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Overexpression of *TsApx1* from *Thellungiella salsuginea* improves abiotic stress tolerance in transgenic *Arabidopsis thaliana*

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Abstract

The halophyte *Thellungiella salsuginea* is a new model plants due to its small genome size, short life cycle, and copious seed production. Although *T. salsuginea* shares a high sequence identity with its close relative *Arabidopsis thaliana*, it shows a greater tolerance to salinity, drought, freezing, heat, and cold. To elucidate the mechanism of abiotic stress resistance in *T. salsuginea*, we characterized its cytosolic *Apx1* gene (*TsApx1*) and established *A. thaliana* transgenic lines overexpressing *TsApx1*. Under 300 mM NaCl, the content of H_2O_2, malondialdehyde, and proline were lower and the activities of superoxide dismutase, catalase, glutathione peroxidase, and ascorbate peroxidase were all higher in the transgenic plants overexpressing *TsApx1* (*35S:TsApx1-GFP*) than in the wild-type plants. The *atapx1* mutant plants of *A. thaliana* had a NaCl/mannitol-sensitive phenotype. The ectopic expression of *TsApx1* in the *atapx1* mutant effectively remedied the phenotype. These results suggest that *TsApx1* plays an important role in scavenging reactive oxygen species in the cytoplasm under salinity or drought. Although *TsApx1* in *T. salsuginea* was constantly expressed at a high level, this gene was clearly inducible. In summary, the high constitutive expression and rapid induction of *TsApx1* may contribute to the tolerance to abiotic stresses in *T. salsuginea*.

Additional key words: antioxidative enzymes, ascorbate peroxidase, ectopic expression, reactive oxygen species.

Introduction

Environmental factors can have a significant impact on the life cycle of a plant. Abiotic stresses, such as drought, salinity, and extreme temperatures, affect normal growth and development of plants, which could result in the reduction of their productivity (Bartels and Sunkar 2005). Abiotic stresses also play major roles in shaping evolutionary paths of plants (Zhu 2002), and plants have developed various mechanisms to cope with a wide range of environmental and climate changes (Frazier *et al.* 2011). Salinity causes osmotic stress and ion toxicity (primary effects) and secondary effects as oxidative stress or inhibition of K^+ uptake (Nounjan *et al.* 2012).

Under salinity and drought, plants can generate reactive oxygen species (ROS), such as superoxide anion ($O_2^{\cdot-}$), hydroxyl free radical ($OH^{\cdot}$), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2) in chloroplasts, mitochondria, and peroxisomes (Laloi *et al.* 2004, Mittler *et al.* 2011, Xu *et al.* 2014), which can cause extensive cell injury or death. To scavenge ROS, plants have developed a complex antioxidant defense system which includes enzymatic and non-enzymatic antioxidants (Hu *et al.* 2012). The antioxidant enzymes include superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (APX, EC 1.11.1.1), glutathione peroxidase (GPX, EC 1.11.1.9), and catalase (CAT, EC 1.11.1.6) (Ray *et al.* 2012, Naliwajski and Sklodowska 2014), whereas

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Abbreviations: APX - ascorbate peroxidase; CaMV - Cauliflower mosaic virus; cAPX - cytosolic APX; CAT - catalase; cDNA - complementary DNA; DAPI - 4',6-diamidino-2-phenylindole; DTNB - dithio-bis-nitrobenzoic acid; EDTA - ethylene diamine tetraacetic acid; GFP - green fluorescent protein; GPX - glutathione peroxidase; GSH - glutathione; H_2O_2 - hydrogen peroxide; mAPX - microbody membrane-bound APX; MDA - malondialdehyde; MS - Murashige and Skoog; NBT - nitroblue tetrazolium; $O_2^{\cdot-}$ - superoxide anion; $OH^{\cdot}$ - hydroxyl free radical; $^1O_2$ - singlet oxygen; ORF - open reading frame; qPCR - quantitative PCR; ROS - reactive oxygen species; RT-PCR - reverse transcriptase-PCR; sAPX - stromal APX; SOD - superoxide dismutase; tAPX - thylakoid membrane-bound APX; TBARS - thiobarbituric acid-reactive-substances; WT - wild-type.
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glutathione and ascorbate are classified as non-enzymatic antioxidants (Noctor and Foyer 1998).

In plants, GPX, APX, and CAT, control H_2O_2 content, and APX has the strongest affinity for H_2O_2 (Miao *et al.* 2005). The APX isoenzymes are distributed in distinct cellular compartments: stromal APX (sAPX) and thylakoid membrane-bound APX (tAPX) in chloroplasts, microbody (including the glyoxysome and peroxisome) membrane-bound APX (mAPX), and cytosolic APX (cAPX) (Shigeoka *et al.* 2002). Eight *Apx* genes have been identified in *Arabidopsis thaliana* (Panchuk *et al.* 2002). An *Arabidopsis Apx* gene family consists of three cytosolic (*Apx1*, *Apx2*, and *Apx6*), three peroxisomal (*Apx3*, *Apx4*, and *Apx5*), and two chloroplastic (*tApx* and *sApx*) isoforms (Chew *et al*. 2003).

Thellungiella salsuginea (syn. *T. halophila*) is a close relative of *A. thaliana* in the family *Brassicaceae* (Sun *et al*. 2013). Its genetic coding sequence shows 92 % congruence to that of *Arabidopsis*. The plants of *T. salsuginea* can tolerate seawater-level salinity and reproduce after an exposure to an extreme salinity of 500 mM NaCl, which is far beyond the capacity of *Arabidopsis* (Zhang *et al.* 2008). Furthermore, *T. salsuginea* has the ability to tolerate chilling, freezing, heat, drought, and ozone (Higashi *et al.* 2013). There are many other desirable traits in this plant species, such as small stature, short life cycle, self-pollination, abundant seed number, and small genome size; therefore, it can serve as a valuable halophytic genetic model species (Zhang *et al.* 2008). Several studies on *T. salsuginea* have provided novel insights into the mechanisms of salt tolerance and many candidate genes for salt tolerance have been isolated (Higashi *et al.* 2013). However, the mechanisms of its high tolerance to abiotic stresses remain to be disclosed.

