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Overexpression of *ERF96*, a small ethylene response factor gene, enhances salt tolerance in *Arabidopsis*

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Abstract

Salt stress is one of the abiotic stresses limiting the yield of crops worldwide. However, the molecular mechanisms underlying the regulation of plant response to salt stress are not completely elucidated. Ethylene response factors (ERFs) are a subfamily of the AP2 (APETALA2)/ERF transcription factor family that regulates multiple aspects of plant growth and development, and plant responses to biotic and abiotic stresses. ERF96 is one of the small ERFs that is involved in plant defense response and abscisic acid signaling in *Arabidopsis*. By using real time quantitative PCR, we found that the expression of *ERF96* in the wild type *Arabidopsis thaliana* (cv. Col-0) seedlings was induced by NaCl treatment. The transgenic plants overexpressing *ERF96* were more tolerant to salt stress in terms of NaCl inhibited seed germination, early seedling development, and fresh mass. Consistent with these observations, elevated expressions of some NaCl-responsive genes including responsive drought 29 (*RD29A*), Δ^1-pyrroline-*5*-carboxylate synthetase (*P5CS*), cold responsive 15A (*COR15A*), and kinase 1 (*KIN1*) were observed in the transgenic plants in the presence of NaCl. We also found that the Na^+ and K^+ content and expressions of genes related to Na^+/K^+ homeostasis including stelar K^+ outward rectifier (*SKOR*) and potassium transport 2/3 (*AKT2/3*) were altered in the *ERF96* transgenic plants in response to NaCl treatment. Taken together, these results showed that overexpression of *ERF96* enhanced plant tolerance to salt stress, indicating that *ERF96* is a positive regulator of salt tolerance in *Arabidopsis*.

Additional key words: NaCl, potassium, sodium, transcription factor, transgenic plants.

Introduction

Environmental stresses, such as salt, drought, cold, wounding, pathogen, and insect attack are growing threats to sustainable agriculture. To survive, plants have to adapt to various stress conditions including abiotic and biotic stresses, which may cause adverse effects on the growth and productivity of plants (Xiong *et al.* 2002). Plants have developed different types of adaptive responses to these stresses, such as physiological and biochemical adaptations, adaptation through complex signaling networks involving hormones, receptors, protein kinase cascades, transcription factors, and regulators of stress-related proteins (Xiong *et al.* 2002, Chakravarthhy *et al.* 2003, Agarwal *et al.* 2006).

Several different types of transcription factors are associated with stress responses in plants (Kang *et al.* 2002, Singh *et al.* 2002, Agarwal *et al.* 2006, 2010, Eulgem and Somssich 2007, Zhang *et al.* 2009). APETALA2/ethylene response factors (AP2/ERF) is one of the most important transcription factor families that regulates plant responses to both biotic and abiotic stresses (Agarwal *et al.* 2006). According to the number and similarity of their DNA binding domains, AP2/ERF superfamily has been classified into four different subfamilies: ERF, AP2, related to ABI3/VP1 (RAV), and Apetala 2 family protein involved in SA mediated disease defense 1 (APD1; Nakano *et al.* 2006, Giri *et al.* 2014). Genes in the AP2 family are mostly involved in the regulation of plant development processes such as flower

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Abbreviations: ABA - avscisic acid; AKT2/3 - potassium transporter 2/3; AP2 - APETALA2; AsA - ascorbic acid; COR15A - cold-responsive 15A; ERF - ethylene response factor; KIN1 - kinase 1; MS - Murashige and Skoog; P5CS - Δ^1-pyrroline-5-carboxylate synthetase; RAV - related to ABI3/VP1; RD29A - responsive drought 29A; SKOR - stelar K^+ outward rectifier.

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development (Jofuku *et al*. 1994, Aukerman *et al*. 2003), somatic embryogenesis (El Ouakfaoui *et al*. 2010), early floral meristem identity (Kirch *et al*. 2003), and primary and lateral roots formation (Oh *et al*. 2007, Kitomi *et al*. 2011). Genes in the RAV family are involved in the regulation of plant response to phytohormones such as ethylene and brassinosteroids (Alonso *et al*. 2003, Hu *et al*. 2004, Salvi *et al*. 2007). Genes in the ERF family have diverse functions, they are involved in the regulation of plant response to biotic and abiotic stresses (Yamaguchi-Shinozaki and Shinozaki 2006, Century *et al*. 2008), as well as plant growth and development (Yin *et al*. 2010), primary and secondary metabolism (Aharoni *et al*. 2004, Broun *et al*. 2004), and hormonal signaling (Ohme-Takagi and Shinshi 1995).

Several trascription factors modulate plant responses to salt stress. For example, overexpression of *Oryza sativa Dehydration Response Element B1A* (*OsDREB1A*; Ito *et al*. 2006), *AP37* and *AP59* (Oh *et al*. 2009), *Jasmonate and Ethylene-Responsive Factor 1* (*JERF1* and *JERF3* (Zhang *et al*. 2010a,b), *Telomeric Repeat Binding Factor 1* (*TERF1*; Gao *et al*. 2008), and *Hordeum vulgare C-Repeat-Binding Factor 4* (*HvCBF4*; Oh *et al*. 2007) increase tolerance to salinity in rice. Transgenic tobacco plants expressing *SodERF3* (Trujillo *et al*. 2008), *JERF3* (Wu *et al*. 2008), *GmERF3* (Zhang *et al*. 2009), *JERF1* (Wu *et al*. 2007), *GmERF7* (Zhai *et al*. 2013) also show enhanced tolerance to salt stress. Overexpression of *HvRAF* (Jung *et al*. 2007), *HARDY* (*HRD*; Karaba *et al*. 2007), *DREB2* (Nakashima *et al*. 2000), *BrERF4* (Seo *et al*. 2010), *Related to AP2.6* (*RAP2.6*; Zhu *et al*. 2010) in *Arabidopsis* result in enhanced resistance to salt stress. Ecotopic expression of *GmDREB1*, a gene from a wild type soybean, enhances salt resistance in transgenic alfalfa (Jin *et al*. 2010). More specifically, TaERF3 has been shown to positively regulate wheat adaptation responses to salt stress through the activation of stress-related genes (Rong *et al*. 2014). However, over-expression of some *ERF* genes has been reported to repress the stress response and render the transgenic plants more sensitive to stresses (Yang *et al*. 2005, Zhang *et al*. 2007).

