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Enhanced salt tolerance in tomato plants constitutively expressing heat-shock protein in the endoplasmic reticulum

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ABSTRACT. The accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER) causes ER stress and activates the unfolded protein response (UPR) signaling pathway. The UPR signaling pathway is associated with plant responses to adverse environmental conditions. Thus, changes in the UPR signaling pathway might affect plant abiotic tolerance. Here, the role of ER small heat-shock protein (ER-sHSP) in improving plant resistance to salt stress was explored. Under salt stress conditions, ER-sHSP transgenic plants were found to have more vigorous roots, maintain a higher relative water content, absorb less Na^+, accumulate more osmolytes and Ca^{2+}, and sustain less damage to the photosystem, compared to wild-type non-transgenic plants. Furthermore, we found that the constitutive expression of ER-sHSP under salt stress depressed the expression of other ER molecular chaperones. These results indicate that the constitutive expression of ER-sHSP enhanced

salinity tolerance of tomato plants significantly, and alleviated the ER stress caused by the salt stress in plant cells.

Key words: Endoplasmic reticulum; Endoplasmic reticulum stress; Endoplasmic reticulum binding protein; Unfolded protein response; Endoplasmic reticulum small heat shock protein; Salt tolerance

INTRODUCTION

The synthesis of secretory and membrane proteins in the endoplasmic reticulum (ER) is a complex process regulated by a protein quality control system. A variety of molecular chaperones and foldases are involved in this system (Brodsky and McCracken, 1999; Halperin et al., 2014). Molecular chaperones, such as the ER binding protein (BiP), help newly synthesized proteins to assume their correct conformations, or associate with unrecoverable proteins to prevent them from exiting the ER until they are digested (Gething, 1999). In addition, protein disulfide isomerase (PDI), which catalyzes the formation or the rearrangement of disulfide bonds, participates in the quality control process of ER proteins (Noiva, 1999).

The concentrations of chaperones and foldases are usually adequate to deal with protein flux across the ER. In the event that protein translocation through the ER is impeded by adverse factors, the protein homeostasis in the ER will be disrupted by protein overload, causing ER stress. In response to ER stress, cells activate a protective signaling cascade, the unfolded protein response (UPR), to relieve the ER protein overload (Schröder and Kaufman, 2005; Wan and Jiang, 2015). The UPR mainly involves three steps: 1) decreasing the burden on the ER by inhibiting synthesis of secretory proteins at the translation stage; 2) inducing chaperones and foldases to assist with folding of protein and trafficking of vesicles; 3) accelerating degradation of unwanted, unfolded proteins (Martínez and Chrispeels, 2003).

Many factors can cause ER stress in plant cells. In the laboratory, chemical reagents, such as tunicamycin and dithiothreitol (DTT), can be applied to trigger ER stress. Under natural conditions, abiotic stresses can disturb the protein-folding process and lead to ER stress. The ER stress signal is closely connected with the signal pathway of environmental stress responses (Liu et al., 2007a; Liu and Howell, 2010). For example, salt stress leads to changes in the redox state in ER, which further regulates signaling of reactive oxygen species and triggers the antioxidant defense in *Arabidopsis thaliana* (Ozgur et al., 2014).

ER-residing molecular chaperones can alleviate ER stress and enhance abiotic tolerance in plants. ER BiP alleviates ER stress, confers drought tolerance, and delays drought-induced leaf senescence (Alvim et al., 2001; Valente et al., 2009). Similarly, a wheat calreticulin has been shown to relieve ER stress in tobacco cells and to improve tobacco drought tolerance (Jia et al., 2008).

Most ER-residing molecular chaperones are significantly induced by tunicamycin and DTT (Martínez and Chrispeels, 2003; Kamauchi et al., 2005), but the ER-located small heat-shock protein (hereafter ER-sHSP) is an exception (Zhao et al., 2007). ER-sHSP has the functional activity of a molecular chaperone (Zhao et al., 2007; Mamedov and Shono,

2008) and its overexpression can alleviate ER stress caused by tunicamycin and DTT exposure (Zhao et al., 2007). The aim of this study was to determine whether the constitutive expression of ER-sHSP would improve the abiotic tolerance of plants, as does overexpression of BiP (Alvim et al., 2001; Valente et al., 2009) and an increased HSP90 level (Ling et al., 2014). To this end, we examined salt tolerance in transgenic tomato plants expressing ER-sHSP.