The aim of this study was to clone *TsApx1* from *T. salsuginea* and to follow its expression pattern under a high salinity. In addition, we generated the transgenic lines of *A. thaliana* that constitutively overexpressed *TsApx1*, and examined their content of H_2O_2, proline, and malondialdehyde, and activities of antioxidant enzymes under the high salt stress. The transgenic *A. thaliana* plants that ectopically expressed *TsApx1* in the *atapx1* loss-of-function mutant were also analyzed.

Materials and methods

Thellungiella salsuginea O.E. Schulz (ecotype Shandong) and the wild-type (WT) *Arabidopsis thaliana* L. (ecotype Columbia) were used in this study. The homozygous *atapx1* T-DNA insertion mutant (SALK_143111) was obtained from the Arabidopsis Biological Resource Center (https://abrc.osu.edu/). After vernalization of seeds at 4 ºC in the dark for 3 d, they were germinated in plastic pots containing a mixture of soil and *Vermiculite* (3:1) in a growth chamber at a temperature of 22 °C, a relative humidity of 70 %, a 16-h photoperiod, and an irradiance of 110 μmol m^{-2} s^{-1}. For *in vitro* culture, seeds were surface-sterilized with a 0.1 % (m/v) $HgCl_2$ solution for 5 min and then rinsed 5 times with sterilized water before vernalization. Seedlings were then grown in a Murashige and Skoog (MS) medium (pH 5.8) supplemented with 3 % (m/v) sucrose, 0.7 % (m/v) agar, and different concentrations of NaCl (50, 100, or 150 mM) or mannitol (100, 200, or 300 mM). After 3 d, germination rates were determined as the percentage of radicle emergence.

In the investigation of the expression profiles of *TsApx1* and *AtApx1* mRNAs in response to NaCl stress within a short time, the wild-type *Arabidopsis* and *Thellungiella* plants were transferred to a mixture of *Vermiculite* and *Perlite* (3:1, v/v), and irrigated with Hoagland nutrient solution every three days. When the seedlings had 4 ~ 6 leaves, they were watered with Hoagland nutrient solution, supplemented with 300 mM NaCl. Then, samples were taken at different time points (0, 2, 4, 6, 8, 10, 12, 24, 36, or 48 h) for examination (for detail see Cho *et al.* 2008).

For investigation of dehydration and recovery, 4-week-old plants were further grown in a mixture of soil and *Vermiculite* (3:1) for 20 d without watering and then rewatered; survival rate was determined after 3 d of recovery.

Detached rosette leaves of 4-week-old plants were placed on open-lid Petri dishes at room temperature with approximately 60 % humidity under a dim light and weighed at the indicated times after their excision (0, 30, 60, 90, 120 min). Water loss rate was calculated as the percentage of initial fresh mass.

For physiological analyses, 20 plants per line were grown in the mixture of soil and *Vermiculite* (3:1) and watered every three days. A half of 4-week-old plants were treated with 300 mM NaCl for 3 d, and the rest control plants (CK) were irrigated with water. All experiments were repeated three times.

Total RNA was isolated with an *RNeasy Plant Mini Kit* (*Qiagen*, Amsterdam, the Netherlands). The RNA quality and integrity were checked before cDNA was synthesized. For RT-qPCR, we firstly accurately quantified RNA concentration using spectrophotometry. Next, cDNA was synthesized using 2 mg of total RNA with a *TransScript*^{TM} *One-Step gDNA* removal and a cDNA synthesis *SuperMix* kit (*TransGen*, Beijing, China). The RT-qPCR was performed with a real-time PCR system *7500* (*Applied Biosystems*, New York, USA) using a *TransStart*^{TM} *Green qPCR SuperMix* kit (*TransGen*). Data were analyzed by the comparative C_T method. Primers specific to *Apx1* and *Actin* are listed in Table 1 Suppl.

The full-length *TsApx1* and *AtApx1* cDNAs were amplified using *Apx1PstI*-FW and *Apx1BlnI*-RV primers (Table 1 Suppl.) for their identical upstream and downstream primer sequences. The PCR products were

digested with *Pst*I and *Bln*I and inserted into the pCAMBIA1302 vector (produced for the C-terminal protein fusions with a green fluorescent protein, GFP) under the control of the CaMV35S promoter. The recombinant plasmids were introduced into the *Agrobacterium tumefaciens* strain GV3101 and then transformed into the WT *Arabidopsis* and *atapx1* knockout mutant plants by the floral-dip method. To obtain independent transgenic lines, seeds were selected on MS plates containing 40 μg cm^{-3} hygromycin. The presence and expression of the transgenes were confirmed by Southern blotting, RT-PCR, and microscopy analysis as described previously (Lee *et al.* 2009). Specific primers are shown in Table 1 Suppl. Homozygous T3 lines were obtained by further self-crossing. Among all the transgenic lines, lines 1 and 7 of *35S:TsApx1-GFP*, lines 13 and 20 of *35S:AtApx1-GFP* and lines 5 and 6 of *atapx1/35S:TsApx1-GFP* were used for phenotypic and physiological analyses.