A total of 147 genes encoding AP2/ERFs in *Arabidopsis* have been identified, and 122 of them encode ERF transcription factors (Nakano *et al*. 2006), which can be further classified into 12 different groups, namely, groups I to X, VI-L and Xb-L (Nakano *et al*. 2006). Four ERFs in group IX, namely ERF95, ERF96, ERF97, and ERF98 contain only 131 - 139 amino acids and do not have an obvious activation or repression domain, thus were named small ERFs (Wang *et al*. 2015a). Among them, ERF95 and ERF98 have been shown to be involved in the regulation of salt tolerance (Zhang *et al*. 2011, 2012), ERF96 and ERF97 (previously named AtERF14) have been shown to regulate plant defense response (Oñate-Sánchez *et al*. 2007, Catinot *et al*. 2015). ERF96 has also been shown to regulate abscisic acid (ABA) response (Wang *et al*. 2015*a*). However, it is unclear if ERF96 may be involved in the regulation of abiotic stresses. Here we provide evidence that ERF96 is involved in the regulation of salt tolerance in *Arabidopsis*.

Materials and methods

Arabidopsis thaliana L. ecotype Columbia (Col-0) was used as wild type (WT). The plants about 5-week-old with several mature flowers on the main inflorescence were used for transformation to generate *35S:HA-ERF95* transgenic plants. To generate *35S:HA-ERF95* construct, the full-length open-reading frame (ORF) of *ERF95* was amplified by reverse transcription (RT)-PCR using RNA isolated from 10-d-old *Arabidopsis* seedlings, and cloned in-frame with an N-terminal HA tag under the control of the double *35S* enhancer promoter of *CaMV* in the *pUC19* vector (Wang *et al*. 2005) The *35S:HA-ERF95* construct was then digested with proper enzymes, and sub-cloned into binary vector *pPZP211* (Hajdukiewicz *et al*. 1994). The primers used to amplify *ERF95* are 5'-ATGGAACGTATAGAGTCTTATAAC-3' and 5'-TAGGGTTTGCGTCGTTAC-3'. The *35S:HA-ERF96* transgenic plants have been described previously (Wang *et al*. 2015a).

Plants were transformed by using the floral dip method (Clough and Bent 1998). Then, T1 seeds were planted on 1/2 Murashige and Skoog (MS) medium containing 50 μg cm^{-3} kanamycin and 100 μg cm^{-3} carbenicillin to select transgenic plants. Overexpression of *ERF95* in the transgenic plants was confirmed by RT-PCR. More than five independent overexpression lines were obtained, and two lines *ERF95* and *ERF96* were selected for further analyses (for more detail see Wang *et al*. 2015*a*).

The seeds of WT and transgenic plants were surface sterilized and sown on 1/2 MS basal medium plates with vitamins, 1 % (m/v) sucrose, and 0.6 % (m/v) phytoagar (*Plantmedia*, USA). The plates were kept in darkness at 4 °C for 2 d before they were transferred to growth room with a temperature of 22 °C, a 14-h photoperiod, and an irradiance of approximately 120 μmol m^{-2} s^{-1}.

Sterilized seeds were sown on 1/2 MS medium with or without 120 mM NaCl. Seed germination was scored 24, 36, and 48 h after the plates had been transferred into the growth room. Green seedlings were scored 10 d after the transfer. For fresh mass assay, 4-d-old seedlings were transferred to 1/2 MS medium with or without the presence of 170 mM NaCl and grown vertically. Fresh mass was measured 4 d after transfer. A minimum 10 seedlings per genotype were used. The best NaCl concentration was selected for different tests.

For RNA isolation, 10-d-old WT and *ERF95* and *ERF96* transgenic seedlings grown vertically on 1/2 MS plates were transferred to and incubated in 1/2 MS liquid medium (without phytoagar) for 90 min, then treated with 100 mM NaCl for 4 h before the seedlings were frozen in liquid N_2. Total RNA was isolated as described previously (Wang *et al*. 2014, 2015b, Guo *et al*. 2015). The absorbance A_{260}/A_{280} ratios of the isolated RNA are listed in Table 1 Suppl. Total RNA (1 µg) was subjected to cDNA synthesis by using *Omniscript* RT kit (*Qiagen*, Germany) and following the manufacture's instructions. Real time quantitative PCR (qPCR) was used to examine the expression of NaCl-responsive genes including responsive drought 29a (*RD29A*; Nakashima *et al*. 2006), Δ^1-pyrroline-5-carboxylate synthetase (*P5CS*; Strizhov *et al*. 1997), cold-responsive 15a (*COR15A*; Liu *et al*. 2014), and kinase 1 (*KIN1*), and genes related to Na^+/K^+ metabolism including stelar K^+ outward rectifier (*SKOR*) and potassium transporter 2/3 (*AKT2/3*; Marten *et al*. 1999). The expression of *actin 2* (*ACT2*) was used as control. The primers used for RT-qPCR examination as well as the efficiency are listed in Table 2 Suppl.

To examine the expression of small *ERFs* to ABA, 10-d-old WT seedlings were treated with 50 µM ABA as described previously (Tian *et al*. 2015).

For determination of proline and Na^+ and K^+ content, WT and *ERF96* transgenic seedlings were grown vertically on 1/2 MS plates containing 100 mM NaCl for 12 d. Seedlings were washed with distilled water and dried in a hot-air oven at 100 °C for 10 min and then at 60 °C to a constant mass. The dried sample was soaked with 10 cm^3 of deionized water and kept at 100 °C for 1 h. The extract was used for determining the content of Na^+ and K^+ using an atomic absorption spectrophotometer (*TAS-990*, *Purkinje General*, Beijing, China). In the same extract, proline content was measured by ninhydrin method following the procedures described by Zhu *et al*. (1983). The assays were repeated three times.

Statistical analysis was performed by using Student-Newman-Keuls *q*-test. The term significant indicates differences at $P \leq 0.05$.