MATERIAL AND METHODS

Plant material and growth conditions

The tomato seeds used in this experiment were from the transgenic lines that contained LeERsHSP cDNA and the 35sCaMV promoter (Zhao et al., 2007). We selected LeERsHSP transgenic lines 10 (OE10) and 13 (OE13), in which the LeERsHSP protein was stably and constitutively expressed for five generations (data not shown). As controls, we used wild-type (WT) and a vector control plant with pROK II (VC). Tomato seeds were sown in moist sand pots. The pots were kept in a greenhouse at 25/20°C day/night temperatures and were irrigated with 50% Hoagland solution twice daily. Meanwhile, the heights of plants were measured weekly, to determine the growth rate of plants. When the plants were 1 month old, salt treatments were initiated with 50 mM NaCl in 50% Hoagland solution for 4 days. Thereafter, we increased the NaCl concentration in 50% Hoagland solution by 50 mM every 4 days until the final 4-day treatment of 200 mM NaCl was completed. At this time, the overground parts and the roots of the plants were photographed, and the biomass of root was measured.

Before the beginning of salt treatment and just after the irritation of 100 and 200 mM NaCl, the middle leaves at half the height of the tomato plants were taken as materials for physiological measurements. From each plant, three leaves were sampled for the assay. The roots at the corresponding periods were obtained, and rinsed 3 times with distilled water, and were used as materials for ion content measurements.

Relative water, chlorophyll, and ion contents

Relative water content (RWC) was determined using the following formula: RWC (%) = 100 x (fresh mass - dry mass) / (turgid mass - dry mass), where turgid mass was determined after the leaf was saturated. Chlorophyll content was determined using the method described by Li et al. (2003). K^+, Na^+, and Ca^{2+} contents in tomato leaves and roots were determined using an atomic absorption spectrophotometer (Z-8000; Hitachi, Tokyo, Japan).

Maximum quantum yield of photosystem II photochemistry

We evaluated cellular damage related to photosystem activity. The maximum quantum yield of photosystem II photochemistry was measured with an FMS-2 modulated chlorophyll fluorometer (Hansatech, Kings Lynn, UK). Samples of intact leaves were inserted in leaf clips and dark-adapted for 30 min before the measurement of minimum fluorescence F_0. They were then exposed to saturation pulse light (15,000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 0.7 s), to measure maximum fluorescence F_m. The maximum quantum yield of photosystem II photochemistry is defined as F_v/F_m, in which $F_v = F_m - F_0$.

Net photosynthetic rate, stomatal conductance, transpiration rate, and soluble proline and sugar content

Wilting is a clear symptom of damaged tomato leaves and the rate of water loss in leaves depends partly on the stomatal conductance (g_s) and the transpiration rate. Therefore, we quantified these two physiological components in addition to net photosynthetic rate. Net photosynthetic rate, g_s, and transpiration rate were measured by IR gas analysis using a portable analyzer (CIRAS-2; PP Systems, Herts, UK) at 600 $\mu mol \cdot m^{-2} \cdot s^{-1}$ irradiance. We also measured the contents of two important osmolytes: proline and soluble sugars. Proline content was determined using a colorimetric method involving ninhydrin (Bates et al., 1973). Measurement of soluble sugar content was done following the method of Marschner et al. (1981), using an ultraviolet spectrophotometer (TU-1810; Persee, Beijing, China).

Quantitative real-time polymerase chain reaction (PCR) of ER stress-related genes

Tunicamycin- or DTT-caused ER stress can be alleviated by constitutive expression of ER-sHSP in tomato plants (Zhao et al., 2007). In this study, we investigated the effect of ER-sHSP on the UPR under salt stress. To acclimate plants to salt stress, we first irrigated 1-month-old tomato plants with 50 mM NaCl for 4 days; the plants were then exposed to 100 mM NaCl. Leaf samples were collected after 0, 24, 48, and 96 h of 100 mM NaCl stress for quantitative real-time PCR analysis. Total RNA was isolated from these leaf samples that had been frozen in liquid nitrogen using the TRIzol reagent (Takara, Tokyo, Japan) according to manufacturer protocol and was converted into cDNA using PrimeScript RT reagent Kit (Takara). Quantitative real-time PCR was performed with SYBR Premix Ex Taq II (Takara), using a Real-Time PCR Detection System (DNA Engine Opticon 2; Bio-Rad, Hercules, CA, USA). For the quantitative real-time PCR, amplification was performed with oligonucleotides specific for the *BiP*, *PDI*, *calnexin*, and *calreticulin* genes. Amplification of the *actin7* gene was used as an internal control. Primer names and sequences are listed in Table 1.

