Available online at www.sciencedirect.com

SciVerse ScienceDirect

South African Journal of Botany 78 (2012) 235–245

SOUTH AFRICAN JOURNAL OF BOTANY

www.elsevier.com/locate/sajb

Overexpression of *Suaeda salsa* stroma ascorbate peroxidase in *Arabidopsis* chloroplasts enhances salt tolerance of plants

K. Li [a], C.-H. Pang [a,b], F. Ding [c], N. Sui [a], Z.-T. Feng [a], B.-S. Wang [a,*]

[a] *Key Laboratory of Plant Stress Research, College of Life Science, Shandong Normal University, Jinan 250014, Shandong, China*
[b] *Shandong Academy of Forestry, Jinan 250014, Shandong, China*
[c] *Shandong Provincial Key Laboratory of Fine Chemicals, College of Food and Bioengineering, Shandong Institute of Light Industry, China*

Received 28 December 2010; received in revised form 15 September 2011; accepted 18 September 2011

Abstract

To clarify the contribution of the stroma ascorbate peroxidase (*sAPX*) to the protection against salt-induced oxidative stress, we cloned the stromal *APX* from the euhalophyte *Suaeda salsa* (*Ss.sAPX*) for transfer into *Arabidopsis*. Confocal and RNA blotting analyses showed that the foreign gene was integrated into the *Arabidopsis* genome and located in chloroplasts. Measurements of isolated chloroplasts indicated that total APX and stromal APX activity were significantly higher in the transgenic plants than in wild-type (WT) plants but that thylakoid APX activity was not altered. The overexpression of *Ss.sAPX* in *Arabidopsis* increased the germination rate, cotyledon growth, survival rate, and tolerance to salt stress. Under NaCl stress, the transgenic plants had longer roots, higher total chlorophyll content, higher total APX activity, less cell membrane damage (as indicated by MDA levels), and lower H_2O_2 content than the WT. Moreover, under either normal or NaCl-stress conditions, sodium and proline contents and SOD and CAT activities did not differ between transgenic and WT plants. SOD and CAT activities in all tested lines declined gradually under salt stress. These results suggest that *Ss.sAPX* can play an important role in the protection against salt-induced oxidative stress in higher plants.

Keywords: Oxidative stress; Salt tolerance; Stroma ascorbate peroxidase; *Suaeda salsa* L.; Transgenic *Arabidopsis thaliana*

1. Introduction

The evolution of aerobic metabolic processes such as photosynthesis and respiration led to the continuous generation of reactive oxygen species (ROS) in chloroplasts, peroxisomes and mitochondria. ROS are scavenged by various antioxidants. However, the balance between production and scavenging of ROS may be perturbed by biotic and abiotic stresses, leading to excessive concentrations of ROS, including damaging concentrations of the superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), the hydroxyl radical ($OH^{\cdot}$), and singlet oxygen ($^1O_2$). H_2O_2, which is a relatively stable ROS, can diffuse across biological membranes (Sairam et al., 2002) and form $OH^{\cdot}$ in the presence of transition metals such as iron and copper (Halliwell and Gutteridge, 1999), causing protein denaturation and lipid peroxidation (Cheeseman and Slater, 1993; Scandalios et al., 1997).

Because plants are frequently subjected to abiotic stress, they have developed several strategies to avoid and alleviate injury by ROS (Foyer et al., 1995). Antioxidative defense systems include non-enzymatic and enzymatic components. Non-enzymatic components include ascorbate, reduced glutathione, vitamin E, phenolic compounds, and several other compounds. Enzymatic components include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR), monoascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), alternative oxidase (AOX), and ascorbate peroxidase (APX).

Abbreviations: CAT, catalase; chlAPX, chloroplastic ascorbate peroxidases; DAB, diaminobenzidine; Hyg, HygromycinB; MDA, malondialdehyde; MV, methylviologen; ROS, reactive oxygen species; SOD, superoxide dismutase; *Ss.sAPX*, stroma ascorbate peroxidase from *Suaeda salsa*; WT, wild type.

* Corresponding author. Tel.: +86 531 86180197; fax: +86 531 86180107.
E-mail address: bswang@sdnu.edu.cn (B.-S. Wang).

0254-6299/$ - see front matter © 2011 SAAB. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.sajb.2011.09.006

In plant cells, CAT and APX can remove excess H_2O_2 (Prasad et al., 1994; Willekens et al., 1995). CAT activity, however, is not found in the chloroplasts, and the chloroplast is the organelle in which most ROS are generated, particularly when environmental stresses are accompanied by high-intensity light. The electron transfer chain of the chloroplasts is the best-documented source of H_2O_2 (Asada, 1999, 2000). To prevent damage, therefore, the plant must scavenge excess H_2O_2 in the chloroplasts, and APXs are the key enzymes responsible for removing H_2O_2 (Noctor and Foyer, 1998). APXs directly contribute to ROS scavenging by converting H_2O_2 to water, with ascorbic acid (ASA) as the specific electron donor.

APX enzymes are found in at least five distinct compartments: the cytosol (cAPX), the microbody (mAPX), the peroxisome (pAPX), the stroma (sAPX) of chloroplasts, and the thylakoid membranes (tAPX) of chloroplasts (Asada, 1992; Caldwell et al., 1998; Ishikawa et al., 1995; Leonardis et al., 2000; Yamaguchi et al., 1995). chlAPXs, i.e., sAPX and tAPX, play important roles in photosynthesis when plants are stressed, and both sAPX and tAPX are involved in the water–water cycle, in which O_2 produced at the photosystem I site by the Mehler reaction is reduced to water in a two-step reaction catalyzed by SOD and APX (Mano and Asada, 1999). The cDNAs encoding the APX isoenzymes were isolated from various plants and have been well characterized by many research groups (Bunkelmann and Trelease, 1996; Mano et al., 1997; Murgia et al., 2004; Yamaguchi et al., 1996). In some species (e.g. *Arabidopsis*) the evolutionary path of chloroplast isoforms probably separated fairly into the isozymes sAPX and tAPX (Jespersen et al., 1997). In other species, chlAPXs are encoded by one gene and their mRNAs are regulated by the alternative splicing of its two 3′-terminal exons (Mano et al., 1997), as in spinach (Yoshimura et al., 1999) and tobacco (Yoshimura et al., 2000).

Researchers recently examined the role of the APX isoforms in protecting against oxidative stress induced by abiotic stress. The results were controversial. Overexpression of *cAPX* in chloroplasts of transgenic tobacco enhanced tolerance to oxidative stress generated by water deficit and salinity (Badawi et al., 2004). Similarly, overexpression of a gene encoding a *tAPX* in tobacco or in *Arabidopsis* significantly increased tolerance to oxidative stress caused by the application of methylviologen (MV) and by low temperature accompanied by high-intensity light (Yabuta et al., 2002) and to nitric oxide-induced cell death (Murgia et al., 2004). In contrast, Torsethaugen et al. (1997) reported that overproduction of pea *cAPX* in tobacco chloroplasts did not protect against ozone-induced stress. In other studies, chlAPXs did not respond to high-intensity light, drought, salinity, MV, or ABA treatment in spinach (Yoshimura et al., 2000), and oxidative stress did not affect the level of *chlAPX* transcripts (Panchuk et al., 2002; Shigeoka et al., 2002). Giacomelli et al. (2007) and Kangasjärvi et al. (2008) found that chlAPXs may not be key enzymes in photo-protection, at least in mature *Arabidopsis* plants. Together, these findings suggest that APX isozymes are individually regulated in each cellular compartment in higher plants under various oxidative stresses and that stress tolerance differs depending on plant species, plant developmental age, and stress severity.

The current study concerns the physiological mechanism of salt tolerance in *Suaeda salsa* L. *S. salsa*, a C_3 plant belonging to the Chenopodiaceae, is native to saline soils and is adapted to high salinity regions in northern China (Wang et al., 2001). Lu et al. (2002, 2003) reported that high salinity (200 mM NaCl) led to a slight increase in the CO_2 assimilation rate and did not affect photosystem II photochemistry in *S. salsa*, suggesting that *S. salsa* may have an effective antioxidant system to protect against oxidative stress induced by salt treatment. Similarly, previous results from our laboratory revealed that differential regulation of SOD isoenzymes and enhancement of thylakoid-bound SOD and chlAPX activity were involved in the salt tolerance of *S. salsa* (Pang et al., 2005; Wang et al., 2004; Zhang et al., 2005). To clarify the contribution of *Ss.sAPX* in the regulation of ROS levels and plant protection against oxidative stress, we cloned *Ss.sAPX*, transferred it into *Arabidopsis* via *Agrobacterium*-mediated transformation, and produced *Arabidopsis* lines overexpressing *Ss.sAPX*. We then investigated the performance of two of these transgenic lines of *Arabidopsis* under different NaCl conditions.