The content of H_2O_2, MDA, and proline, and the activities of SOD, APX, GPX, and CAT were measured using kits that were provided by the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Fresh leaf samples (0.2 g) were homogenized in ice-cold potassium phosphate buffer (50 mM, pH 7.8). For the APX assay, 2 mM ascorbic acid was added to the enzyme extraction buffer. The homogenate was centrifuged at 12 000 *g* and 4 °C for 15 min. The supernatants were used as the crude extracts for assay. The assay of H_2O_2 was carried out according to the method described by Liu *et al*. (2012). The supernatant (1 cm^3) was mixed with 0.1 cm^3 of molybdic acid. The content of H_2O_2 was determined at 405 nm. Malondiadehyde was measured by determining the thiobarbituric acid-reactive-substances (TBARS) according to the method described by Hodges *et al*. (1999). Acidic ninhydrin method was applied to quantify the proline content (Yıldıztugay *et al.* 2011). Catalase and glutathione peroxidase activities were measured according to Naliwajski and Sklodowska (2014) with a slight modification. For the CAT assay, the reaction mixture (2.3 cm^3) contained 50 mM potassium phosphate buffer, pH 7.0, 15 mM H_2O_2, and the enzyme extract. Decomposition of H_2O_2 can be interrupted by ammonium molybdate immediately, and the product of H_2O_2 and ammonium molybdate was measured at 405 nm. To detect GPX activity, the glutathione (GSH) as a substrate was used. The reaction mixture (2.5 cm^3) comprised 50 mM potassium phosphate buffer, pH 7.0, 2 mM ethylene diamine tetraacetic acid (EDTA), 0.2 μM GSH, and the enzyme extract. Reaction of GSH and dithio-bis-nitrobenzoic acid (DTNB) was recorded at 412 nm. The activity of SOD was analyzed using the method described by Xu *et al*. (2014). One unit of SOD activity was defined as the amount of enzyme required to inhibit the reduction of nitroblue tetrazolium (NBT) by 50 % as monitored at 560 nm. For the APX assay, the reaction mixture (3 cm^3) contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM EDTA and 0.1 cm^3 of enzyme extract. The reaction started by adding H_2O_2 to a final concentration of 0.1 mM. The absorbance of the mixture was measured at 290 nm. The APX activity was calculated using the coefficient of absorbance of 2.8 mM^{-1} cm^{-1} (Nakano and Asada 1981). Protein content was estimated by the Bradford (1976) method. All spectrophotometric analyses were conducted using a *Navaspec1100* spectrophotometer (*Navaspec*, Stockholm, Sweden).

For investigation of subcellular localization of the TsAPX1 protein, the transgenic *Arabidopsis* seeds carrying *TsApx1-GFP* or the control vector were transferred to agar plates after surface sterilization. Seedlings (5-d-old) were dyed with 200 μg cm^{-3} 4',6-diamidino-2-phenylindole (DAPI; *Sigma*, Munich, Germany) and then examined microscopically. Stained roots were placed on a slide glass and observed using a fluorescence microscope (model *LSM700*, Zeiss, Jena, Germany).

All the data were presented as means ± standard deviations (SD). Statistical analysis was performed by one-way *ANOVA* followed by the Fisher's LSD test using the statistical program *SPSS 20.0 for Windows* (www.spss.com). Values were considered statistically different when $P < 0.05$. All figures were drawn using the *Sigma Plot 12.5* software.

Results

The *TsApx1* cDNA (GenBank acc. No. AK 352780) consisted of 1 057 nucleotides, including a 753 bp open reading frame (ORF). The amino acid sequence of TsApx1 was similar to the sequence of AtApxs and had the highest similarity with *AtApx1* (94 %) among eight *AtApx* genes. Based on the protein database search (http://ipsort.hgc.jp/index.html), we found no signal, mitochondrial targeting, or chloroplast transit sequence in the N-terminus of TsAPX1 protein indicating that this protein was likely to be in the cytoplasm. We generated stable transgenic plants expressing the GFP (TsAPX1-GFP) fusion protein. The fluorescence signal of the TsAPX1-GFP fusion protein was predominantly found in the cytosolic fraction (Fig. 1*F*). This differed from the localization pattern of the positive control 35S:GFP which was in both the nucleus and the cytoplasm. Based on these results, we concluded that TsAPX1 is a cytosolic protein.

The results of RT-qPCR demonstrate that the transcription of *TsApx1* was rapidly elevated in response to the 300 mM NaCl treatments, with a 1.3- to 18.3-fold induction at 36 h (Fig. 2), whereas *AtApx1* mRNA showed a 1.1- to 1.8-fold induction by the NaCl stress. These results suggest that *TsApx1* may play a vital role in the salt stress response of *T. salsuginea.*

The *atapx1* mutant was selected as the NaCl and

mannitol-sensitive phenotype, therefore we compared seed germination rates of the WT, *atapx1*, *35S:TsApx1-GFP*, and *atapx1/35S:TsApx1-GFP* in the presence or absence of NaCl (50, 100, or 150 mM) or mannitol (100, 200, or 300 mM). The seeds of all lines fully germinated on the MS medium without NaCl or mannitol. On the NaCl/mannitol-containing media, the germination rates of the WT were concomitantly reduced as the concentrations of NaCl/mannitol increased (Fig. 3*A* and Fig. 1*A* Suppl.). In the presence of 100 mM NaCl, 19 % of the WT seeds and over 27 % of the *atapx1* knockout mutant seeds did not germinate (Fig. 3*A*), and approximately 47 % of the WT seeds germinated on the medium containing 150 mM NaCl. In contrast, the seeds

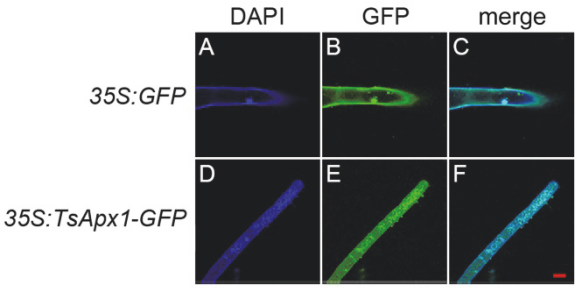

Fig. 1. Localization of TsAPX1-GFP fusion proteins in *Arabidopsis thaliana* (*bottom row*, *D-F*). The GFP protein served as a positive control. Green fluorescence from GFP (*B* and *E*) and blue fluorescence of DAPI (*A* and *D*). *C* and *F* are the merged images of *A* and *B* and *D* and *E*, respectively.