Table 1. Sequences of quantitative real-time PCR primers.

Primers	Sequences (5'→3')	GenBank accession number
BiP-F	gaagcacttgaatggttggacg	XM_004234937
BiP-R	gccgtgataactgggttgca	
PDI-F	acaagctccaggcaagtgaga	XM_004241984
PDI-R	tgattacagactagggtaagaagggt	
calnexin-F	cctgctagagctagtgccgagac	AB218598
calnexin-R	gcatcctcactctgctcttccttc	
calreticulin-F	ttattagtgaattagtagccccttcc	XM_004230251
calreticulin-R	gacatcaaagcaatcagccata	
actin7-F	attgccctcttctgtctggctacac	XM_004235020
actin7-R	agacgaggagaaaacatcacaatcac	

Statistical analysis

All the experiments were performed using three independent biological replicates, and all values are reported as means ± SD. Statistical analysis was performed using the SPSS 16.0 software with the Duncan multiple range test ($P \leq 0.05$).

RESULTS

When the tomato plants were irrigated with 50% Hoagland solution, transgenic plants (hereafter abbreviated OE lines) and the control plants grew normally and at the same rate (data not shown). This indicated that the constitutive expression of ER-sHSP did not affect normal development of the tomato plants.

Following the two final 4-day treatments of 150 and 200 mM NaCl solutions, respectively, the control plants displayed damage. The most expanded leaves had withered and turned yellow and the top leaves were slightly wilted. In contrast, the expanded leaves in the OE lines crumpled slightly but their color remained green (Figure 1A). The roots of the OE lines appeared more vigorous (Figure 1B) and root biomass was >30% greater than the control plants (Figure 1C).