Results

It has been previously reported that *ERF95* is able to regulate plant response to salt stress (Zhang *et al*. 2012), Because *ERF96* is closely related to *ERF95*, and *ERF96* and *ERF95* have similar expression pattern in *Arabidopsis* (Wang *et al*. 2015a), we wanted to examine if *ERF96* may be also involved in the salt response in *Arabidopsis*. To do that, we first determined the expression of *ERF96* in response to NaCl, and a 12-fold elevated expression of *ERF96* was observed in response to NaCl treatment (Fig. 1). We also examined the expression of the other small *ERF* genes in response to salt; we found that all of them were induced by NaCl with a 40-, 8-, and 21-fold increased expressions of *ERF95*, *ERF97*, and *ERF98,* respectively, when compared with wild-type (Col WT). Because *ERF96* is involved in the regulation of the ABA response in *Arabidopsis* (Wang *et al*. 2015a), we also examined the response of small *ERFs* including *EFRF96* to ABA, however, only slightly increased expressions were observed (Fig. 1).

Having shown that the expressions of both *ERF96* and *ERF95* were elevated after NaCl treatment (Fig. 1), we examined the responses of the transgenic plant lines to NaCl. Firstly we examined seed germination of the transgenic plants on ½ MS medium containing 120 mM NaCl. We found that both *ERF96* and *ERF95* transgenic plant seeds geminated better than the Col WT seeds on medium with 120 mM NaCl (Fig. 2). After 48 h, nearly all the transgenic plant seeds were germinated, but only about 70 % of the Col WT seeds were germinated (Fig. 2). Further, we found that 10 d after the seeds were sown on NaCl plates, all the *ERF96* and *ERF95* transgenic seedlings were green, but some of the Col WT seedlings showed a white colour. Quantitative results showed that green seedling ratio for the transgenic plants was 100 %, but that of the Col WT plants was only about 60 % (Fig. 3*A*). We also examined the effect of NaCl on seedling growth. After transferring 4-d-old seedlings on plates with 170 mM NaCl, plant fresh mass was greatly decreased but more in the Col WT seedlings than in the transgenic plant seedlings (Fig. 3*B*). It should be noted that the *ERF96* and *ERF95* transgenic seedlings was largely indistinguishable in terms of NaCl inhibited seed germination and seedling establishment (Figs. 2, 3*A*).

Because *ERF96* transgenic plants displayed enhanced

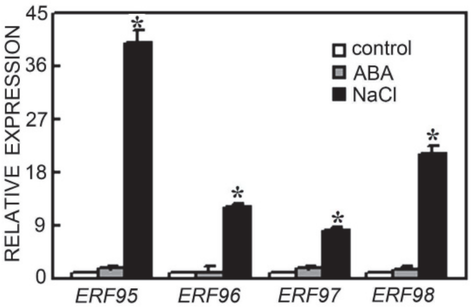

Fig. 1. Expression of *ERF95*, *ERF96*, *ERF97*, and *ERF98* in response to NaCl and ABA treatments. Wilde-type *Arabidopsis* seedlings were treated with 100 mM NaCl or 50 µM ABA for 4 h, then total RNA was isolated, and the expressions of small *ERF* genes were examined. The expression of *ACT2* was used as an internal control, and the expression of corresponding *ERF* genes in control seedlings was set at 1. Means ± SDs of three biological replicates; * - significantly different from control ($P < 0.001$).

tolerance to salt (Figs. 2, 3), we decided to examine if the expression of other small *ERFs* may be affected (Fig. 1 Suppl.) We found that the expression of *ERF95* increased 2 to 4-fold, expression of *ERF97* increased 8 to 10-fold, whereas the expression of *ERF98* remained largely unchanged in the *ERF96* transgenic plants.

We further examined expression of some NaCl-responsive genes in transgenic plants treated with 100 mM NaCl for 4 h (Fig. 4). Without addition of NaCl, the expression of *RD29A* was increased in the *ERF96* transgenic plants, whereas expressions of the other three genes remained largely unchanged when compared to the Col WT plants. The expressions of *RD29A* and *KIN1* were strongly induced by NaCl treatment in the Col WT seedlings, and further elevated expressions were observed in the transgenic seedlings. On the other hand, NaCl treatment had little, if any effects on the expressions of *P5CS* and *COR15A* in the Col WT seedlings, but dramatically induced their expressions in the *ERF96* transgenic plants (Fig. 4).

Proline is one of the osmolytes which accumulates in plants in response to salt stress, and the accumulation of proline could enhance tolerance to salt (Leigh 1997). Therefore, we examined proline content in the *ERF96* transgenic plants. We found that proline content increased in both WT and the transgenic plants in response to salt treatment, however, relatively more in the transgenic plants (Fig. 5*A*).

Having shown that transgenic plants overexpressing *ERF96* showed enhanced tolerance to NaCl, we further examined whether they displayed altered Na^+ and K^+ content in response to NaCl treatment. Under control conditions, Na^+ and K^+ content in the *ERF96* transgenic plants was largely consistent with that in the Col WT plants. A more than 4-fold increase and a more than 2-fold decrease of Na^+ and of K^+, respectively, was observed in the Col WT plants after NaCl treatment (Fig. 5*B*). In the *ERF96* transgenic plants, however, Na^+ content only increased about 3-fold, and K^+ decreased about 20 % (Fig. 5*B*), *i.e.*, under salt treatment *ERF96* transgenic plants contained lower amount of Na^+ and higher content of K^+ than the wild-type. These results indicate that ERF96 may be involved in the regulation of Na^+ and K^+ homeostasis. Consistent with this, RT-qPCR