of both the *35S:TsApx1-GFP* and *atapx1/35S:TsApx1-GFP* transgenic plants were more resistant to NaCl, and more than 50 % of the seeds were able to germinate in the presence of 150 mM NaCl (Fig. 3*A*). Unlike the NaCl treatment, mannitol had a less effect on the radicle emergence of the seeds. Except for *atapx1*, whose germination rate was 59 % under the 300 mM mannitol treatment, more than 80 % of the transgenic and the WT seeds were able to germinate (Fig. 1*A* Suppl.). Thus, *TsApx1* efficiently rescued the NaCl- or mannitol-sensitive phenotype of the loss-of-function *atapx1* mutant.

At 7 d after germination, approximately 65 % of the *35S:TsApx1-GFP* and 52 % of the *atapx1/35S:TsApx1-GFP* transgenic seedlings developed true green cotyledons in the presence of 100 mM NaCl (Fig. 3*B*). In contrast, under the same concentration of NaCl, the growth of 87 % of the *atapx1* seedlings was completely arrested, and true green cotyledons failed to develop 7 d after germination (Fig. 3*B*). Similar to NaCl treatment, mannitol had different effects on the cotyledon greening of different seedlings. Over 35 % of the *35S:TsApx1-GFP* and 10 % of the *atapx1/35S:TsApx1-GFP* transgenic seedlings developed true green cotyledons in the presence of 300 mM mannitol (Fig. 1*B* Suppl.). However, under the same concentration of mannitol, only 5 % of the WT seedlings developed true green cotyledons, the growth of almost all *atapx1* seedlings was restrained, and true green cotyledons failed to develop 7 d after germination (Fig. 1*B* Suppl.). This result shows that the *TsApx1*-overexpressing plants are more tolerant to NaCl or mannitol in terms of both radicle emergence and cotyledon development.

Ten days after germination, the effects of NaCl or mannitol on root growth were identifiable in all the plants (Fig. 3*C*, and Fig. 1*C* Suppl.). No significant differences in phenotype and growth were observed among plants grown under normal conditions. The growth of *35S:TsApx1-GFP* roots was generally unaffected by 50 mM NaCl or 100 mM mannitol. The elongation of *atapx1* mutant roots was significantly inhibited by a low concentration of NaCl (50 mM) or mannitol (100 mM)

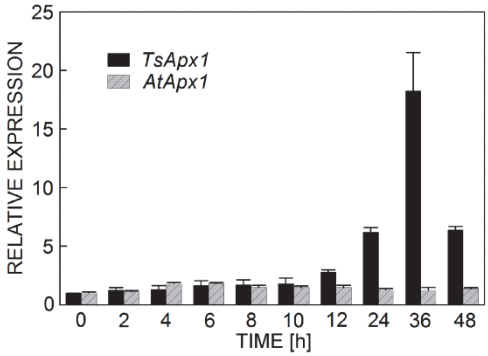

Fig. 2. Effect of salinity on *TsApx1* and *AtApx1* expression. Total RNAs were prepared from 4-week-old *Arabidopsis* or 6-week-old *Thellungiella* plants treated with 300 mM NaCl for 0 (control), 2, 4, 6, 8, 10, 12, 24, 36, or 48 h. Induction patterns of *TsApx1* and *AtApx1* were investigated by RT-qPCR. Relative amounts were calculated and normalized with respect to *Act2* (= 100 %). The means of three independent replicates of three biological samples ± SD.

($P < 0.05$), indicating an increased sensitivity to NaCl or mannitol. In the presence of 100 mM NaCl or 300 mM mannitol, the growth of most *atapx1* roots was severely retarded in contrast to the *35S:TsApx1-GFP* roots, which were able to elongate under the same conditions. The phenotypes of WT and *atapx1/35S:TsApx1-GFP* roots were in the middle between *atapx1* and *35S:TsApx1-GFP* overexpressing plants (Fig. 3*C*, and Fig. 1*C* Suppl.). Thus, concerning root growth, the *atapx1* mutant plants were sensitive to NaCl or mannitol and the *35S:TsApx1-*

GFP (lines 1 and 7) transgenic roots displayed a lower sensitivity. In addition, the root lengths of *atapx1/35S:TsApx1-GFP* (lines 5 and 6) were greater than of the WT roots. These data suggest that the expression of *TsApx1* was related to development during the germination and post-germination stages in the presence of NaCl or mannitol in the *Arabidopsis* plants.