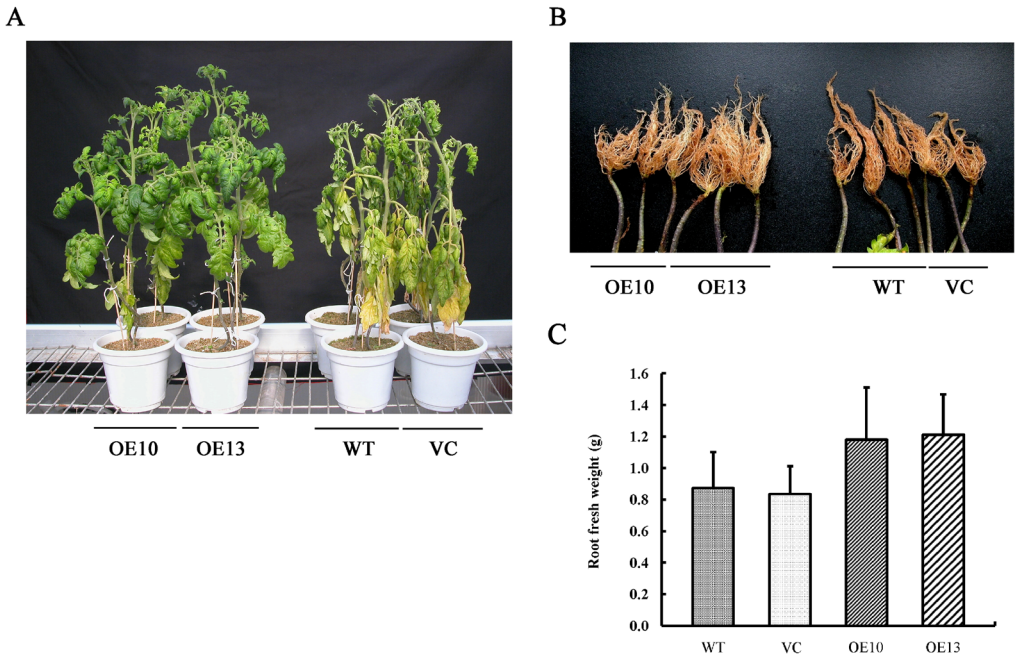

Figure 1. Tomato plants with constitutive expressed ER-sHSP exhibited improved tolerance to salt. **A.** Phenotype of tomato plants after a series of NaCl treatments. **B.** Roots of transgenic tomato plants are more vigorous than control plants. **C.** Root biomass of transgenic tomato and control plants. Values are reported as means ± SD from three replicates. OE = transgenic plants; WT = wild-type plants; VC = vector control plants.

Following the first 4-day treatment with 50 mM NaCl solution, no visible damage was evident in any plant (including OE lines, WT, and VC) (data not shown). The subsequent 4-day treatment with 100 mM NaCl solution caused the chlorophyll and RWC of the control (WT and VC) plants to decline significantly compared with the OE lines (Figure 2). During the 200 mM NaCl treatment period both chlorophyll and RWC in the leaves of the OE lines declined more slowly compared to the control plants (Figure 2). The phenotypic and physiological data all indicated that the OE lines had superior tolerance to salt stress.

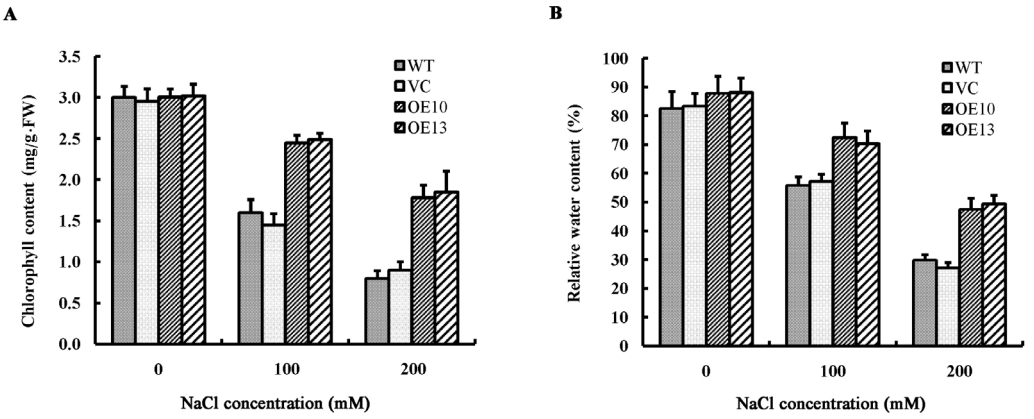

Figure 2. Effects of NaCl treatment on relative chlorophyll (**A)** and relative water (**B)** content in tomato leaves. Values are reported as means ± SD from three replicates. FW = fresh weight. For other abbreviations, see legend to Figure 1.

The plant ion (Na^+, K^+, and Ca^{2+}) levels were greatly affected by salt stress (Figure 3). After treatment of 100 mM NaCl, the tissues of the OE lines, including leaves and roots, accumulated more K^+ and Ca^{2+}, but less Na^+, compared to the control lines. Likewise, the ratio of K^+/Na^+ in OE lines was significantly higher than that in control plants (Figure 3). At the end of the 200 mM NaCl treatment, the ion level differences were greater than at the lower NaCl exposures. All ion data consistently indicated that the OE lines absorbed less Na^+, and had higher levels of K^+ and Ca^{2+}.