2. Materials and methods

2.1. Plant materials

The plants of *S. salsa* L. were grown in sand and were watered daily according to Pang et al. (2005). Three-week-old seedlings were subjected to NaCl treatment by exposing them to 200 mM NaCl dissolved in Hoagland nutrient solution for 24 h (NaCl was applied to increase the transcription of the *Ss.sAPX* gene). RNA was then extracted, and mRNA was used to isolate the *Ss.sAPX* gene as described in Section 2.2.

2.2. RNA isolation and gene cloning

Total RNAs were isolated from 3-week-old seedlings (see Section 2.1) using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The cDNA was synthesized using the SMART™ RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA).

A PCR product (about 280 bp) of *Ss.sAPX* was amplified from *S. salsa* cDNA with degenerate primers: forward 5′ GAR(A/G)TGGCCRCW(A/T)RM(A/C)GAGGTGGAGC 3′ and reverse 5′ GCWGGK(T/G)GAWGGAGGN(A/T/C/G) CCAGCATCWGG 3′. The 5′- and 3′-ends of the sequence were obtained using 5′-RACE and 3′-RACE amplification with the SMART™ RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA). The full-length sequence was then obtained by PCR with primer pair: 5′ CCATATCATCAGCTAGTCAA ATCAGC 3′ and 5′ TGGTCATTTGATGCTTTATGAAAA 3′. All RT-PCR products were sequenced on both strands.

Sequences were analyzed using DNASIS software, and databank searches were conducted through the BLAST program. Sequences were aligned and phylogenetic trees were constructed with CLUSTALX and DNAMAN software.

2.3. Vector construction and Arabidopsis transformation

For the construction of transgenic vectors, *Ss.sAPX* cDNA was ligated into the pCAMBIA1300 vector under the control of the CaMV 35S promoter, which contained a hygromycin B (Hyg) selectable marker. The GATEWAY vector (produced for C-terminal protein fusions with green fluorescent protein—GFP) under the control of the CaMV *Ss.sAPX* 35S promoter was used to investigate the intracellular targeting of *Ss.sAPX*. The recombinant plasmids were introduced into *Agrobacterium tumefaciens* strain GV3101, and *Arabidopsis* transformation was conducted using the floral dip method (Clough and Bent, 1998).

After the self-pollinated *Arabidopsis* transformants (T_0) were harvested, the seeds were plated on MS solid medium supplemented with 30 mg/L Hyg, and transgenic lines (T_1) were selected. The integration and expression of each transgene in different homozygous lines (T_2) were then confirmed by PCR and northern analysis (see Section 2.4).

2.4. Detection of Ss.sAPX in transgenic Arabidopsis by PCR and Northern blot

Genomic DNA samples were isolated from the WT and transgenic plants according to Yabuta et al. (2002). The transgenic plants were represented five lines: s-4, s-7, s-9, s-12, and s-13. About 100 ng of genomic DNA was used to amplify the *Ss.sAPX* transgene by PCR with primer pair: 5′ GTCGTCAAACCCAACCAACCTCCTC 3′ and 5′ GTGAGCTGTCGGATTCTATGAGGCA 3′. RNAs were isolated using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNAs (15 μg each) were electrophoretically separated on a 1.2% (w/v) agarose gel in 3-(N-morpholino) propanesulphonic acid (MOPS) buffer, then transferred to Hybond-N+ membranes (Amersham).The probe was amplified with the primer pair: 5′ GTCGTCAAACCCAACCAACCTCCTC 3′ and 5′ GTGAGCTGTCGGATTCTATGAGGCA 3′. The RNA blots were hybridized with ^{32}p-labeled gene-specific probes for *Ss.sAPX* in church buffer (1 mM EDTA, 0.25 M Na_2HPO_4–NaH_2PO_4 pH 7.2, 7% SDS) at 65 °C. They were also probed with 18*S* rRNA to check for equal loading. The hybridized blots were washed at 65 °C while gradually decreasing the salt concentration to 0.5×SSC/0.1% SDS, then exposed to X-ray film.

2.5. Isolation of chloroplasts

Fresh-cut leaves were obtained from WT and transgenic plants growing on MS medium without added NaCl, and chloroplasts were isolated from 1 g of these fresh-cut leaves according to Jeannette et al. (2010) with some modifications. The leaves were washed with cold double-distilled water, and homogenized in a blender in 10 ml of ice-cold 1× chloroplast (Clp) buffer containing 0.3 M sorbitol, 50 mM Hepes/Tris (pH 7.5), 5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM ethyleneglycoltetraacetic acid (EGTA), 1 mM $MgCl_2$, 2 mM sodium ascorbate, and freshly added 0.5 mM dithiothreitol (DTT). Before chloroplasts were isolated from a sample, a 50% Percoll layer was established in each of two polypropylene tubes (13 ml, Beckman, USA) by mixing 3.1 ml of Percoll and 3.1 ml of 2× Clp buffer (DTT included) in each tube. The solutions were cooled to 0–4 °C. After the homogenate was filtered through a polyester mesh (pore size 38 μm), the homogenate was carefully loaded onto the 50% Percoll layers with the help of a pipette, and the tubes were centrifuged for 10 min at 2000×*g* in a swing-out rotor (Beckman, USA). Upper layers containing broken chloroplasts were removed using a pipette, and the chloroplast pellet was washed once by carefully adding 14 ml of 1× Clp buffer and inverting the tubes. After centrifugation for 5 min at 1000×*g*, the supernatant was removed and the lower layer containing intact chloroplasts was collected. One aliquot of chloroplasts was resuspended with 1 ml 1× Clp buffer containing 0.1% Triton X-100, and this suspension was used in the total APX activity assay (see Section 2.8). A second aliquot was resuspended in hypotonic medium (the same as the 1× Clp buffer except that the sorbitol concentration was 4 mM) to break the chloroplasts and to release the stromal enzymes. After centrifugation at 20,000×*g* for 5 min, the supernatant was used to analyze the stromal APX activity (see Section 2.8), and thylakoids were included in the pellet for solubilizing membrane-bound APX. Thylakoid-bound APX, which was obtained by incubating the membranes with 0.5 ml 1× Clp buffer containing 0.1% Triton X-100 and 1.5 mM DTT for 30 min, was also used in the quantification of tAPX activity (see Section 2.8). All steps were carried out at 4 °C.

2.6. Analysis of salt stress-resistance of transgenic Arabidopsis

For observation of phenotypic differences between transgenic (s-4 and s-7) and WT plants, transgenic and WT seeds were placed in MS medium containing 0, 100, 120, and 140 mM NaCl for 1 week (16-h light/8-h dark at 24±2 °C, 80 μmol photons m^{-2} s^{-1}, 70% humidity) after vernalization for 3 days at 4 °C. Salt tolerance of the plants was estimated by measuring germination rate, cotyledon formation, survival rate, root length, and total chlorophyll content. The fresh leaves of WT and transgenic plants (s-4 and s-7) growing in MS medium with 0 and 120 mM NaCl for 1 week were used to determine malondialdehyde (MDA) levels (Section 2.7), H_2O_2 content (Section 2.7), staining of H_2O_2 with 3,3-diaminobenzidine (DAB) (Section 2.7), and enzyme activity assays (Section 2.8).

Chlorophyll content was measured according to Lichtenthaler (1987) with some modifications. Fresh leaves (0.1 g) were briefly rinsed in deionized water and then soaked in 80% acetone (v/v) in the dark for more than 40 h. Total chlorophyll content, expressed as mg g^{-1} DW, was determined by measuring the absorbance at 663 and 646 nm of the acetone containing the released chloroplasts.