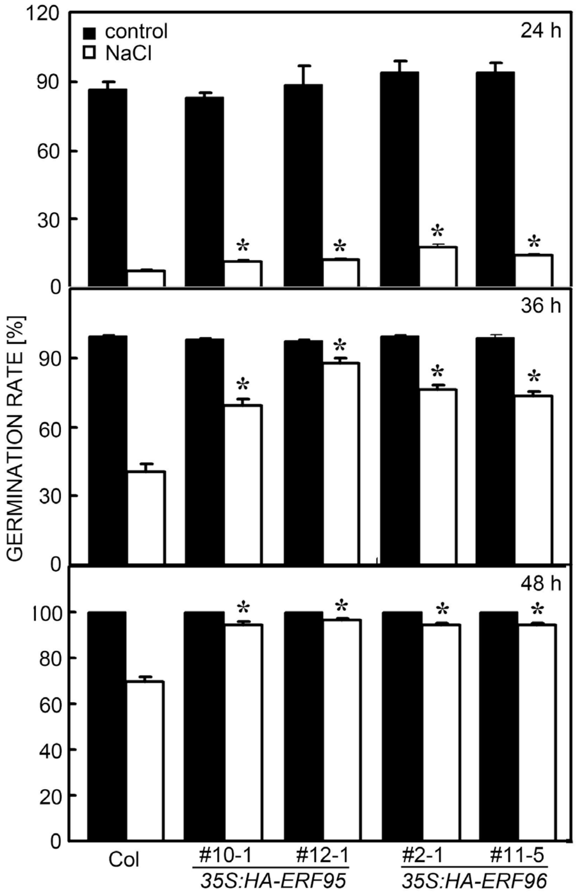

Fig. 2. Effects of NaCl on seed germination of the transgenic plants. Seeds of the Col wild type and the *35S:HA-ERF95* and *35S:HA-ERF96* transgenic plants were sown on plates in the presence or absence of 120 mM NaCl and the percentage of seed germination was scored after 24, 36, and 48 h. Means ± SDs of three replicates; * - significantly different from the WT in the presence of NaCl ($P < 0.05$).

results show that the expressions of *SKOR* and *AKT2/3* genes related to Na^+/K^+ homeostasis (Maathuis 2006, Marten *et al*. 1999) were elevated in *ERF96* transgenic plants in response to salt treatment, when compared with those in the Col WT seedlings (Fig. 6).

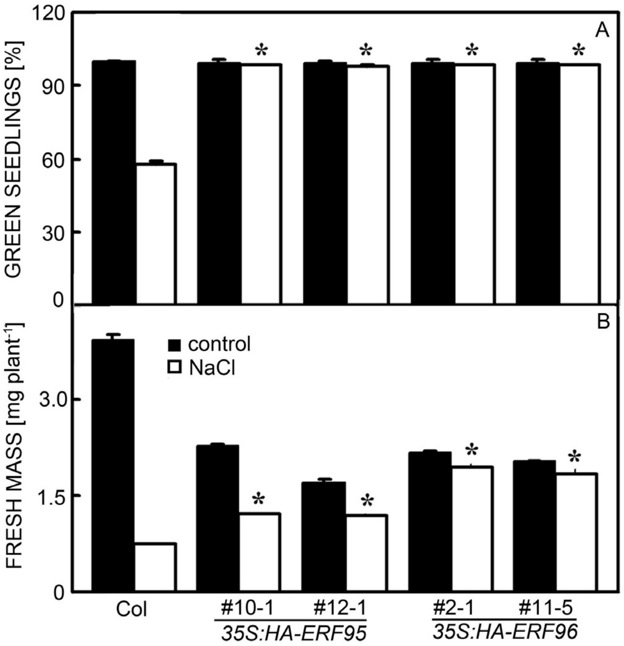

Fig. 3. Effects of NaCl on seedling greening and fresh mass. *A* - Seeds were sown on plates with or without 120 mM NaCl and after 10 d the percentage of green seedlings was scored. *B* - The fresh mass of Col WT and transgenic plants was measured 4 d after the seedlings were transferred to 1/2 MS medium containing 170 mM NaCl. Means ± SDs, $n = 3$ in *A* or 10 in *B*; * - significantly different from the Col WT in the presence of NaCl ($P < 0.05$).

Discussion

The expression of some *ERFs* such as *RAP2.6* has been shown to be regulated by both ABA and abiotic stresses including salinity, and transgenic *Arabidopsis* seedlings overexpressing *RAP2.6* showed increased sensitivity to ABA and abiotic stresses (Zhu *et al*. 2010). ERF96 has been shown to regulate plant defense response and ABA response (Catinot *et al*. 2015, Wang *et al*. 2015*a*). In this study, we provide evidence that expression of *ERF96* was regulated by salt, and ERF96 played a role in the regulation of salt tolerance in *Arabidopsis*. However, it is still unclear if ERF96 regulation of salt tolerance is independent of ABA signaling.

Previous studies have shown that ERF95 and ERF98 are involved in the regulation of plant response to salt stress (Zhang *et al*. 2011, 2012). *ERF95* is one of the target genes of the ethylene signaling component EIN3, which can bind to the *ERF95* promoter, and *ERF95* can bind to the *DRE* and the *GCC* box to activate the expression of salt-related genes, leading to enhanced tolerance to salt stress (Zhang *et al*. 2011). Whereas ERF98 enhances the salt tolerance of *Arabidopsis* by mediating the activation of ascorbic acid (AsA) synthesis, it can directly interact with the *DRE*-containing region of the AsA synthesis gene *VTC1* promoter and activate its expression, which results in increased content of AsA, allowing plant cells to avoid damage caused by oxidative stress (Zhang *et al*. 2012).

ERF96-RNAi plants have been reported to demonstrate a wild type defense response (Catinot *et al*. 2015), and the *erf96-1* mutant seedlings show a wild type ABA response (Wang *et al*. 2015*a*). Although we examined the salt response of the *erf96-1* mutant seedlings, and found it had a near wild-type response in all the above mentioned assays, our data showed that *ERF96* transgenic plants have enhanced tolerance to salt stress (Figs. 2, 3). Further, the expressions of some NaCl-responsive genes including *RD29A*, *P5CS*, *COR15A,* and *KIN1* were elevated in the transgenic plants under salt stress (Fig. 4). Among them, *P5CS* gene encodes Δ^1-pyrroline-5-carboxylate synthetase, which is the key enzyme in proline biosynthesis in plants, and the elevated expression of *P5CS* was consistent with the increased proline content (Fig. 5*A*).