In order to explore plant ability to adjust to environmental stresses, we examined the content of H_2O_2, proline, and MDA and activities of antioxidant enzymes in all the plants experiencing the 300 mM NaCl stress. Under the control conditions, the content of H_2O_2 in leaves did not differ among the WT, *atapx1* mutant, and transgenic plants (Fig. 4*A*). Under the high salinity, the H_2O_2 content was significantly lower in the *35S:TsApx1-GFP* and *atapx1/35S:TsApx1-GFP* transgenic plants than in the WT plants ($P < 0.01$); however, the H_2O_2 content was much higher in the *atapx1* mutant than in the WT plants ($P < 0.01$). These findings strongly suggest that *TsApx1* was involved in salt-induced H_2O_2 elimination in *Arabidopsis*. The salt-stressed leaves had more MDA in all plants compared to the control (Fig. 4*B*) and the MDA content was prominently higher in the WT and *atapx1* mutant plants than in the *35S:TsApx1-GFP* transgenic plants ($P < 0.01$), and the MDA content of *atapx1/35S:TsApx1-GFP* was between the WT and *35S:TsApx1-GFP* transgenic plants. These data indicate a high rate of lipid peroxidation for the WT and *atapx1* mutant plants due to the salt stress, and the salt-tolerant *TsApx1*-overexpressing transgenic plants might have a better protection against oxidative damage. The effect of NaCl on proline content in all the lines was also investigated. There was a slight difference in all the lines under the normal conditions (Fig. 4*C*). However, after 3 d of the NaCl treatments, the proline content in the *atapx1* mutant line was 2.02, 6.06, and 3.31 folds higher than in the WT, *35S:TsApx1-GFP*, and *atapx1/35S:TsApx1-GFP* lines, respectively. In addition, we found that under the normal growth conditions, the SOD, CAT, and GPX activities of the transgenic lines and the *atapx1* mutant were similar to those of WT (Fig. 4*D,E,F*); however, the APX activity was higher in the overexpressing lines, particularly in *35S:TsApx1-GFP*, than in WT and the *atapx1* mutant (Fig. 4*G*). After the treatment with 300 mM NaCl, the SOD, CAT, GPX, and APX activities of all plants significantly increased ($P < 0.01$), and the enzyme activities in the transgenic lines were significantly higher than those in the WT and *atapx1* mutant plants. In particular, the APX activity was significantly greater in the *35S:TsApx1-GFP* transgenic line, mainly in line 7, than in the WT and *atapx1/35S:TsApx1-GFP* lines and especially in the *atapx1* mutant. Surprisingly, the insertion of *TsApx1* led also to the coordinated upregulation of SOD, CAT, and GPX activities under the stress conditions.

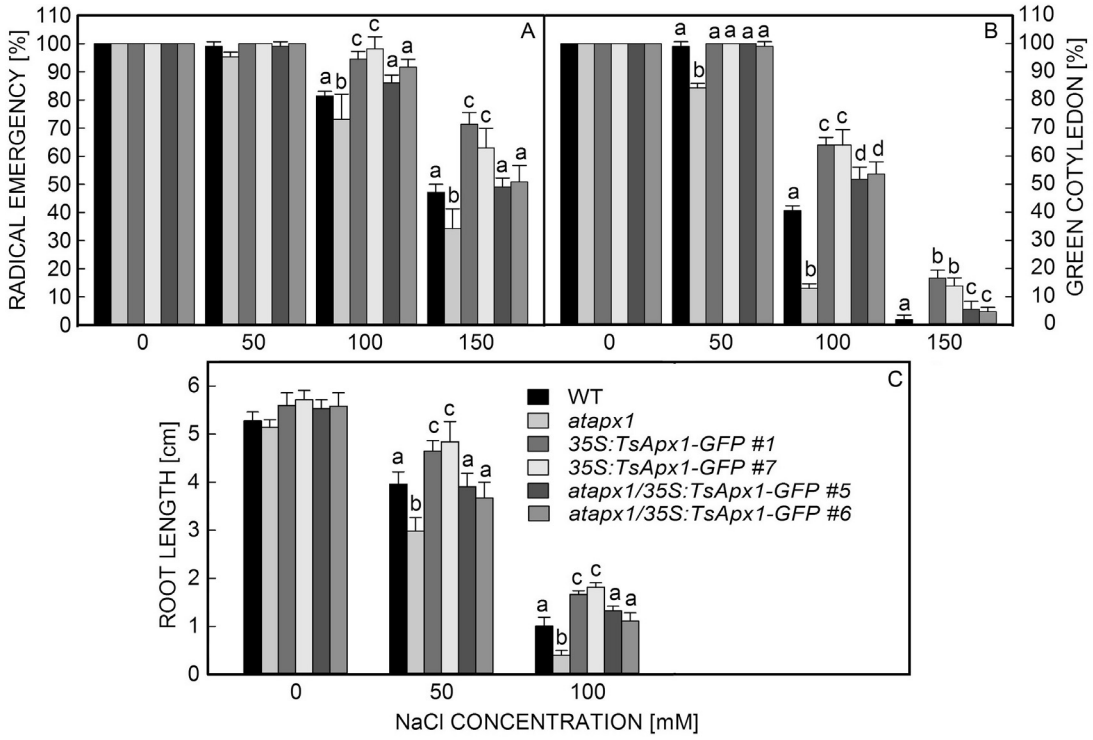

Fig. 3. Effect of NaCl (0, 50, 100, and 150 mM) in MS medium on WT, *atapx1* loss-of-function mutant, *35S:TsApx1-GFP*, and *atapx1/35S:TsApx1-GFP* plants in the germination and early seedling growth stage. *A, B* - radicle emergence (3 d after sowing) and cotyledon greening (7 d). Means ± SD from 4 biological with 36 technical replicates ($n = 144$). *C* - root length (10 d). Means ± SD from three biological and ten technical replicates ($n = 30$), significant differences among all of them were assigned with specific letters. Groups without letters indicate no statistic differences among them.

