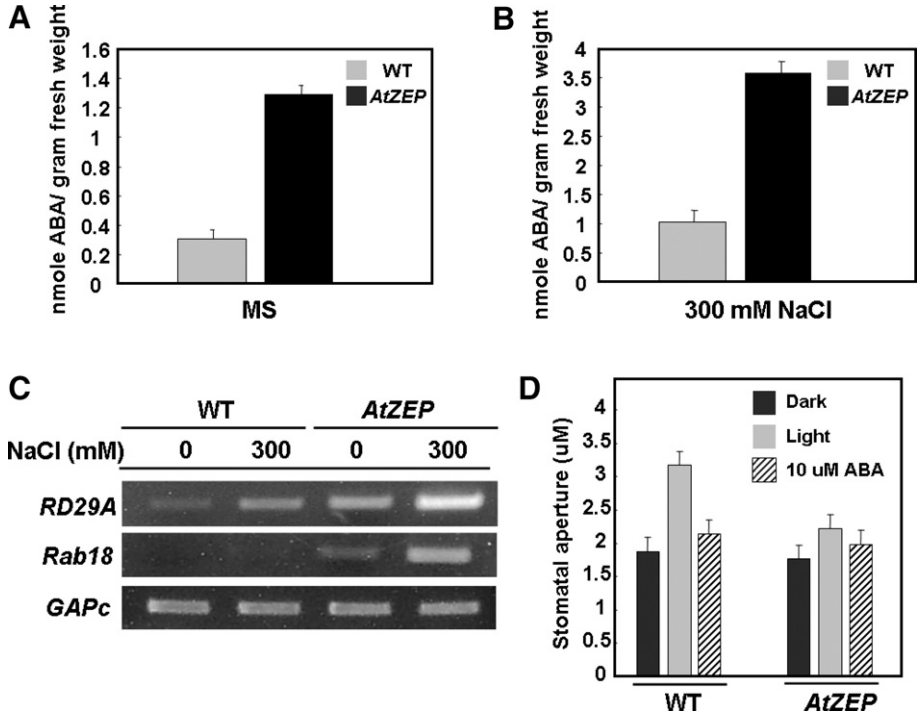

Fig. 4. Analysis of endogenous ABA levels, expression patterns of stress-responsive genes, and stomatal aperture size in wild-type and *AtZEP*-overexpressing plants (T-5 line). (A) ABA levels were determined in 2-week-old seedlings grown on MS medium. Error bars represent standard deviation (n = 4 experiments). (B) ABA levels were determined in 2-week-old seedlings under salt stress. Error bars represent standard deviation (n = 4 experiments). (C) *RD29A* and *Rab18* transcripts were determined by semi-quantitative RT-PCR in wild-type and *AtZEP*-overexpressing plants. (D) Stomatal aperture measurements of wild-type and *AtZEP*-overexpressing plants. Results shown are averages of three independent experiments. Error bars represent ±SE (n = 40–60 stomata).

responsive genes. To confirm this hypothesis, we measured ABA induction following salt stress using an immunoassay. The ABA level in *AtZEP*-overexpressing plants was 4.2 times higher than that in wild-type plants (Fig. 4A). Following salt stress, the ABA levels in *AtZEP*-overexpressing plants were 2.76 times higher than that in wild-type plants (Fig. 4B). These results suggested that enhanced ABA induction might be responsible for osmotic stress tolerance in these plants.

In addition, our results demonstrated that the expression of endogenous *RD29A* and *Rab18* was much higher in *AtZEP*-overexpressing plants than in wild-type plants, under both normal and salinity condition (Fig. 4C). These results suggested that osmotic stress tolerance in the *AtZEP*-overexpressing plants might be due to enhanced expression of stress-responsive genes.

To clarify whether salt tolerance in these plants operated via the ABA signaling pathway, we treated them with fluridone, a potent inhibitor of ABA biosynthesis. *AtZEP*-overexpressing seedlings of the T-5 line treated with 1 μM fluridone did not display tolerance to 150 mM NaCl, but untreated *AtZEP*-overexpressing seedlings did (Figs. 2D and A). Also, seedlings of the T-4 transgenic plants showed similar results (data not shown). These results supported the hypothesis that the salt tolerance of *AtZEP*-overexpressing plants might be due to an increase in *de novo* biosynthesis and accumulation of ABA.

Previous studies reported that in the *los6*/*aba1* mutant, the ABA levels were lower than in wild-type [34], and the expression levels of stress-responsive genes *RD29A*, *RD29B*, *COR15A*, *KIN1*, *COR47*, *RD19*, *RD22*, *ADH*, and *P5CS* were all reduced to various extents under osmotic stress [19]. Also, NaCl treatment increased the expression level of a *RD29A*-driven *LUC* transgene in *LOS6*-overexpressing plants by nearly 2-fold compared to wild-type [19]. Taken together, these results suggested that the stress tolerance in *AtZEP*-overexpressing plants may be due to the increase of ABA-regulated stress-responsive genes mediated by higher ABA biosynthesis.

Overexpression of AtZEP enhanced stomatal closure

ABA signaling plays a crucial role in reducing water loss by regulating stomatal aperture [35]. Since we had demonstrated that *AtZEP* overexpression led to increased ABA levels and decreased water loss, we hypothesized that *AtZEP* overexpression might reduce stomatal aperture size and therefore mediate higher drought and salt tolerance. The results indicated that the stomatal aperture of *AtZEP*-overexpressing plants was smaller than that in wild-type plants following exposure to light (Fig. 4D). In particular, *AtZEP* overexpression decreased light-induced stomatal opening approximately 1.4-fold compared to wild-type. These results suggest that *AtZEP* overexpression in *Arabidopsis* enhances closure of stomatal aperture to reduce water loss upon exposure to drought.

In summary, our results revealed that *AtZEP*-overexpressing transgenic plants show higher tolerance to salt and drought stresses, and that this tolerance is due to enhanced expression of ABA-responsive genes as a result of increased ABA levels under the osmotic stress. Taken as a whole, our results have revealed that *AtZEP* plays important roles during the osmotic stress response.

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