2.7. MDA levels, H_2O_2 content, and staining of H_2O_2 with DAB

Lipid peroxidation was determined by measuring the amount of MDA produced by the thiobarbituric acid reaction as described by Heath and Packer (1968). The MDA concentration was

calculated by its molar extinction coefficient (155 mM^{-1} cm^{-1}), and the results were expressed as μmol (MDA) g^{-1} FW.

H_2O_2 was quantified spectrophotometrically according to the modified method of Wolfe (1962). Leaf samples (0.5 g) were homogenized in 5 ml of acetone. The homogenate was centrifuged at 2000 × *g* for 10 min at 10 °C. The supernatant (1.0 ml) was mixed with 0.1 ml of 20% titanium chloride and 0.2 ml of 28% ammonia, and the mixture was then centrifuged at 2000 × *g* for 10 min at 10 °C. The sediment was dissolved by adding 5 ml of 2 mM H_2SO_4, and the intensity of yellow color of the supernatant was measured at 410 nm.

The leaves were stained with DAB (Thordal-Christensen et al., 1997) When the salt treatments described in Section 2.6 were completed, plant materials were soaked in 1 mg ml^{-1} of DAB (pH 3.8) for 8 h, washed, and bleached in a mixture containing acetic acid, glycerol, and ethanol (1:1:3) by heating at 100 °C for 5–10 min.

2.8. Preparation of crude enzyme extracts and enzyme activity assays

Crude enzymes were extracted from plants in Section 2.6 as described by Rao et al. (1997) with some modifications. Leaf tissues (0.2 g) were ground to a fine powder in liquid N_2 and then homogenized in 2 ml of 50 mM potassium phosphate buffer (pH 7.0) and 1% PVP using a chilled pestle and mortar. The homogenate was centrifuged at 15,000 × *g* for 10 min at 2–4 °C. The supernatant was used as crude extract for enzyme activity except for the measurement of APX, in which case the plant tissue was homogenized in 50 mM sodium phosphate (pH 7.8) containing 1 mM EDTA and 5 mM ascorbate. All operations were carried out at 4 °C.

APX activity was assayed from plant materials in Sections 2.5 and 2.6 according to Jiménez et al. (1997). Activity was determined in a reaction mixture consisting of 50 mM phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM EDTA, and 0.1 mM H_2O_2, and activity was indicated by the change in absorbance at 290 nm (E = 2.8 mM^{-1} cm^{-1}). The results were calculated in terms of micromoles of ascorbate oxidized per minute.

CAT activity of leaf tissues (see Section 2.6) was measured by following the decomposition of H_2O_2 at 240 nm (E = 39.4 mM^{-1} cm^{-1}) in a reaction mixture containing the appropriate extract in 100 mM phosphate buffer (pH 7.0) according to Rao et al. (1997). The reaction was initiated by addition of 10% H_2O_2, and 1 U of CAT activity was defined as micromoles of H_2O_2 degraded per minute.

SOD activity was assayed from plants in Section 2.6 by monitoring the inhibition of the photochemical reduction of nitro blue tetrazolium (NBT) according to Giannopolitis and Ries (1977). One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition in the reduction of NBT at 560 nm, expressed in units per milligram of protein.

2.9. Confocal microscopy and statistical analysis

For observation of GFP localization, whole seedlings obtained from WT and transgenic plants growing on MS medium without added NaCl (see Section 2.6) were mounted in water under glass cover slips and the GFP signals was visualized using a confocal microscope (Eclipse E-800 C1; Nikon, Instrument Inc, NY, USA) equipped with a krypton/argon laser.

All experiments were carried out in triplicate, and 15 plants per transgenic line were examined each time. All data are presented as means ± standard deviation (SD). Comparisons between transgenic plants and WT plants were performed using one-way analysis of variance and Duncan's multiple range test with a 5% level of significance.

3. Results

3.1. Characterization of cDNAs encoding the sAPX from S. salsa

Based on sequence homology between the *sAPX* genes or transcripts mainly from *Mesembryanthemum crystallinum*, *Nicotiana tabacum*, *Spinacia oleracea*, *Cucurbita* cv. *Kurokawa Amakuri*, *Chlamydomonas reinhardtii* and *Arabidopsis thaliana*, reverse transcriptase (RT)-PCR amplification and 5′- and 3′-RACE amplification were used to obtain the full-length transcript sequences of *Ss.sAPX*. *Ss.sAPX* (GenBank accession number: GU228491) cDNA consists of 1726 nucleotides with an 1137-bp open reading frame (ORF) of 378 amino acids, and has a predicted molecular mass of 40.96 kDa. The N-terminal 378 amino acids of *Ss.sAPX* are identical with those of *Ss.tAPX*, whereas *Ss.tAPX* is 49 amino acids more than *Ss.sAPX* in C-terminal.

To detect similarities and differences in individual amino acid sequence positions, the deduced amino acid sequence of *sAPX* from six plant species were aligned. The deduced amino acid sequence of *Ss.sAPX* was closest to that of the halophyte *M. crystallinum* L. (Fig. 1). *M. crystallinum* is a facultative halophyte that responds to water stress in the form of drought or high salinity by switching from C_3 photosynthesis to crassulacean acid metabolism, a physiological adaptation that increases water conservation.

3.2. Localization of sAPX proteins from S. salsa

Sequence analysis of the *sAPX* proteins suggested the presence of an N-terminal signal peptide. To determine the subcellular location of *sAPX*, we generated stable transgenic *Arabidopsis* plants expressing the green fluorescent protein: *Ss.sAPX*+GFP fusion protein. As shown in Fig. 2, the fusion protein is indeed localized exclusively in chloroplasts.

3.3. Molecular characterization and chlAPX activity of the transgenic Arabidopsis plants

The transmission and stable integration of the *Ss.sAPX* gene in the genome of *Arabidopsis* plants were confirmed by PCR (Fig. 3A). To examine the expression of the *Ss.sAPX* in T_2 transgenic plants, the total RNA was isolated from WT plants and plants of five transgenic lines (s-4, s-7, s-9, s-12, and s-13) to RNA blot analysis; mRNA transcripts were detected