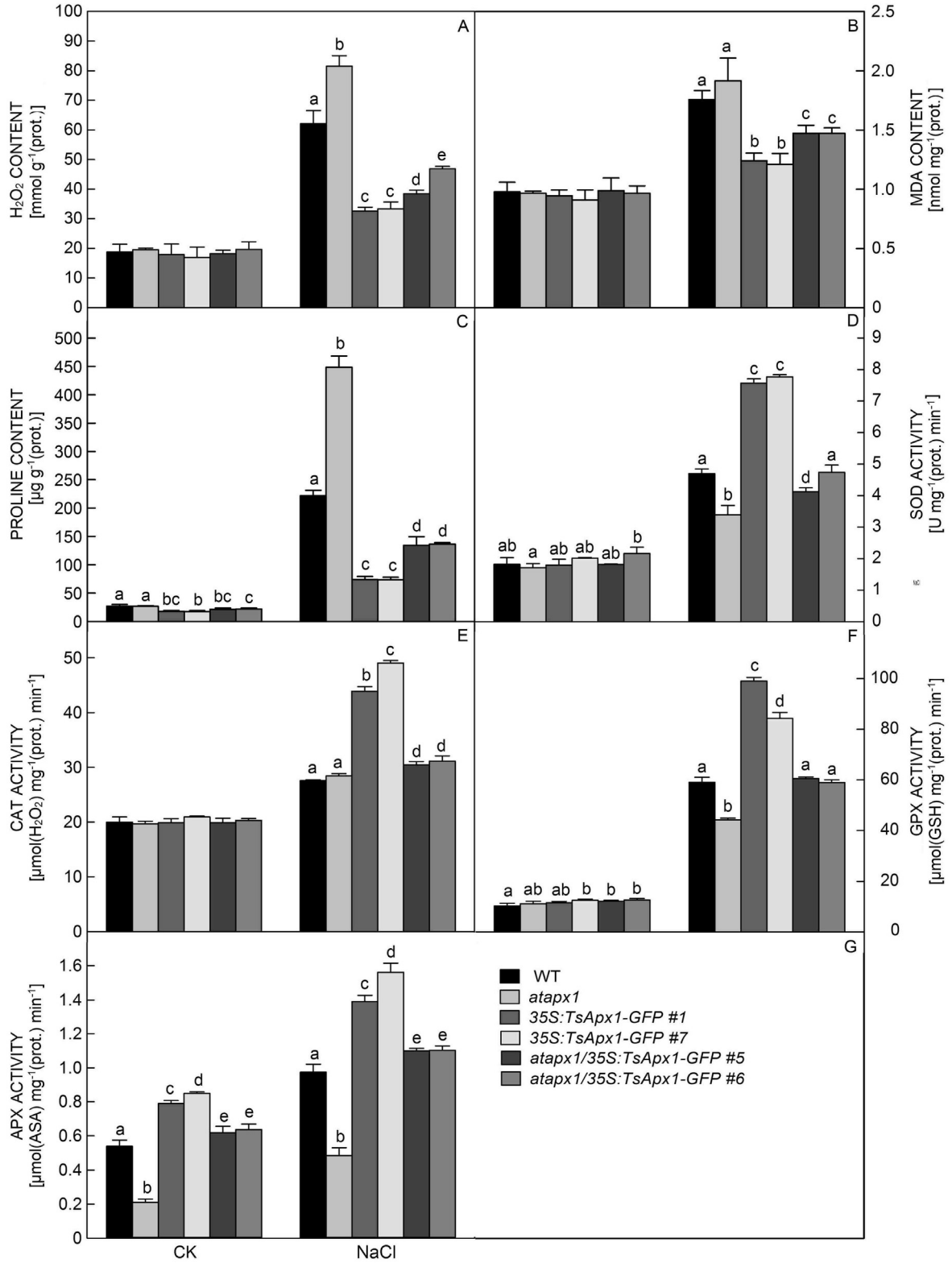

Fig. 4. Effect of 300 mM NaCl for 3 d on the H_2O_2, MDA, and proline content and SOD, CAT, GPX, and APX activities in leaves of WT, *atapx1* loss-of-function mutant, *35S:TsApx1-GFP* and *atapx1/35S:TsApx1-GFP* plants. Means ± SD from three biological replicates of ten plants (n = 30), significant differences among all of them were assigned with specific letters. Groups without letters indicate no statistic differences among them.