Salt stress affects the ionic homeostasis in plant cells, especially Na^+ and K^+ content (Miller *et al*. 2010). In general, low Na^+ and high K^+ in the cytoplasm are essential for the maintenance of a number of enzymatic processes (Munns and Tester 2008). However, because of the similarity of the hydrated ionic radii of Na^+ and K^+, Na^+ can compete with K^+ for major binding sites in some key enzymes involved in the metabolic processes in the cytoplasm (Marschner 1995). With over 50 cytoplasmic enzymes being activated by K^+, the effects of disruption of ionic homeostasis to metabolism is severe (Munns and Tester 2008). Indeed, keeping low Na^+ and high K^+ content has been suggested as a key determinant of plant salt tolerance (Colmer *et al.* 2006). In this study, we

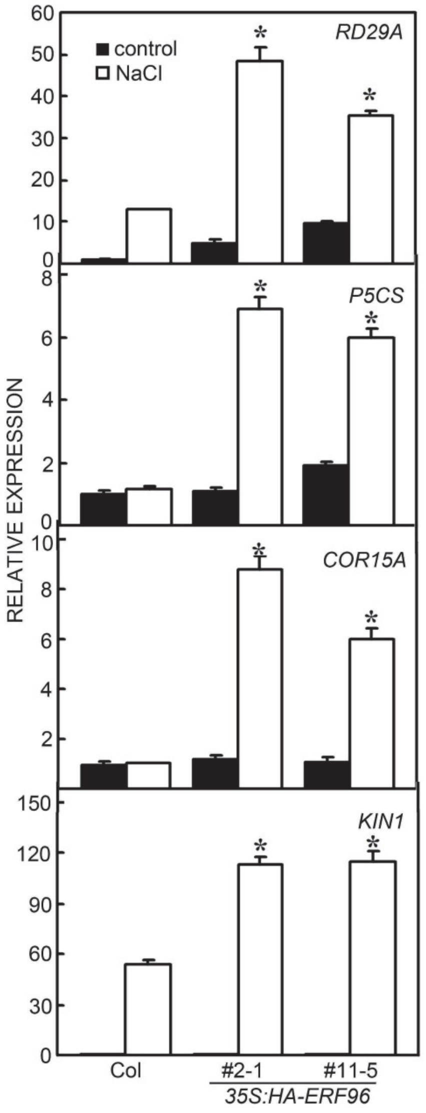

Fig. 4. Expressions of NaCl-responsive genes in the *35S:HA-ERF96* transgenic 10-d-old seedlings treated with 100 mM NaCl for 4 h. The RT-qPCR was used to examine the expression of *RD29A*, *P5CS*, *COR15A*, and *KIN1* genes. The expression of *ACT2* was used as an internal control, and the expression of the corresponding gene in the Col WT seedlings was set to 1. Means ± SDs of three biological replicates; * - significantly different from expression in the Col WT seedlings in the presence of NaCl ($P < 0.05$).

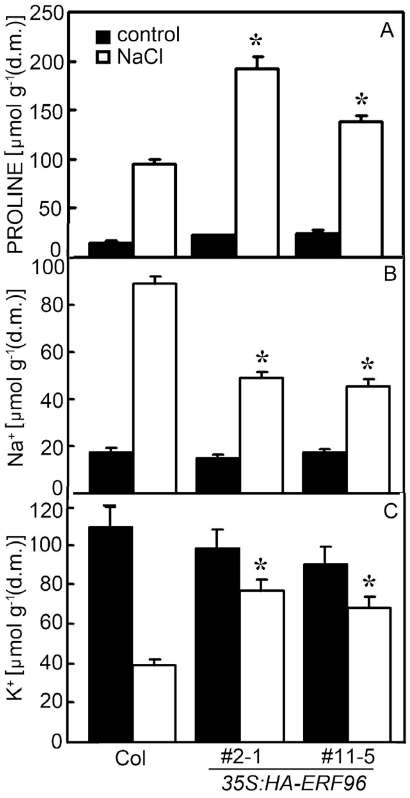

Fig. 5. Effects of 100 mM NaCl applied for 12 d on proline (*A*), Na^+ (*B*, *upper panel*), and K^+ (*B*, *lower panel*) content in the Col wild type and the *35S:HA-ERF96* transgenic plant seedlings. Means ± SDs of three replicates; * - significantly different from that in the Col WT seedlings in the presence of NaCl ($P < 0.05$).

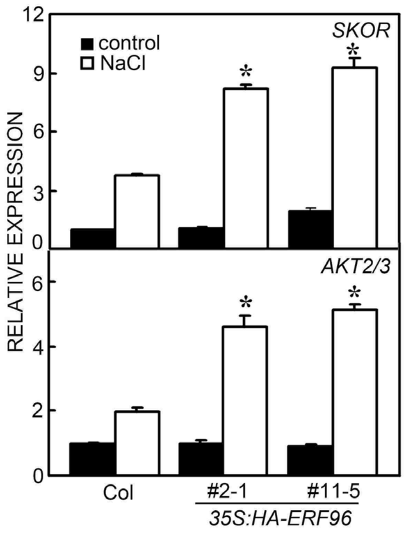

Fig. 6. Expressions of genes related to Na^+/K^+ homeostasis in the Col wild type and the *35S:HA-ERF96* transgenic 10-d-old seedlings treated with 100 mM NaCl for 4 h. The expression of *ACT2* was used as an internal control, and the expression of the corresponding gene in the control Col WT seedlings was set to 1. Means ± SDs of three biological replicates; * - significantly different from that in the Col WT seedlings in the presence of NaCl ($P < 0.05$).

found that *ERF96* transgenic plants contained lesser content of Na^+ and higher content of K^+ under salt treatment (Fig. 5*B*). Thus ERF96 may regulate NaCl tolerance by affecting the homeostasis of Na^+ and K^+. Because SKOR is Na^+/K^+ transporter and AKT2/3 functions as K^+ channel protein (Marten *et al.* 1999, Maathuis 2006), they may affect Na^+/K^+ homeostasis in plants. Our results showed that the expression of *SKOR* and *AKT2/3* was induced by salt treatment in the Col WT plants and their expression was further elevated in the *ERF96* transgenic plants in response to salt stress (Fig. 6). These results demonstrated that ERF96 regulate the homeostasis of Na^+ and K^+ possibly by affecting the expression of genes related to Na^+/K^+ metabolism. In conclusion, our results suggest a positive role of ERF96 in the regulation of salt tolerance in *Arabidopsis*.

References

Agarwal, P., Agarwal, P.K., Joshi, A.J., Sopory, S.K., Reddy, M.K.: Overexpression of *PgDREB2A* transcription factor enhances abiotic stress tolerance and activates downstream stress-responsive genes. - Mol. Biol. Rep. **37**: 1125-1135, 2010.