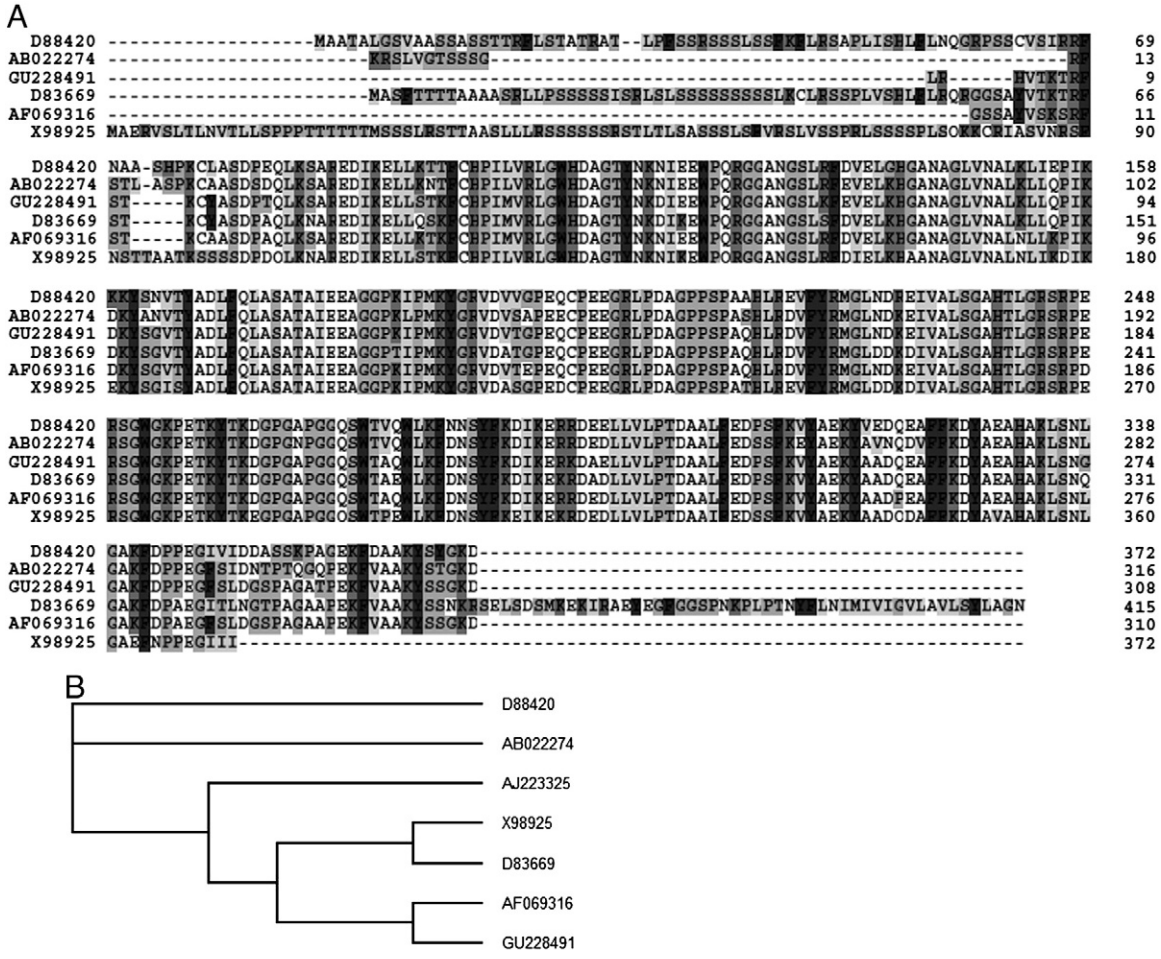

Fig. 1. Deduced amino acid sequence alignment of *sAPX* from six plant species. (A) Homology of *Ss.sAPX* amino acid sequence with other *sAPX* amino acid sequences. Identical and similar amino acid residues are shaded black, and dashes indicate gaps introduced to optimize alignment. (B) Phylogenic tree of *Ss.sAPX* amino acid sequence with other *sAPX* amino acid sequences. D88420 (*Cucurbita* cv. Kurokawa Amakuri), AB022274 (*Nicotiana tabacum*), AJ223325 (*Chlamydomonas reinhardtii*), X98925 (*Arabidopsis thaliana*), D83669 (*Spinacia oleracea*), AF069316 (*Mesembryanthemum crystallinum*), and GU228491 (*Suaeda salsa*).

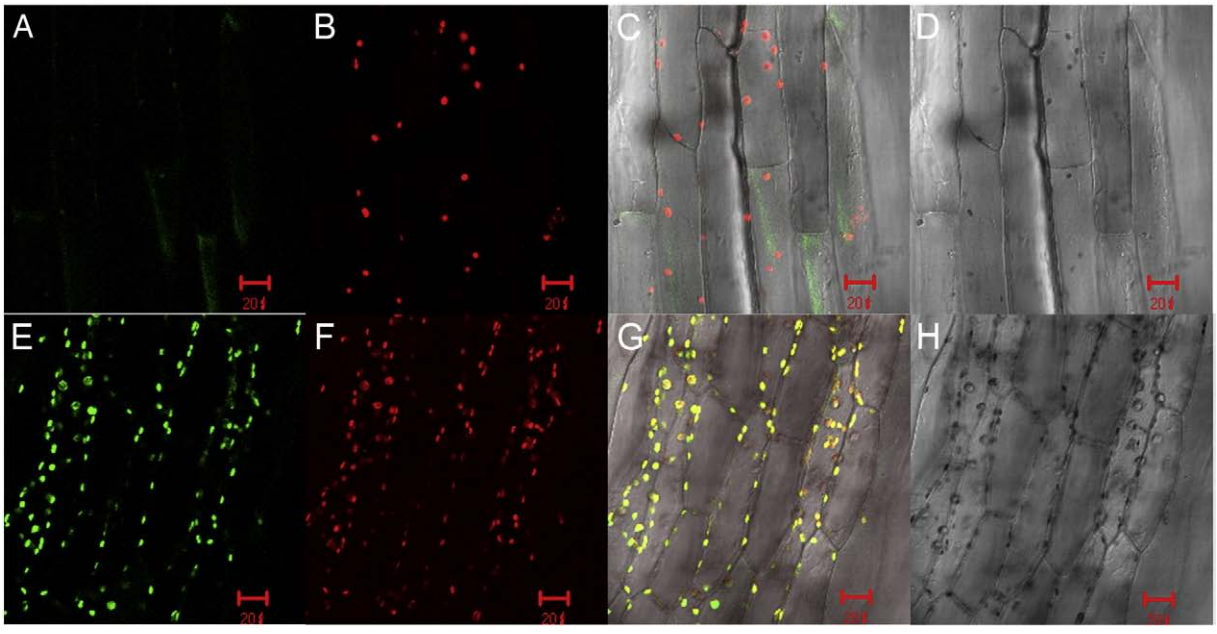

Fig. 2. Localization of *sAPX* proteins of *S. salsa* in WT (col, A–D) and transgenic (sAPX+GFP, E–H) *Arabidopsis* plants. Green fluorescence from GFP (A, E), red fluorescence of chlorophyll (B, F), and white light (D, H) images were monitored separately by confocal laser microscopy; c is the merged image of A and B, and G is the merged image of E and F.

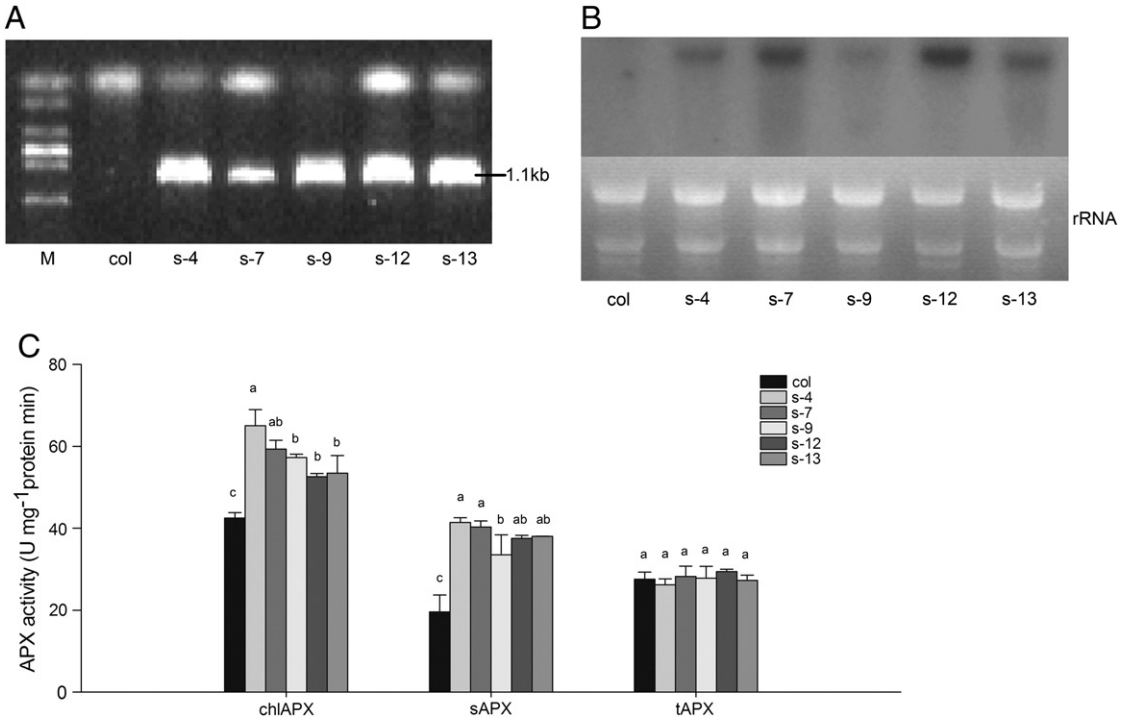

Fig. 3. Molecular analysis and chlAPX activity in WT and five transgenic *Arabidopsis* lines (T_2) overexpressing *Ss.sAPX* (s-4, s-7, s-9, s-12, s-13) grown in MS medium without NaCl for 30 days. (A) PCR analysis of WT (col) and transgenic plants overexpressing *Ss.sAPX* (s-4, s-7, s-9, s-12, s-13). (B) RNA blot analysis of WT and transgenic lines. (C) The activity assay of the chlAPX, sAPX, and tAPX in transgenic and WT plants. Values are the means (n = 3) ± SD of four independent experiments. For each kind of APX in panel C, values with different letters are significantly different ($P < 0.05$).

at various levels in the transgenic plants, indicating that *Ss.sAPX* was expressed in the T_2 transgenic plants (Fig. 3B). To confirm the role of *Ss.sAPX*, chlAPX activity of transgenic and WT plants under normal conditions was quantified. As shown in Fig. 3C (left), chlAPX activity in chloroplasts was substantially higher in the transgenic plants than in the WT plants. Similar results were obtained for sAPX activity in chloroplasts (Fig. 3C, middle). In contrast, tAPX activity did not differ between transgenic and WT plants (Fig. 3C, right). These results indicate that sAPX activity constituted the major part of total chloroplast APX activity in the transgenic lines and that *Ss.sAPX* was expressed in transgenic *Arabidopsis*.