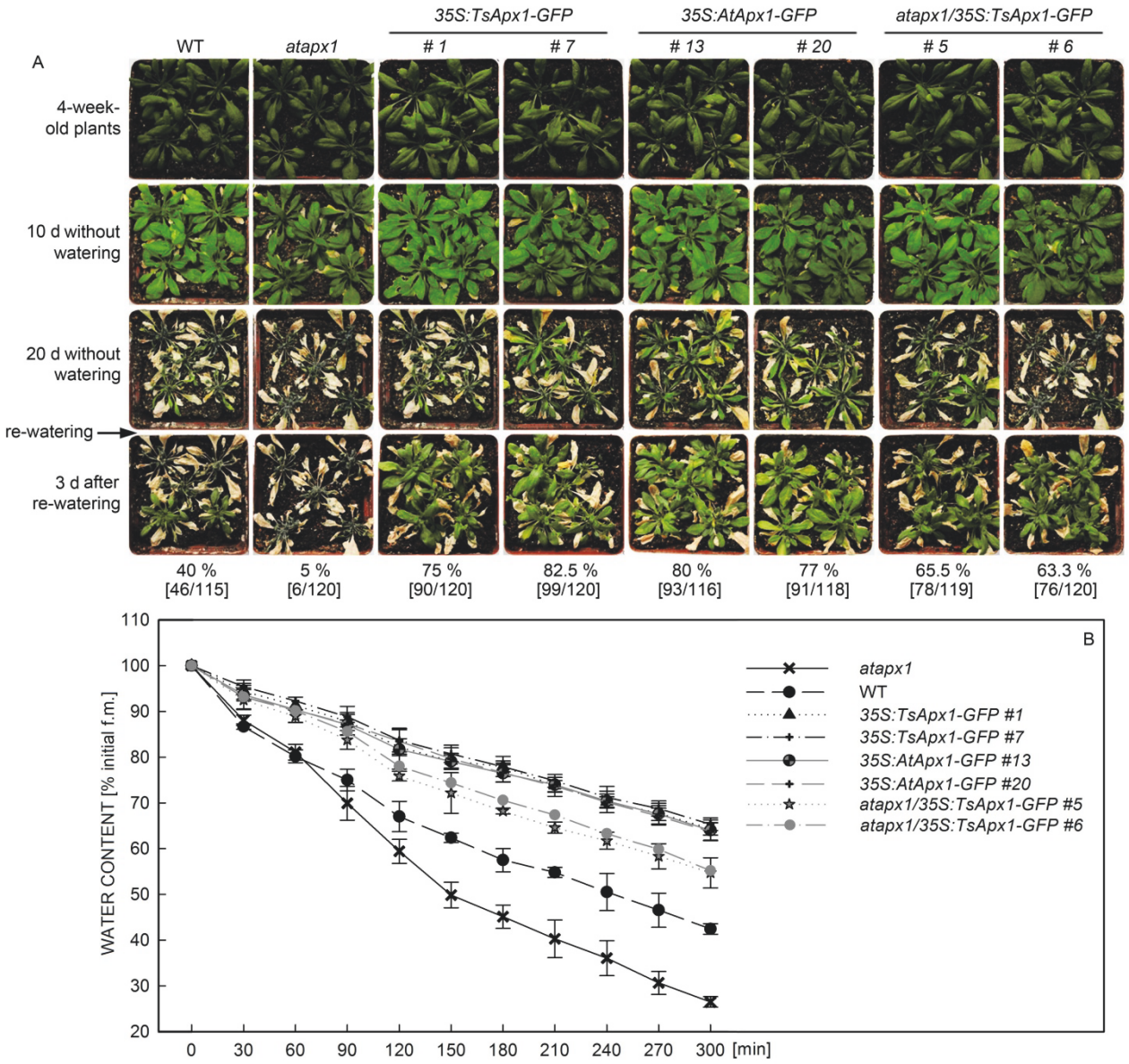

Fig. 5. Overexpression of *TsApx1* enhanced tolerance to drought stress in *Arabidopsis*. *A* - drought tolerance of WT, *atapx1* loss-of-function mutant, *35S:TsApx1-GFP*, *35S:AtApx1-GFP*, and *atapx1/35S:TsApx1-GFP* plants. Four-week-old plants were grown without watering for 20 d and survival rates were determined after 3 d of rehydration. *B* - measurements of cut rosette water loss rates at the indicated times after their excision. The water loss was calculated as the percentage of initial fresh mass. Means ± SD of thirty leaves arranged into six replicates.

We hypothesized that *TsApx1* may be also involved in a response to water deficits. After dehydration for 20 d, almost all the mutant plants and most WT plants were seriously wilted (Fig. 5*A*). These plants were then rewatered, and the phenotypes were compared. After 3 d of rewatering, 40 % (46 of 115) of the WT plants were able to resume their growth and survived, whereas only 5 % (6 of 120) of the mutant plants resumed their growth and survived (Fig. 5*A*). However, all the *35S:TsApx1-GFP*, *35S:AtApx1-GFP*, and *atapx1/35S:TsApx1-GFP* lines appeared as relative healthy after recovery from this severe drought. After 3 d of rewatering, the survival rates of the *35S:TsApx1-GFP*, *35S:AtApx1-GFP*, and *atapx1/35S:TsApx1-GFP* plants were 75 % (90 of 120 for line 1) to 82.5 % (99 of 120 for line 7), 77 % (91 of 118 for line 20) to 80 % (93 of 116 for line 13) and 63.3 % (76 of 120 for line 6) to 65.5 % (78 of 119 for line 5), respectively (Fig. 5*A*). This result strongly suggests that the *TsApx1*-overexpressing transgenic plants, as opposed to the *atapx1* mutant plants, highly tolerated the severe water stress. In addition, *TsApx1* effectively rescued the loss-of-function susceptible phenotype of *atapx1*.

To further evaluate the responses to the drought stress, the water loss rates of cut leaf rosettes of 4-week-

old plants were estimated. The results indicate that the detached rosettes of the WT and *atapx1* mutant plants lost water continuously, and their fresh masses were approximately 50 and 45 % of their starting masses, respectively, after the 5-h incubation (Fig. 5*B*). In contrast, the detached leaf rosettes of *35S:TsApx1-GFP*, *35S:AtApx1-GFP*, and *atapx1/35S:TsApx1-GFP* lost water more slowly and after 5-h of incubation, the fresh masses of the *Apx1* overexpressors were from 55 % (*atapx1/35S:TsApx1-GFP*) to 65 % (*35S:TsApx1-GFP* and *35S:AtApx1-GFP*) of the starting masses (Fig. 5*B*). These results show that the *TsApx1*-overexpressing transgenic plants displayed highly drought tolerant phenotypes, whereas the *atapx1* plants were hypersensitive to the water stress. We concluded that the expression of *Apx1* highly correlated with the responses to the water deficit, suggesting the importance of *TsApx1* in responses to drought stress in *Arabidopsis*.

Discussion

The role of APX in protecting plants from oxidative stress that is induced by abiotic stresses has been examined with inconsistent outcomes. The over-expression of pea cytosolic APX (cAPX) in tobacco chloroplasts reduces oxidative stress induced by a paraquat treatment (Yabuta *et al.* 2002). An *APX1*-deficient mutant (*apx1*) accumulates more H_2O_2 and is significantly more sensitive to combined drought and heat stresses than a wild-type (Koussevitzky *et al.* 2008). The overexpression of *OsAPX2* has been confirmed to confer a strong tolerance to drought stress in *Oryza sativa* (Zhang *et al.* 2013). However, the overproduction of APX in tobacco chloroplasts does not protect plants against ozone-induced stress (Torsethaugen *et al.* 1997).