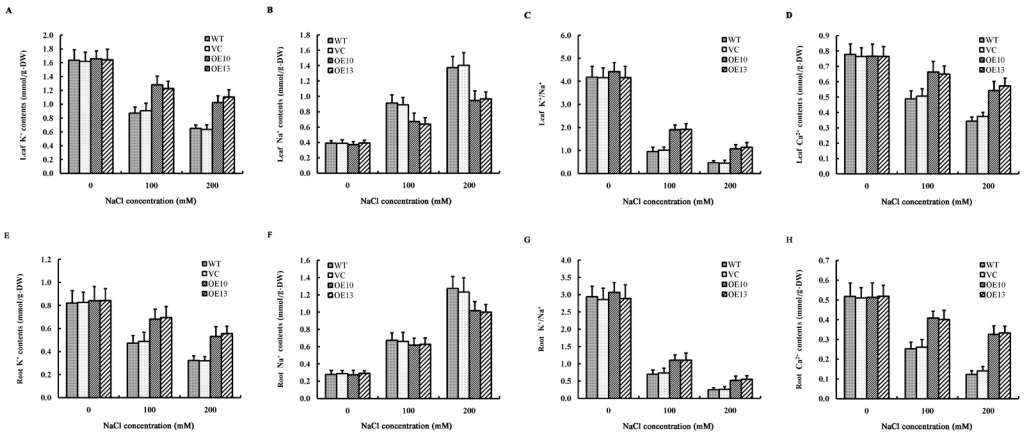

Figure 3. Effects of NaCl treatment on K^+ (**A** and **E**), Na^+ (**B** and **F**), Ca^{2+} (**D** and **H**) levels and K^+/Na^+ (**C** and **G**) ratio in tomato leaves and roots. Values are reported as means ± SD from three replicates. DW: dry weight. For other abbreviations, see legend to Figure 1.

After the 4-day exposure to 100 mM NaCl, the net photosynthetic rate (Figure 4A) and the F_v/F_m ratios of photosystem II (Figure 4B) in the control plants decreased more than the OE lines. Following the 200 mM NaCl treatment, photosynthetic activity had almost ceased in the control plants and their F_v/F_m ratios declined to 0.1-0.2. At the same time, the OE lines maintained their photosynthetic activity and the F_v/F_m ratio had only declined to 0.6 (Figure 4B). Thus, constitutive expression of ER-sHSP reduced photosystem damage under salt stress. Compared to the control plants, OE lines had greater leaf g_s and transpiration rate at all time points (Figure 4).