3.4. Salt tolerance in transgenic Arabidopsis overexpressing Ss.sAPX

WT and two transgenic lines (s-4 and s-7) were treated with different concentrations of NaCl. As shown in Fig. 4A, the transgenic plants grew as well as WT plants under normal conditions (no NaCl added) in MS medium. Although the growth of both WT and transgenic lines was inhibited when NaCl was added, their growth was similar when 100 mM NaCl was applied. At higher NaCl concentrations, however, the growth of WT plants were more significantly inhibited than transgenic plants (Fig. 4A).

When NaCl was not added to the MS medium, germination, cotyledon growth, seedling survival, root length, and total chlorophyll content did not significantly differ between the WT plants and the two transgenic lines (Fig. 4B–F). When NaCl was added, germination, cotyledon growth, and survival were greater in the transgenic lines than in the WT (Fig. 4B–D). At 120 mM NaCl, germination rate, cotyledon formation, and survival rate were at least 1.3-fold greater in the transgenic plants than in the WT plants. Subsequent experiments concerning root length and total chlorophyll used two levels of NaCl, 0 and 120 mM, in the MS medium. Salt stress caused by 120 mM NaCl significantly decreased root length and total chlorophyll content in both WT and transgenic plants but the reduction was less with the transgenic plants than with the WT plants (Fig. 4E, F).

3.5. Determination of MDA levels

MDA is used as an indicator of lipid peroxidation caused by oxidative stress. When NaCl was not added to the MS medium, MDA content did not significantly differ between transgenic and WT plants (Fig. 5). Although addition of NaCl caused the level of MDA to increase in both transgenic and WT plants, the increase was substantially less in the transgenic plants than in the WT plants (Fig. 5).

3.6. Effects of salt stress on the activities of antioxidative enzymes

To test whether the lower MDA content in the transgenic lines than in the WT was due to an increase of antioxidant

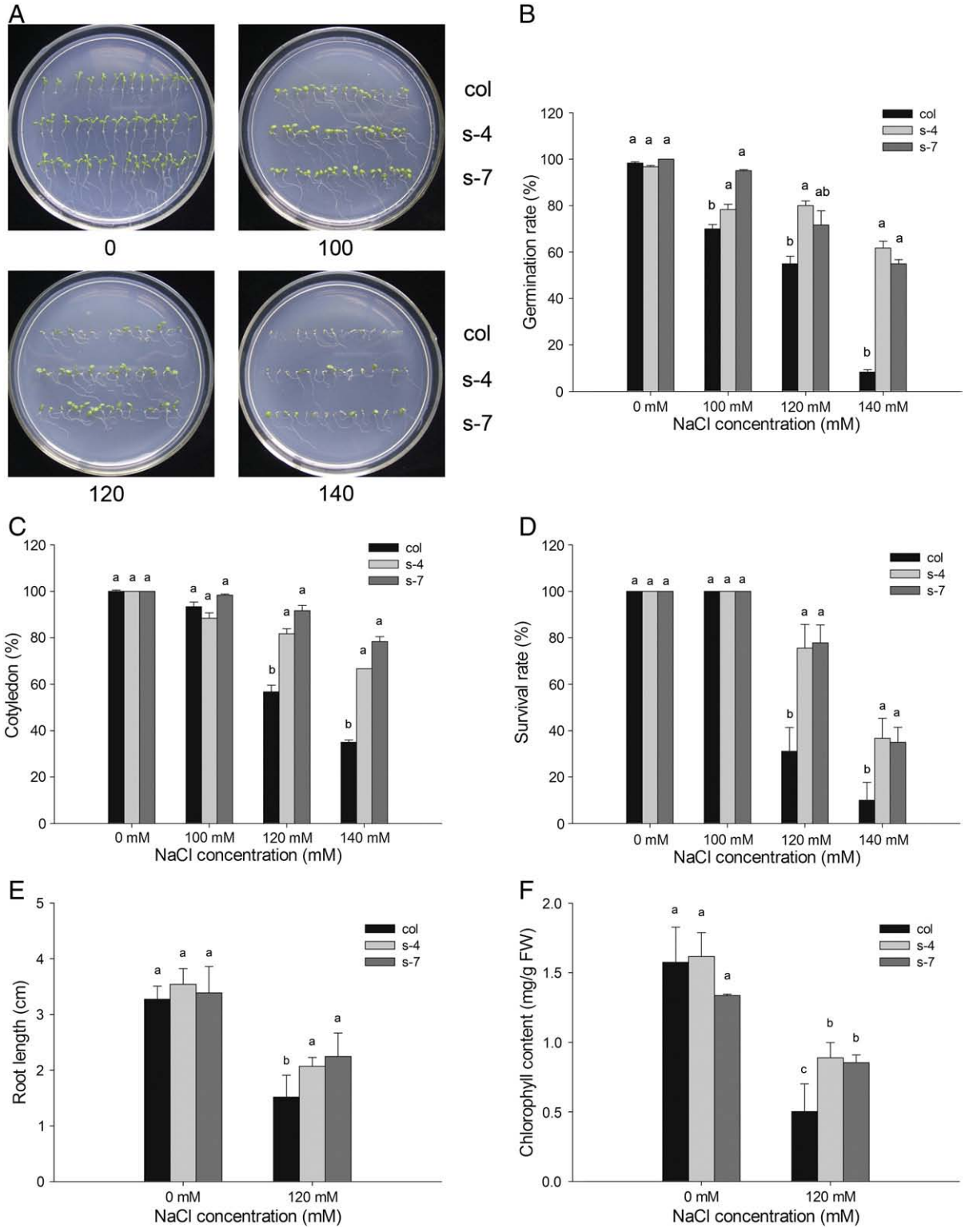

Fig. 4. Effect of salt stress on early growth (A), germination rate (B), cotyledon formation (C), survival (D), root length (E), and chlorophyll content (F) of WT (col) and transgenic *Arabidopsis* overexpressing *Ss.sAPX* (s-4 and s-7). After vernalization, plants were grown on MS medium supplemented with 0 (control), 100, 120, or 140 mM NaCl for 1 week in the greenhouse. Germination, cotyledon formation, and survival were measured after 1, 4, and 7 days, respectively. Root length and total chlorophyll content were measured for plants grown in 0 or 120 mM NaCl for 7 days (E, F). Values are means (n=3)±SD. Within each concentration of NaCl in panels B–F, values with different letters are significantly different ($P<0.05$).

enzymes in general, the activities of CAT, SOD, and APX were measured. When NaCl was not added to the MS medium, there is just a very small increase in activities of CAT, SOD and total APX in the two transgenic lines compared to WT. When the MS medium contained 120 mM NaCl, the activity of total APX was 1.1-fold to 1.2-fold greater in the transgenic plants

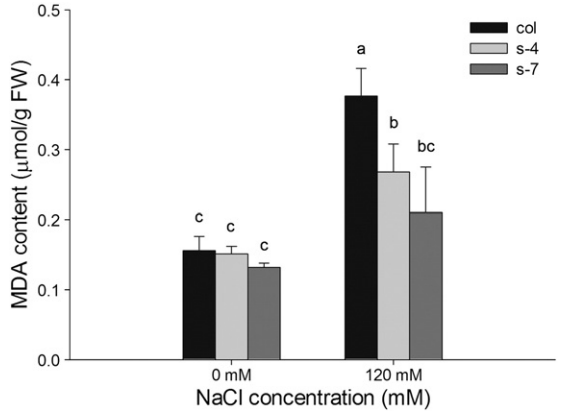

Fig. 5. Levels of MDA in cotyledons of WT (col) and transgenic *Arabidopsis thaliana* (s-4 and s-7, overexpressing *Ss.sAPX*) grown for 7 days in MS medium supplemented with 0 and 120 mM NaCl. Values are means (n=3)±SD. Within each NaCl concentration, bars with different letters are significantly different ($P<0.05$).

than in the WT. However, SOD and CAT activities in all lines declined in 120 mM NaCl and did not differ among the plants (Table 1).