Agarwal, P.K., Agarwal, P., Reddy, M.K., Sopory, S.K.: Role of DREB transcription factors in abiotic and biotic stress tolerance in plants. - Plant Cell Rep. **25**: 1263-1274, 2006.

Aharoni, A., Dixit, S., Jetter, R., Thoenes, E., Van Arkel, G., Pereira, A.: The SHINE clade of AP2 domain transcription factors activates wax biosynthesis, alters cuticle properties, and confers drought tolerance when overexpressed in *Arabidopsis*. - Plant Cell **16**: 2463-2480, 2004.

Alonso, J.M., Stepanova, A.N., Leisse, T.J., Kim, C.J., Chen, H., Shinn, P., Stevenson, D.K., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C.C., Parker, H., Prednis, L., Ansari, Y., Choy, N., Deen, H., Geralt, M., Hazari, N., Hom, E., Karnes, M., Mulholland, C., Ndubaku, R., Schmidt, I., Guzman, P., Aguilar-Henonin, L., Schmid, M., Weigel, D., Carter, D.E., Marchand, T., Risseeuw, E., Brogden, D., Zeko, A., Crosby, W.L., Berry, C.C., Ecker, J.R.: Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. - Science **301**: 653-657, 2003.

Aukerman, M.J., Sakai, H.: Regulation of flowering time and floral organ identity by a microRNA and its *APETALA2*-like target genes. - Plant Cell **15**: 2730-2741, 2003.

Broun, P., Poindexter, P., Osborne, E., Jiang, C.Z., Riechmann, J.L.: WIN1, a transcriptional activator of epidermal wax accumulation in *Arabidopsis*. - Proc. nat. Acad. Sci. USA **101**: 4706-4711, 2004.

Catinot, J., Huang, J.B., Huang, P.Y., Tseng, M.Y., Chen, Y.L., Gu, S.Y., Lo, W.S., Wang, L.C., Chen, Y.R., Zimmerli, L.: ETHYLENE RESPONSE FACTOR 96 positively regulates *Arabidopsis* resistance to necrotrophic pathogens by direct binding to GCC elements of jasmonate- and ethylene-responsive defence genes. - Plant Cell Environ. **38**: 2721-2734, 2015.

Century, K., Reuber, T.L., Ratcliffe, O.J.: Regulating the regulators: the future prospects for transcription-factor-based agricultural biotechnology products. - Plant Physiol. **147**: 20-29, 2008.

Chakravarthy, S., Tuori, R.P., D'Ascenzo, M.D., Fobert, P.R., Despres, C., Martin, G.B.: The tomato transcription factor Pti4 regulates defense-related gene expression *via* GCC box and non-GCC box *cis* elements. - Plant Cell **15**: 3033-3050, 2003.

Clough, S.J., Bent, A.F.: Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. - Plant J. **16**: 735-743, 1998.

Colmer, T.D., Flowers, T.J., Munns, R.: Use of wild relatives to improve salt tolerance in wheat. - J. exp. Bot. **57**: 1059-1078, 2006.

El Ouakfaoui, S., Schnell, J., Abdeen, A., Colville, A., Labbé, H., Han, S., Baum, B., Laberge, S., Miki, B.: Control of somatic embryogenesis and embryo development by AP2 transcription factors. - Plant mol. Biol. Rep. **74**: 313-26, 2010.

Eulgem, T., Somssich, I.E.: Networks of WRKY transcription factors in defense signaling. - Curr. Opin. Plant Biol. **10**: 366-371, 2007.

Gao, S., Zhang, H., Tian, Y., Li, F., Zhang, Z., Lu,X., Chen, X., Huang, R.: Expression of *TERF1* in rice regulates expression of stress-responsive genes and enhances tolerance to drought and high-salinity. - Plant Cell Rep. **27**: 1787-1795, 2008.

Giri, M.K., Swain, S., Gautam, J.K., Singh, S., Bhattacharjee, L., Nandi, A.K.: The *Arabidopsis thaliana At4g13040* gene, a unique member of the AP2/EREBP family is a positive regulator for salicylic acid accumulation and basal defense against bacterial pathogens. - J. Plant Physiol. **171**: 860-967, 2014.

Guo, H., Zhang, W., Tian, H., Zheng, K., Dai, X., Liu, S., Hu, Q., Wang, X., Liu, B., Wang, S.: An auxin responsive *CLE* gene regulates shoot apical meristem development in *Arabidopsis*. - Front. Plant Sci. **6**: 295, 2015.

Hajdukiewicz, P., Svab, Z., Maliga, P.: The small, versatile *pPZP* family of *Agrobacterium* binary vectors for plant transformation. - Plant mol. Biol. **25**: 989-994, 1994.

Hu, Y.X., Wang, Y.H., Li, X.F., Li, J.Y.: *Arabidopsis RAV1* is down-regulated by brassinosteroid and may act as a negative regulator during plant development. - Cell Res. **14**: 8-15, 2004.

Ito, Y., Katsura, K., Maruyama, K., Taji, T., Kobayashi, M., Seki, M., Shinozaki, K., Yamaguchi-Shinozaki, K.: Functional analysis of rice DREB1/CBF-type transcription factors involved in cold-responsive gene expression in transgenic rice. - Plant Cell Physiol. **47**: 141-153, 2006.

Jin, T., Chang, Q., Li, W., Yin, D., Li, Z., Wang, D., Liu, B., Liu, L.X.: Stress-inducible expression of *GmDREB1* conferred salt tolerance in transgenic alfalfa. - Plant Cell Tissue Organ Cult. **100**: 219-227, 2010.

Jofuku, K.D., Den Boer, B.G., Van Montagu, M., Okamuro, J.K.: Control of *Arabidopsis* flower and seed development by the homeotic gene *APETALA2*. - Plant Cell **6**: 1211-1225, 1994.

Jung, J., Won, S.Y., Suh, S.C., Kim, H., Wing, R., Jeong, Y., Hwang, I., Kim, M.: The barley ERF-type transcription factor HvRAF confers enhanced pathogen resistance and salt tolerance in *Arabidopsis*. - Planta **225**: 575-588, 2007.