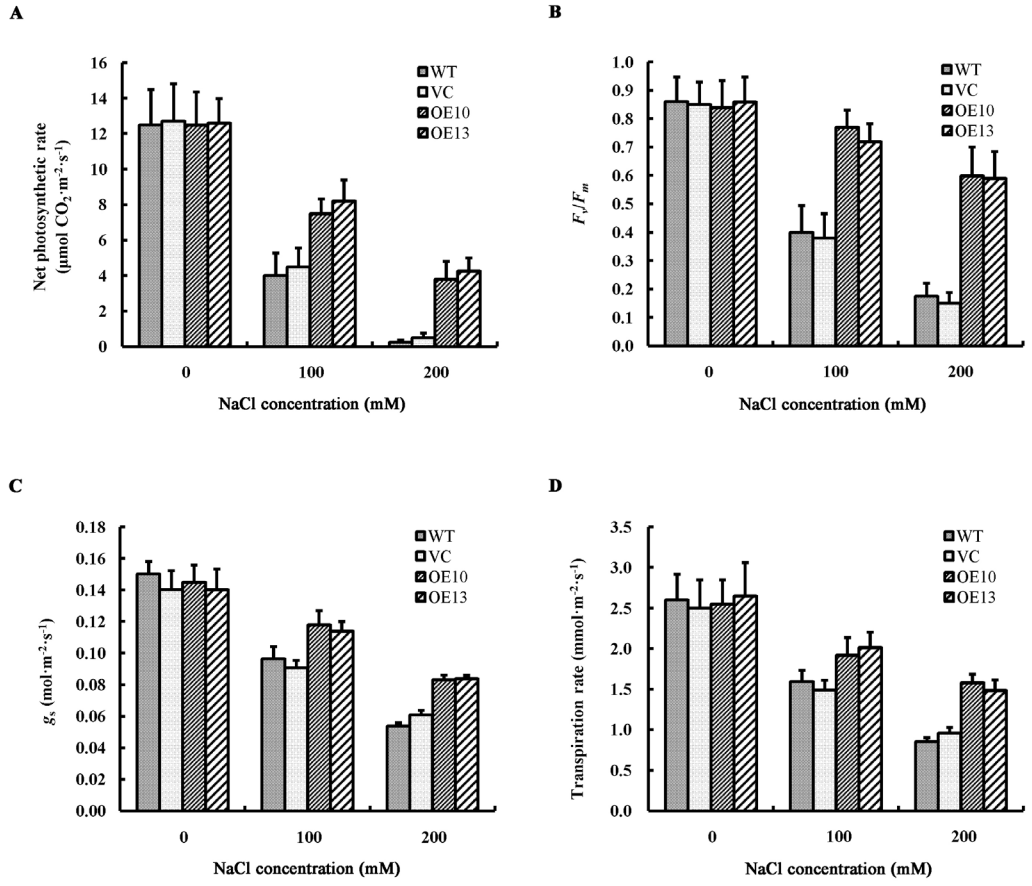

Figure 4. Net photosynthetic rate (**A**), maximum quantum yield of photosystem II photochemistry (F_v/F_m; **B**), stomatal conductance (g_s; **C**), and transpiration rate of tomato leaves (**D**). Values are reported as means ± SD from three replicates. For abbreviations, see legend to Figure 1.

Under salt stress, OE lines accumulated more osmolytes and soluble sugars than control plants (Figure 5). The large accumulation of osmolytes in the OE lines is associated with non-wilting leaves that have higher water content.

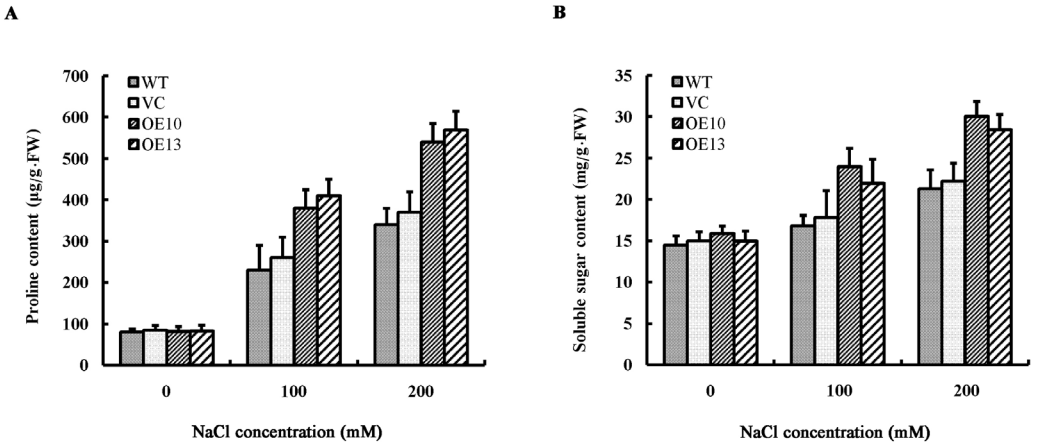

Figure 5. Salt-induced accumulation of proline and soluble sugars. Proline (**A**) and soluble sugar (**B**) content of tomato leaves. Values are reported as means ± SD from three replicates. FW = fresh weight. For other abbreviations, see legend to Figure 1.

The high salt stress-induced expression of *BiP*, *PDI*, *calnexin*, and *calreticulin* increased in all plants, but this increase was less evident in the OE line plants compared to the control plants (Figure 6).