3.7. Effects of salt stress on H_2O_2 content

To clarify whether the increase in APX activity in the transgenic lines might be sufficient to limit the increase in H_2O_2 levels induced by salt stress, H_2O_2 content was quantified. Under stress imposed by 120 mM NaCl, H_2O_2 content increased in the WT and in the transgenic lines but the increase was significantly greater in the WT (Fig. 6A). This finding was confirmed by DAB staining, which was less intense in the transgenic lines, indicating that H_2O_2 accumulation was lower in the transgenic *Arabidopsis* overexpressing *Ss.sAPX* than in the WT plants (Fig. 6B).

4. Discussion

Elevated salt levels play a major in soil degradation, and salt stress is an important limiting factor in agricultural production

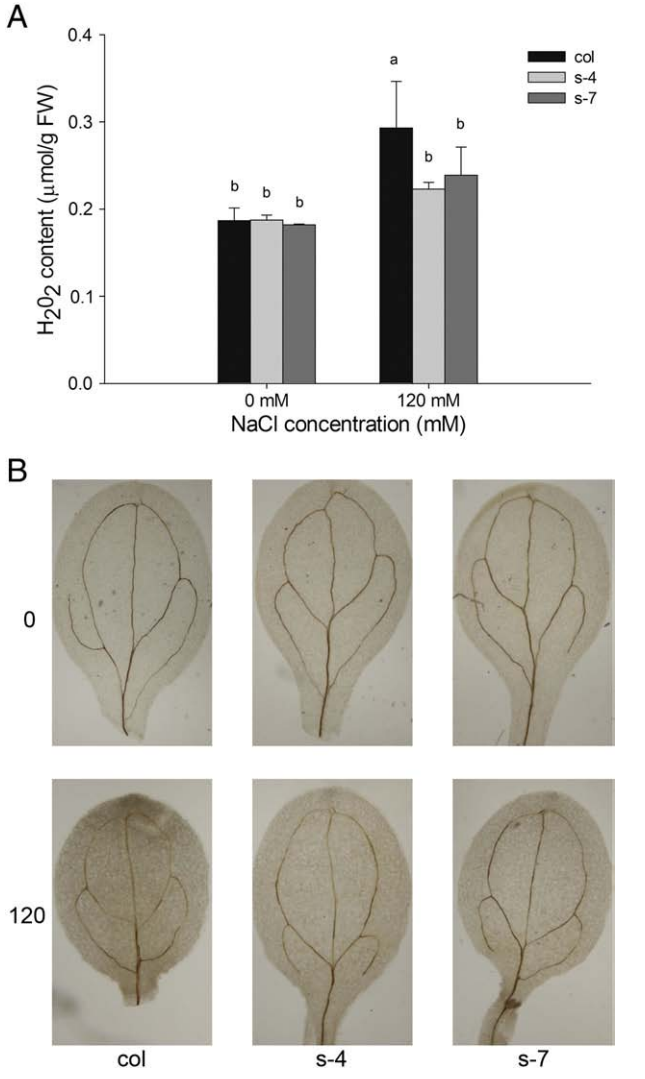

Fig. 6. Levels of H_2O_2 (A) and staining with DAB (B) in transgenic *Arabidopsis* overexpressing *Ss.sAPX* (s-4 and s-7) and WT (col) plants as affected by NaCl (0 or 120 mM) for 7 days. DAB-stained leaves were examined and photographed with a light microscope at 40× magnification. Values are means (n=3)±SD. For each NaCl concentration in panel A, bars with different letters are significantly different ($P<0.05$).

Table 1
Effect of NaCl on the activities of CAT, APX and SOD in WT (col) and transgenic overexpressing *Ss.sAPX* plants. WT and transgenic *Arabidopsis* over-expressing *Ss.sAPX* (s-4 and s-7) plants were grown on MS medium supplemented with 0 and 120 mM NaCl for 7 days.

Plant	Treatments	APX	CAT	SOD
	NaCl (mM)	Activity (U mg^{-1}protein·min)		
col	0	25.90±3.59b	150.56±24.63a	87.04±11.62a
	120	30.15±0.73ab	138.79±6.97a	75.36±12.21a
s-4	0	27.45±2.36ab	154.34±22.85a	88.46±13.10a
	120	32.76±4.72ab	142.93±15.97a	79.20±13.55a
s-7	0	27.49±3.01ab	154.79±21.08a	91.97±3.22a
	120	35.23±1.53a	145.21±15.84a	86.50±11.09a

Values are means (n=3)±SD.
For each level of NaCl values followed by different letters are significantly different ($P<0.05$).

worldwide. Salinization affects a large land area, especially in irrigated fields (Bartels and Sunkar, 2005; Zhu, 2001, 2002). The inhibition of plant growth by salt stress involves both osmotic and ionic components (Munns et al., 2006) as well as secondary oxidative stress (Sairam et al., 2002). The chloroplast is one of the organelles in which ROS is generated, and it is exceptionally susceptible to ROS damage. APX enzymes, and especially the chlAPX pathway, constitute an important mechanism that protects plants from the damage caused by the H_2O_2 resulting from salt stress in this organelle (Azevedo Neto et al., 2006; Shigeoka et al., 2002; Tsugane et al., 1999). sAPX can also be envisioned as the first line of defense against diffusion of H_2O_2 away from thylakoid membranes to the stroma. In this study, we cloned the *Ss.sAPX* from a C_3 euhalophyte *S. salsa*. To determine the role of *Ss.sAPX*, this gene in *Arabidopsis* was overexpressed and examined

effects of the transgene under salt stress. The results showed that the overexpressing *Ss.sAPX* lines had high tolerance to saline condition, which was consistent with the hypothesis that salt-induced oxidative stress could be reduced in *Arabidopsis* carrying *Ss.sAPX*.

The cDNA sequence of *Ss.sAPX* consists of 1726 nucleotides, including an 1137-bp open reading frame (ORF) of 378 amino acids, and has a predicted molecular mass of 40.96 kDa. The N-terminal 378 amino acids of *Ss.sAPX* are identical with those of *Ss.tAPX*, whereas *Ss.tAPX* is 49 amino acids more than *Ss.sAPX* in C-terminal. It suggests that *Ss.sAPX* and *Ss.tAPX* are encoded by one gene and their mRNAs are regulated by the alternative splicing of its two 3′-terminal exons as in spinach (Yoshimura et al., 1999), tobacco (Yoshimura et al., 2000). Interestingly, amino acid sequence alignment analysis suggested that the *Ss.sAPX* is more closely related to the halophyte *M. crystallinum* (90.6% amino acid identity) than to *Arabidopsis* (76.8% amino acid identity). In addition, sAPX contains three conserved amino acids residues (Asn, Asp, and Ser) (Jespersen et al., 1997) and further comparison of the patterns of conserved residues showed that Ser-60, Ser-80, Ser-93, Asn-236, Asp-166, Asp-228, Asp-269, Asp-293, and Asp-350 differed between *S. salsa* and *Arabidopsis* but did not differ between *S. salsa* and *M. crystallinum*. Our previous transcript analysis had shown that chlAPX activity of *S. salsa* markedly increased under salt stress (Pang et al., 2005). Perhaps the changes of amino acids residues in *S. salsa* and *M. crystallinum* explain the higher chlAPX activity under salt stress.