A number of recent analyses of the *A. thaliana Apx* gene family indicated an incontestable link between the control of ROS intracellular content and many physiological processes ranging from plant development to responses to biotic and abiotic stresses (Rizhsky *et al*. 2002, Pnueli *et al*. 2003, Davletova *et al*. 2005, Rossel *et al*. 2006, Lu *et al*. 2007, Miller *et al*. 2007, Locato *et al*. 2008). Understanding how antioxidant metabolism is regulated in plant cells is challenging since these enzymes have substrates that also function as signal molecules and are represented by several isoforms targeted to different intracellular compartments (Bowler and Fluhr 2000, Mullineaux *et al*. 2000). In *A. thaliana*, the eight members of the *Apx* gene family appear to play different roles during plant development and in response to abiotic stresses. The expression of eight members of the *AtApx* gene family appears to be differentially regulated during leaf senescence (Panchuk *et al*. 2005). Lack of a chloroplastic H_2O_2 scavenging enzyme triggers a specific signal in cells that results in an enhanced tolerance to heat stress, whereas lack of a cytosolic H_2O_2 removal enzyme triggers a different signal which results in stunted growth and enhanced sensitivity to oxidative stress (Miller *et al*. 2007). The *Apx2* is upregulated over 20-fold upon a heat shock, about 10-fold by a high irradiance and also after a drought stress, and about 20-fold in response to an H_2O_2 treatment (Panchuk *et al*. 2002, Rossel *et al*. 2006, Volkov *et al*. 2006). Plants lacking APX2 are more sensitive to heat stress at the seedling stage but more tolerant to heat stress at the reproductive stage (Suzuki *et al*. 2012). Kangasjärvi *et al*. (2008) reported that sAPX is particularly important for photoprotection during an early greening process. In mature leaves, tAPX and sAPX are functionally redundant, and crucial upon a sudden onset of oxidative stress. Moreover, chloroplast APXs contribute to chloroplast retrograde signalling pathways upon slight fluctuations in the accumulation of H_2O_2 in chloroplasts. Cytosolic APX1 has been demonstrated to play a key role for protecting the chloroplast during an excessive radiation and cross-compartment protection of thylakoid and stromal/mitochondrial APXs. The chloroplast contains at least three different isozymes of APX: a thylakoid-bound APX, a lumen APX, and a stromal APX; stromal APX was recently shown to be dually targeted to the stroma and mitochondria in *Arabidopsis* and would be referred to as stromal/mitochondrial APX. In the absence of APX1, the entire chloroplastic H_2O_2-scavenging system of *Arabidopsis* collapses, H_2O_2 content increases, and protein oxidation occurs (Davletova *et al*. 2005) To increase the data related to the expression pattern of the eight members of the *AtApx* gene family, we have tested their response to the drought and high salinity treatments. Generally, upon the drought and salt stress treatments, the *AtApx* genes presented a significant but weak induction. Thus, different molecular mechanisms participated in a coordinative expression of individual *AtApx* genes which were involved in a complex regulatory network controlling H_2O_2 content very precisely in different cell compartments upon the stress and during recovery.

As previously mentioned, *T. salsuginea* exhibits a much higher tolerance to diverse abiotic stresses than *A. thaliana*. Although *T. salsuginea* is salt tolerant and can grow at 500 mM NaCl, this species does not have salt glands or other morphological alterations either before or after salt adaptation (Taji *et al.* 2004). This suggests that the salt tolerance in *T. salsuginea* results from other mechanisms. We hypothesized that ROS-scavenging enzymes of halophytes may have more efficiency in the removal of H_2O_2 than those of non-halophytes under stress conditions. In this study, we have made a comprehensive analysis including patterns of gene expression, function of the TsAPX1, and relations of the ROS-scavenging enzymes to maintaining ROS balance.

We hypothesized that the overexpression of *TsApx1* and the resulting upregulation of SOD, CAT, GPX, and APX activities would enhance the tolerance of transgenic *A. thaliana* to salinity or drought. This hypothesis is

based on the following reasons. First, the *TsApx1* overexpressing plants were insensitive to NaCl or mannitol in terms of radicle emergence, cotyledon development, and root elongation. Second, the *TsApx1* transgenic plants showed less ROS production, a lower MDA and proline accumulation, and were highly resistant to the severe water stress. Third, the *atapx1* mutant phenotype was sufficiently complemented by the expression of *TsApx1*. Taken together, our results suggest that the accumulation of H_2O_2 in the short-term salt stress was primarily due to the osmotic stress induced by external NaCl (Cho *et al.* 2008). A lower content of H_2O_2 in the transgenic plants compared to WT or the *atapx1* mutants under the salt stress might be attributed to the relatively more efficient detoxifying enzymes CAT, GPX, and APX.

The *AtApx1* is a cytosolic APX. We hypothesized that ROS scavenging enzymes of *T. salsuginea* may be more efficient in H_2O_2 removal than those of *A. thaliana* under stress conditions. Our phenotypic data indicate that the drought resistance of the *35S:TsApx1-GFP* lines was similar to that of the *35S:AtApx1-GFP* lines. Furthermore, the dehydration rates of the *35S:TsApx1-GFP* and *35S:AtApx1-GFP* plants were approximately the same indicating that *TsApx1* and *AtApx1* had equal efficiency of H_2O_2 removal. The gene expression profiles of the *TsApx1* and *AtApx1* transgenic plants were also performed at the transcriptional level. The *TsApx1* transcripts were significantly upregulated in response to the high-salinity stress, whereas the transcription of *AtApx1* had no noticeable change. Interestingly, the *TsApx1* expression was always higher than the *AtApx1* one even in the absence of stress, which suggests that *TsApx1* may contribute to the higher abiotic stress tolerance in *T. salsuginea*. Thus, we could investigate some of the cellular signalling pathways regulating the *TsApx1* expression in *T. salsuginea*. For example, there are some known special *cis*-elements in the promoter region of *TsApx1* and some trans-acting factors that interact with them.

In summary, our results provide evidence that TsAPX1 was an effective H_2O_2-scavenging enzyme and that the overexpression of *TsApx1* conferred protection against oxidative stress that was induced by the high salinity or drought stress *via* a well-coordinated upregulation of antioxidant systems. Furthermore, the stress tolerance of *T. salsuginea* may be due to the constitutive overexpression and/or rapidly inducible expression of *TsApx1* and downstream genes which function in stress tolerance.

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