Karaba, A., Dixit, S., Greco, R., Aharoni, A., Trijatmiko, K.R., Marsch-Martinez, N., Krishnan, A., Nataraja, K.N., Udayakumar, M., Pereira, A.: Improvement of water use efficiency in rice by expression of *HARDY*, an *Arabidopsis* drought and salt tolerance gene. - Proc. nat Acad. Sci. USA **104**: 15270-15275, 2007.

Kang, J.Y., Choi, H.I., Im, M.Y., Kim, S.Y.: *Arabidopsis* basic leucine zipper proteins that mediate stress-responsive abscisic acid signaling. - Plant Cell **14**: 343-357, 2002.

Kirch, T., Simon, R., Grünewald, M., Werr, W.: The *DORNRÖSCHEN/ENHANCER OF SHOOT REGENERATION1* gene of *Arabidopsis* acts in the control of meristem cell fate and lateral organ development. - Plant Cell **15**: 694-705, 2003.

Kitomi, Y., Ito, H., Hobo, T., Aya, K., Kitano, H., Inukai, Y.: The auxin responsive AP2/ERF transcription factor *CROWN ROOTLESS 5* is involved in crown root initiation in rice through the induction of *OsRR1*, a type-A response regulator of cytokinin signaling. - Plant J. **67**: 472-484, 2011.

Leigh, R.A.: Solute composition of vacuoles. - Adv. Bot. Res. **25**: 171-194, 1997.

Liu, D., Li, W., Cheng, J., Hou, L.: Expression analysis and functional characterization of a cold-responsive gene *COR15A* from *Arabidopsis thaliana*. - Acta Physiol. Plant. **36**: 2421-2432, 2014.

Maathuis, F.J.: The role of monovalent cation transporters in plant responses to salinity. - J. exp. Bot. **57**: 1137-1147, 2006.

Marschner, H.: The Mineral Nutrition of Higher Plants. 2nd Ed. - Academic Press, London 1995.

Marten, I., Hoth, S., Deeken, R., Ache, P., Ketchum, K.A., Hoshi, T., Hedrich, R.: AKT3, a phloem-localized K^+ channel, is blocked by protons. - Proc. nat. Acad. Sci. USA **96**: 7581-7586, 1999.

Miller, G.A.D., Suzuki, N., Ciftci-Yilmaz, S., Mittler, R.O.N.: Reactive oxygen species homeostasis and signalling during drought and salinity stresses. - Plant Cell Environ. **33**: 453-467, 2010.

Munns, R., Tester, M.: Mechanisms of salinity tolerance. - Annu. Rev. Plant Biol. **59**: 651-681, 2008.

Nakano, T., Suzuki, K., Fujimura, T., Shinshi, H.: Genome-wide analysis of the *ERF* gene family in *Arabidopsis* and rice. - Plant Physiol. **140**: 411-432, 2006.

Nakashima, K., Shinwari, Z., Sakuma, Y., Seki, M., Miura, S., Shinozaki, K., Yamaguchi-Shinozaki, K.: Organization and expression of two *Arabidopsis DREB2* genes encoding DRE-binding proteins involved in dehydration- and high-salinity-responsive gene expression. - Plant mol. Biol. **42**: 657-665, 2000.

Nakashima, K., Fujita, Y., Katsura, K., Maruyama, K., Narusaka, Y., Seki, M., Shinozaki, K., Yamaguchi-Shinozaki, K.: Transcriptional regulation of ABI3- and ABA-responsive genes including *RD29B* and *RD29A* in seeds, germinating embryos, and seedlings of *Arabidopsis*. - Plant mol. Biol. **60**: 51-68, 2006.

Oh, S., Kwon, C., Choi, D., Song, S., Kim, J.: Expression of barley *HvCBF4* enhances tolerance to abiotic stress in transgenic rice. - Plant Biotechnol. J. **5**: 646-656, 2007.

Oh, S.J., Kim, Y.S., Kwon, C.W., Park, H.K., Jeong, J.S., Kim, J.K.: Overexpression of the transcription factor *AP37* in rice improves grain yield under drought condition. - Plant Physiol. **150**: 1368-1379, 2009.

Ohme-Takagi, M., Shinshi, H.: Ethylene-inducible DNA binding proteins that interact with an ethylene-responsive element. - Plant Cell **7**: 173-182, 1995.

Oñate-Sánchez, L., Anderson, J.P., Young, J., Singh, K.B.: AtERF14, a member of the ERF family of transcription factors, plays a non-redundant role in plant defense. - Plant Physiol. **43**: 400-409, 2007.

Rong, W., Qi, L., Wang, A., Ye, X., Du, L., Liang, H., Xin, Z., Zhang, Z.: The ERF transcription factor TaERF3 promotes tolerance to salt and drought stresses in wheat. - Plant Biotechnol. J. **12**: 468-479, 2014.

Salvi, S., Sponza, G., Morgante, M., Tomes, D., Niu, X., Fengler, K.A., Meeley, R., Ananiev, E.V., Svitashev, S., Bruggemann, E., Li, B., Hainey, C.F., Radovic, S., Zaina, G., Rafalski, J.A., Tingey, S.V., Miao, G.H., Phillips, R.L., Tuberosa, R.: Conserved noncoding genomic sequences associated with a flowering-time quantitative trait locus in maize. - Proc. nat. Acad. Sci. USA **104**: 11376-11381, 2007.

Singh, K., Foley, R.C., Oñate-Sánchez, L.: Transcription factors in plant defense and stress responses. - Curr. Opin. Plant Biol. **5**: 430-436, 2002.

Seo, Y., Park, J.B., Cho, Y.J., Jung, C., Seo, H., Park, S.K., Nahm, B.H., Song, J.T.: Overexpression of the ethylene-responsive factor gene *BrERF4* from *Brassica rapa* increases tolerance to salt and drought in *Arabidopsis* plants. - Mol. Cells **30**: 271-277, 2010.

Strizhov, N., Abrahám, E., Okrész, L., Blickling, S., Zilberstein, A., Schell, J., Koncz, C., Szabados, L.: Differential expression of two *P5CS* genes controlling proline accumulation during salt-stress requires ABA and is regulated by *ABA1*, *ABI1* and *AXR2* in *Arabidopsis*. - Plant J. **12**: 557-569, 1997.