Under non-stressed conditions, growth and development were similar for overexpression lines with higher APX activity and the WT. Although germination, cotyledon growth, and survival of all lines were influenced by salt stress, the transgenic lines were less inhibited by NaCl than the WT. In the presence of 120 mM NaCl, root length and total chlorophyll content were also greater for the *Ss.sAPX* lines than for the WT. These phenomena were also observed by Lu et al. (2007) and Xu et al. (2008) in their studies of NaCl tolerance in *Arabidopsis* that overexpressed rice *cAPX* and barley *pAPX*. To investigate the mechanism of the increased salt tolerance in transgenic *Arabidopsis* lines, MDA and H_2O_2 contents in cotyledons were measured, and no significant difference was found between the tested lines when NaCl was not added. MDA and H_2O_2 contents were higher, however, in the WT than in transgenic *Arabidopsis* at 120 mM NaCl. Although the high H_2O_2 contents confirmed that salt stress produces excessive H_2O_2 that reduced the growth of both transgenic and WT *Arabidopsis*, H_2O_2 contents were lower and chloroplasts less damaged in the transgenic lines. In contrast, sodium and proline contents and the ratio of K^+ to Na^+ did not differ between transgenic lines and the WT after treatment with 120 mM NaCl for 7 days (data not shown). These latter results suggested that the salt tolerance in the transgenic lines was not due to maintenance or subsequent reestablishment of cellular ion homeostasis and osmotic adjustment. Salt stress can produce excess ROS including H_2O_2, resulting in oxidative damage and lipid peroxidation (Sairam and Tyagi, 2004). Therefore, we infer that salt tolerance conferred by *Ss.sAPX* can be explained, at least in part, by a reduction of oxidative stress, an inference that is consistent with previous reports (Lu et al., 2007; Murgia et al., 2004).

The ability of higher plants to scavenge ROS seems to be a very important determinant of their tolerance to environmental stress. Under conditions of elevated NaCl, many antioxidative enzyme activities are known to increase in plants but there are also reports indicating that some of these scavenging enzymes actually decline (Lee et al., 2001; Tsai et al., 2005). To obtain more information about the role of *Ss.sAPX* and its relationship to other antioxidant systems, the activities of APX, CAT, and SOD in transgenic *Arabidopsis* under salt stress were also measured. The activity of total APX was a little higher in overexpression *Ss.sAPX* plants than in the WT plants exposed to 120 mM NaCl, but the activities of CAT and SOD in transgenic *Arabidopsis* slightly declined. Moreover, activities of chlAPX and sAPX in chloroplasts were substantially higher in the transgenic plants than in the WT plants, but tAPX activity did not differ between transgenic and WT plants (Fig. 3). These results indicate that the increase in the stromal APX was responsible for the increased salt tolerance, even if it is responsible only for 5–10% increase of the total APX activity. The increased scavenging of H_2O_2 by APX evidently compensated in part for the decreased activities of SOD and CAT in all lines under saline condition. These results confirm that our NaCl treatment did induce oxidative stress, which was also confirmed by the increases in MDA and H_2O_2. Most importantly, the higher APX activity, in particular, the increased stromal APX activity, was the basis of the enhanced tolerance to salt stress, highlighting the important played by *Ss.sAPX* in supporting higher salt tolerance.

In this study, we found that the reduced growth of salt-stressed, WT *Arabidopsis* was correlated with increased levels of ROS. In contrast, the overexpression of *Ss.sAPX* in transgenic *Arabidopsis* markedly enhanced salt tolerance by maintaining a low level of H_2O_2, which consequently protected the transgenic plants from damage caused by oxidative stress. These results suggest that overexpression of the *Ss.sAPX* transcript in *Arabidopsis* plays an essential in preventing the over-accumulation of ROS. This study helps to explain the roles of *Ss.sAPX* in adaptive responses of plants to environmental stress.

Acknowledgments

We thank Prof. Hans Bohnert (Department of Plant Biology, University of Illinois at Urbana-Champaign) and Prof. T.J. Flowers (School of Biological Sciences, University of Sussex, UK) for critically reading and revising the manuscript. We are grateful for the financial support from the NSFC (National Natural Science Research Foundation of China, project Nos. 30870138 and 30670177) and the HI-Tech Research and Development (863) Program of China (2007AA091701). We also thank Prof. Zhizhong Gong (Department of Plant Sciences, College of Biological Science, China Agricultural

University) for providing the facilities for *Ss.sAPX* isolation and localization.

References

Asada, K., 1992. Ascorbate peroxidase — a hydrogen peroxide-scavenging enzyme in plants. Physiologia Plantarum 85, 235–241.

Asada, K., 1999. The water–water cycle in chloroplasts: scavenging of active oxygen and dissipation. Annual Review of Plant Physiology and Plant Molecular Biology 50, 601–639.

Asada, K., 2000. The water–water cycle as alternative photon and electron sinks. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences 355, 1419–1431.

Azevedo Neto, A.D., Prisco, J.T., Eneas-Filho, J., Abreu, C.E.B., Gomes-Filho, E., 2006. Effect of salt stress on antioxidative enzymes and lipid peroxidation in leaves and roots of salt-tolerant and salt-sensitive maize genotypes. Environmental and Experimental Botany 56, 87–94.

Badawi, G.H., Kawano, N., Yamauchi, Y., Shimada, E., Sasaki, R., Kubo, A., Tanaka, K., 2004. Overexpression of ascorbate peroxidase in tobacco chloroplasts enhances the tolerance to salt stress and water deficit. Physiologia Plantarum 121, 231–238.

Bartels, D., Sunkar, R., 2005. Drought and salt tolerance in plants. Critical Reviews in Plant Sciences 24, 23–58.

Bunkelmann, J.R., Trelease, R.N., 1996. Ascorbate peroxidase: a prominent membrane protein in oilseed glyoxysomes. Plant Physiology 110, 589–598.

Caldwell, C.R., Turano, F.J., McMahon, M.B., 1998. Identification of two cytosolic ascorbate peroxidase cDNAs from soybean leaves and characterization of their products by functional expression in *E. coli*. Planta 204, 120–126.

Cheeseman, K.H., Slater, T.F., 1993. An introduction to free radical biochemistry. British Medical Bulletin 49, 481–493.

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

Foyer, C.H., Souriau, N., Perret, S., Lelandais, M., Kunert, K.J., Pruvost, C., Jouanin, L., 1995. Overexpression of glutathione reductase but not glutathione synthetase leads to increases in antioxidant capacity and resistance to photoinhibition in poplar trees. Plant Physiology 109, 1047–1057.

Giacomelli, L., Masi, A., Ripoll, D.R., Lee, M.J., Van Wijk, K.J., 2007. *Arabidopsis thaliana* deficient in two chloroplast ascorbate peroxidases shows accelerated light-induced necrosis when levels of cellular ascorbate are low. Plant Molecular Biology 65, 627–644.

Giannopolitis, C.N., Ries, S.K., 1977. Superoxide dismutases. I. Occurrence in higher plants. Plant Physiology 59, 309–314.

Halliwell, B., Gutteridge, J.M.C., 1999. Free Radicals in Biology and Medicine. Oxford University Press, New York, pp. 1–90.

Heath, R.L., Packer, L., 1968. Photoperoxidation in isolated chloroplasts. I. Kinetics and stochiometry of fatty acid peroxidation. Archives of Biochemistry and Biophysics 125, 189–198.

Ishikawa, T., Sakai, K., Takeda, T., Shigeoka, S., 1995. Cloning and expression of cDNA encoding a new type of ascorbate peroxidase from spinach. FEBS Letters 367, 28–32.

Jeannette, K., Martin, H., Alexander, M., Ales, S., Wilhelm, B., 2010. Isolating intact chloroplasts from small *Arabidopsis* samples for proteomic studies. Analytical Biochemistry 398, 198–202.

Jespersen, H.M., Kjaersgård, I.V., Ostergaard, L., Welinder, K.G., 1997. From sequence analysis of three novel ascorbate peroxidases from *Arabidopsis thaliana* to structure, function and evolution of seven types of ascorbate peroxidase. Biochemical Journal 326, 305–310.

Jiménez, A., Hernández, J.A., del Río, L.A., Sevilla, F., 1997. Evidence for the presence of the ascorbate–glutathione cycle in mitochondria and peroxisomes of pea leaves. Plant Physiology 114, 275–284.

Kangasjärvi, S., Lepistö, A., Hännikäinen, K., Piippo, M., Luomala, E.M., Aro, E.M., Rintamäki, E., 2008. Diverse s for chloroplast stromal and thylakoid-bound ascorbate peroxidases in plant stress responses. Biochemical Journal 412, 275–285.