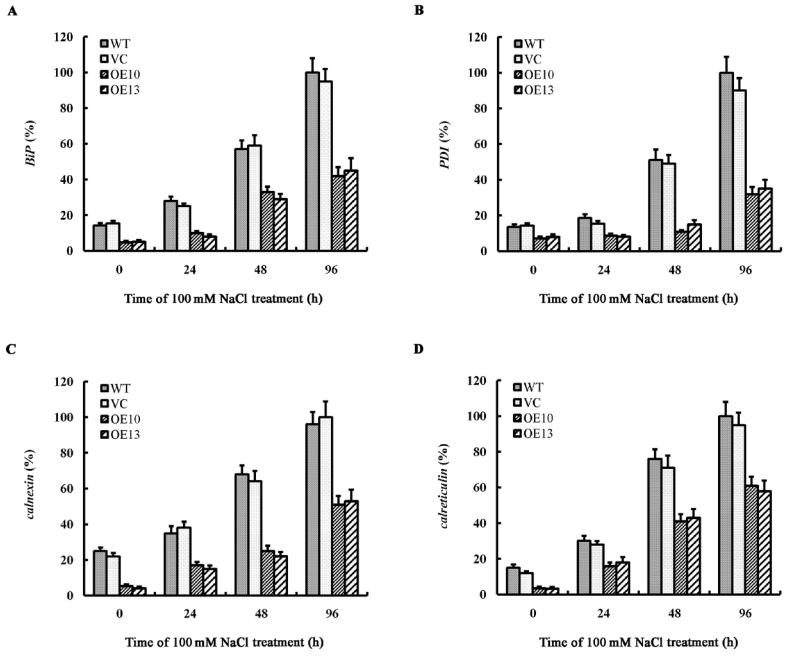

Figure 6. Expression of UPR responsive genes under salt stress. Relative expression levels of *BiP* (**A**), *PDI* (**B**), *calnexin* (**C**), and *calreticulin* (**D**) as determined by quantitative real-time PCR analysis. Within each gene, the maximum was normalized to 100% and the rest were scaled in proportion. Values are reported as means ± SD from five replicates. For abbreviations, see legend to Figure 1.

DISCUSSION

Tunicamycin blocks glycosylation and DTT alters the redox environment in the ER. Both can trigger the UPR and induce expression of chaperones and foldases (Mori et al., 1992; Travers et al., 2000). Environmental stress can also affect the ER. Drought stress can increase the expression of BiP and calreticulin (Cascardo et al., 2000; Jia et al., 2008). Salt stress can also upregulate BiP transcription (Liu et al., 2011). If plant cells have a defect in the UPR signal pathway, salt stress can provoke a stronger UPR and greatly boost BiP expression (Howell, 2013). In contrast, overexpression of ER molecular chaperones has been shown to relieve stress under extreme abiotic conditions (Alvim et al., 2001; Jia et al., 2008; Valente et al., 2009). Previously, it has been demonstrated that ER-sHSP reduces ER stress in tomato plants (Zhao et al., 2007). In the present experiment, OE lines were shown to have increased salinity tolerance and reduced UPR, as indicated by the lower expression of ER resident molecular chaperones.

BiP is the most abundant chaperone in the ER and it is often used as a molecular marker of ER stress (Kamauchi et al., 2005). For ER protein mutants, fluctuating BiP levels do not correlate with changes in salinity tolerance. For example, under NaCl stress, *hrd3a* and *stt3a* increased the expression of BiP (Koiwa et al., 2003; Liu et al., 2011), *atztp29* downregulated the level of BiP (Wang et al., 2010), whereas the BiP content in *atbzip17* remained unchanged (Liu et al., 2007b). In the present experiment, overexpression of ER-sHSP depressed the salt-induced expression of BiP and PDI, but OE lines still displayed improved NaCl tolerance. This indicates that the interaction between the UPR pathway and the salt stress response pathway is complex (Humbert et al., 2012).

BiP transgenic soybean (Alvim et al., 2001; Valente et al., 2009) and ER-sHSP transgenic tomato plants both have improved tolerance to salt stress. However, each has a distinctive phenotype and different physiological changes occur in the two plant species. Under salt stress, the roots of ER-sHSP OE lines were more robust and had a greater biomass than control plants (Figure 2). In contrast, the roots of BiP transgenic soybeans were smaller than WT plants (Valente et al., 2009). Under NaCl stress, ER-sHSP OE lines had increased accumulation of osmolytes, such as proline, soluble sugars, and K^+ ions (Figures 3 and 5), whereas the osmolyte level in BiP transgenic soybean was unchanged (Valente et al., 2009). With the vigorous root system and the large accumulation of osmolytes, ER-sHSP OE lines were able to absorb and hold more water. In addition, constitutive expression of ER-sHSP reduced salt damage to the photosystem (Figure 4). These findings explain why the ER-sHSP OE lines did not display a withered phenotype even under high salt stress.

CONCLUSION

In this study, the role of ER-sHSP in improving plant resistance to salt stress was explored. The results showed that the constitutive expression of ER-sHSP significantly increased salinity tolerance in tomato plants and reduced the expression of ER resident molecular chaperones. We suggest that the incorporated ER-sHSP in the OE lines performed functions that normally are performed by ER molecular chaperones such as BiP.

Conflicts of interest

The authors declare no conflict of interest.

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