Tian, H., Guo, H., Dai, X., Cheng, Y., Zheng, K., Wang, X., Wang, S.: An ABA down-regulated bHLH transcription repressor gene, *bHLH129* regulates root elongation and ABA response when overexpressed in *Arabidopsis*. -Sci. Rep. **5**: 17587, 2015.

Tähtiharju, S., Sangwan, V., Monroy, A.F., Dhindsa, R.S., Borg, M.: The induction of *kin* genes in cold-acclimating *Arabidopsis thaliana*. Evidence of a role for calcium. - Planta **203**: 442-447, 1997.

Trujillo, L.E., Sotolongo, M., Menendez, C., Ochogavia, M.E., Coll, Y., Hernandez, I., Borrás-Hidalgo, O., Thomma, B.P., Vera, P., Hernández, L.: SodERF3, a novel sugarcane ethylene responsive factor (ERF), enhances salt and drought tolerance when overexpressed in tobacco plants. - Plant Cell Physiol. **49**: 512-525, 2008.

Wang, S., Li, E., Porth, I., Chen, J.G., Mansfield, S.D., Douglas, C.J.: Regulation of secondary cell wall biosynthesis by poplar R2R3 MYB transcription factor PtrMYB152 in *Arabidopsis*. - Sci. Rep. **4**: 5054, 2014.

Wang, S., Tiwari, S.B., Hagen, G., Guilfoyle, T.J.: AUXIN RESPONSE FACTOR7 restores the expression of auxin-responsive genes in mutant *Arabidopsis* leaf mesophyll protoplasts. - Plant Cell **17**: 1979-1993, 2005.

Wang, X., Liu, S., Tian, H., Wang, S., Chen, J.G.: The small Ethylene Response Factor ERF96 is involved in the regulation of the abscisic acid response in *Arabidopsis*. - Front. Plant Sci. **6**:1064, 2015a.

Wang, X., Wang, X., Hu, Q., Dai, X., Tian, H., Zheng, K., Wang, X., Mao, T., Chen, J.G., Wang, S.: Characterization of an activation-tagged mutant uncovers a role of

GLABRA2 in anthocyanin biosynthesis in *Arabidopsis*. - Plant J. **83**: 300-311, 2015b.

Wu, L., Chen, X., Ren, H., Zhang, Z., Zhang, H., Wang, J,. Wang, X.C., Huang, R.: ERF protein JERF1 that transcriptionally modulates the expression of abscisic acid biosynthesis-related gene enhances the tolerance under salinity and cold in tobacco. - Planta **226**: 815-825, 2007.

Wu, L., Zhang, Z., Zhang, H., Wang, X., Huang, R.: Transcriptional modulation of ethylene response factor protein JERF3 in the oxidative stress response enhances tolerance of tobacco seedlings to salt, drought, and freezing. - Plant Physiol. **148**: 1953-1963, 2008.

Xiong, L., Schumaker, K.S., Zhu, J.K.: Cell signaling during cold, drought, and salt stress. - Plant Cell **14** (Suppl): S165-S183, 2002.

Yamaguchi-Shinozaki, K., Shinozaki, K.: Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. - Annu. Rev. Plant Biol. **57**: 781-803, 2006.

Yang, Z., Tian, L., Latoszek-Green, M., Brown, D., Wu, K.: *Arabidopsis* ERF4 is a transcriptional repressor capable of modulating ethylene and abscisic acid responses. - Plant mol. Biol. **58**: 585-596, 2005.

Yin, X., Allan, A.C., Chen, K., Ferguson, I.B.: Kiwifruit *EIL* and *ERF* genes involved in regulating fruit ripening. - Plant Physiol. **153**: 1280-1292, 2010.

Zhai, Y., Wang, Y., Li, Y., Lei, T., Yan, F., Su, L., Li, X., Zhao, Y., Sun, X., Li, J., Wang, Q.: Isolation and molecular characterization of *GmERF7*, a soybean ethylene-response factor that increases salt stress tolerance in tobacco. - Gene **513**: 174-183, 2013.

Zhang, G., Chen, M., Li, L., Xu, Z., Chen, X., Guo, J., Ma, Y.: Overexpression of the soybean *GmERF3* gene, an AP2/ERF type transcription factor for increased tolerances to salt, drought, and diseases in transgenic tobacco. - J. exp. Bot. **60**: 3781-3796, 2009.

Zhang, H., Li, W., Chen, J., Yang, Y., Zhang, Z., Zhang, H., Wang, X.C., Huang, R.: Transcriptional activator TSRF1 reversely regulates pathogen resistance and osmotic stress tolerance in tobacco. -Plant Mol. Biol. **63**: 63-71, 2007.

Zhang, H., Liu, W., Wan, L., Li, F., Dai, L., Li, D., Zhang, Z., Huang, R.: Functional analyses of ethylene response factor JERF3 with the aim of improving tolerance to drought and osmotic stress in transgenic rice. - Transgenic Res. **19**: 809-818, 2010b.

Zhang, L., Li, Z., Quan, R., Li, G., Wang, R., Huang, R.: An AP_2 domain-containing gene, *ESE_1*, targeted by the ethylene signaling component EIN_3 is important for the salt response in *Arabidopsis*. - Plant Physiol. **157**: 854-865, 2011.

Zhang, Z., Li, F., Li, D., Zhang, H., Huang, R.: Expression of ethylene response factor JERF1 in rice improves tolerance to drought. - Planta **232**: 765-774, 2010a.

Zhang, Z., Wang, J., Zhang, R., Huang, R.: The ethylene response factor AtERF98 enhances tolerance to salt through the transcriptional activation of ascorbic acid synthesis in *Arabidopsis*. - Plant J. **71**: 273-287, 2012.

Zhu, G.L., Deng, X.W., Zuo, W.N.: Determination of free proline in plants. - Plant Physiol. **1**: 35-37, 1983.

Zhu, Q., Zhang, J., Gao, X., Tong, J., Xiao, L., Li, W., Zhang, H.: The *Arabidopsis* AP2/ERF transcription factor *RAP2.6* participates in ABA, salt and osmotic stress responses. - Gene **457**: 1-12, 2010.