Lee, D.H., Kim, Y.S., Lee, C.B., 2001. The inductive responses of the antioxidant enzymes by salt stress in the rice (*Oryza sativa* L.). Journal of Plant Physiology 158, 737–745.

Leonardis, S.D., Dipierro, N., Dipierro, S., 2000. Purification and characterization of an characterization of an ascorbate peroxidase from potato tuber mitochondria. Plant Physiology and Biochemistry 38, 773–779.

Lichtenthaler, H.K., 1987. Chlorophylls and carotenoids — pigments of photosynthetic biomembranes. Methods in Enzymology 148, 350–382.

Lu, C.M., Qiu, N.W., Lu, Q.T., Wang, B.S., Kuang, T.Y., 2002. Does salt stress lead to increased susceptibility of photosystem II to photoinhibition and changes in photosynthetic pigment composition in halophyte *Suaeda salsa* grown outdoors? Plant Science 163, 1063–1068.

Lu, C.M., Qiu, N.W., Wang, B.S., Zhang, J.H., 2003. Salinity treatment shows no effects on photosystem II photochemistry, but increases the resistance of photosystem II to heat stress in halophyte *Suaeda salsa*. Journal of Experimental Botany 54, 851–860.

Lu, Z.Q., Liu, D., Liu, S.K., 2007. Two rice cytosolic ascorbate peroxidases differentially improve salt tolerance in transgenic *Arabidopsis*. Plant Cell Report 26, 1909–1917.

Mano, S., Yamaguchi, K., Hayashi, M., Nishimura, M., 1997. Stromal and thylakoid-bound ascorbate peroxidases are produced by alternative splicing in pumpkin. FEBS Letter 413, 21–26.

Munns, R., James, R.A., Lauchli, A., 2006. Approaches to increasing the salt tolerance of wheat and other cereals. Journal of Experimental Botany 57, 1025–1043.

Murgia, I., Tarantino, D., Vannini, C., Bracale, M., Carrabvieri, S., Soave, C., 2004. *Arabidopsis thaliana* plants overexpressing thylakoidal ascorbate peroxidase show resistance to paraquat-induced photo-oxidative stress and to nitric oxide-induced cell death. The Plant Journal 38, 940–995.

Noctor, G., Foyer, C.H., 1998. Ascorbate and glutathione: keeping active oxygen under control. Annual Review of Plant Physiology and Plant Molecular Biology 49, 249–279.

Panchuk, I.I., Volkov, R.A., Shoffl, F., 2002. Heat stress and heat shock transcription factor-dependent expression and activity of ascorbate peroxidase in *Arabidopsis*. Plant Physiology 129, 838–853.

Pang, C.H., Zhang, S.J., Gong, Z.Z., Wang, B.S., 2005. NaCl treatment markedly enhances H_2O_2-scavenging system in leaves of halophyte *Suaeda salsa*. Physiologia Plantarum 125, 490–499.

Prasad, T.K., Anderson, M.D., Martin, B.A., Stewart, C.R., 1994. Evidence for chilling-induced oxidative stress in maize seedlings and a regulatory for hydrogen peroxide. The Plant Cell 6, 65–74.

Rao, M.V., Paliyath, G., Ormrod, D.P., Murr, D.P., Watkins, C.B., 1997. Influence of salicylic acid on H_2O_2 production, oxidative stress, and H_2O_2-metabolizing enzymes. Plant Physiology 115, 137–149.

Sairam, R.K., Tyagi, A., 2004. Physiology and molecular biology of salinity stress tolerance in plants. Current Science 86, 407–421.

Sairam, R.K., Rao, K.V., Srivastava, G.C., 2002. Differential response of wheat genotypes to long term salinity stress in relation to oxidative stress, antioxidant activity and osmolyte concentration. Plant Science 163, 1037–1046.

Scandalios, J.G., Guan, L., Polidoros, A.N., 1997. Catalase in plants: gene structure, properties, regulation, and expression. In: Scandalios, J.G. (Ed.), Oxidative Stress and the Molecular Biology of Antioxidant Defenses. Cold Spring Harbor Laboratory Press, New York, pp. 353–406.

Shigeoka, S., Ishikawa, T., Tamoi, M., Miyagawa, Y., Takeda, T., Yabuta, Y., Yoshimura, K., 2002. Regulation and function of ascorbate peroxidase isoenzymes. Journal of Experimental Botany 53, 1305–1319.

Thordal-Christensen, H., Zhang, Z., Wei, Y., Collinge, D.B., 1997. Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley–powdery mildew interaction. The Plant Journal 11, 1187–1194.

Torsethaugen, G., Pitcher, L.H., Zilinskas, B.A., Pell, E.J., 1997. Overproduction of ascorbate peroxidase in the tobacco chloroplast does not provide protection against ozone. Plant Physiology 114, 529–537.

Tsai, Y.C., Hong, C.Y., Liu, L.F., Kao, C.H., 2005. Expression of ascorbate peroxidase and glutathione reductase in roots of rice seedlings in response to NaCl and H_2O_2. Journal of Plant Physiology 162, 291–299.

Tsugane, K., Kobayashi, K., Niwa, Y., Ohba, Y., Wada, K., Kobayashi, H., 1999. A recessive *Arabidopsis* mutant that grows photoautotrophically

under salt stress shows enhanced active oxygen detoxification. The Plant Cell 11, 1195–1206.

Wang, B.S., Lüttge, U., Ratajczak, R., 2001. Effects of salt treatment and osmotic stress on V-ATPase and V-PPase in leaves of the halophyte *Suaeda salsa*. Journal of Experimental Botany 52, 2355–2365.

Wang, B.S., Lüttge, U., Ratajczak, R., 2004. Specific regulation of SOD isoforms by NaCl and osmotic stress in leaves of C3 halophyte. Journal of Plant Physiology 161, 285–293.

Willekens, H., Inzeo, D., Van Montagu, M., Van Camp, W., 1995. Catalase in plants. Molecular Breeding 1, 207–228.

Wolfe, W.C., 1962. Spectrophotometric determination of hydroperoxide in diethyl ether. Analytical Chemistry 34, 1328–1330.

Xu, W.F., Shi, W.M., Ueda, A., Takabe, T., 2008. Mechanisms of salt tolerance in transgenic *Arabidopsis thaliana* carrying a peroxisomal ascorbate peroxidase gene from barley. Pedosphere 18, 486–495.

Yabuta, Y., Motoki, T., Yoshimura, K., Takada, T., Ishikawa, T., Shigeoka, S., 2002. Thylakoid membrane-bound ascorbate peroxidase is a limiting factor of antioxidative systems under photo-oxidative stress. The Plant Journal 32, 912–925.

Yamaguchi, K., Mori, H., Nishimura, M., 1995. A novel isoenzyme of ascorbate peroxidase localized on glyoxysomal and leaf peroxisomal membranes in pumpkin. Plant & Cell Physiology 36, 1157–1162.

Yamaguchi, K., Hayashi, M., Nishimura, M., 1996. cDNA cloning of thylakoid-bound ascorbate peroxidase in pumpkin and its characterization. Plant & Cell Physiology 37, 405–409.

Yoshimura, K., Yabuta, Y., Tamoi, M., Ishikawa, T., Shigeoka, S., 1999. Alternatively spliced mRNA variants of chloroplast ascorbate peroxidase isoenzymes in spinach leaves. Biochemical Journal 338, 41–48.

Yoshimura, K., Yabuta, Y., Ishikawa, T., Shigeoka, S., 2000. Expression of spinach ascorbate peroxidase isozymes in response to oxidative stress. Plant Physiology 123, 223–233.

Zhang, Q.F., Li, Y.Y., Pang, C.H., Lu, C.M., Wang, B.S., 2005. NaCl stress enhances thylakoid-bound SOD activity in the leaves of C3 halophyte *Suaeda salsa* L. Plant Science 168, 423–430.

Zhu, J.K., 2001. Plant salt tolerance. Trends in Plant Science 6, 66–71.

Zhu, J.K., 2002. Salt and drought stress signal transduction in plants. Annual Review of Plant Physiology and Plant Molecular Biology 53, 247–273.

Edited by E Balazs