Overexpression of SsCHLAPXs confers protection against oxidative stress induced by high light in transgenic Arabidopsis thaliana

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To evaluate the physiological importance of chloroplastic ascorbate peroxidase (CHLAPX) in the reactive oxygen species (ROS)-scavenging system of a euhalophyte, we cloned the CHLAPX of Suaeda salsa (SsCHLAPX) encoding stromal APX (sAPX) and thylakoid-bound APX. The stromal APX of S. salsa (Ss.sAPX) cDNA consists of 1726 nucleotides including an 1137-bp open reading frame (ORF) and encodes 378 amino acids. The thylakoid-bound APX of S. salsa (Ss.tAPX) cDNA consists of 1561 nucleotides, including a 1284-bp ORF, and encodes 427 amino acids. The N-terminal 378 amino acids of Ss.sAPX are identical with those of Ss.tAPX, whereas the C-terminal 49 amino acids differ. Arabidopsis thaliana lines overexpressing Ss.sAPX and Ss.tAPX were constructed using Agrobacterium tumefaciens transformation methods. Under high light (1000 μmol m⁻² s⁻¹), malondialdehyde (MDA) content was lower in transgenic plants than in the wild type. Under high light, Fv/Fm and chlorophyll contents of both overexpressing lines and the wild type declined but were significantly higher in the overexpressing lines than in the wild type. The activities of APX (EC 1.11.1.11), catalase (CAT 1.11.1.6) and superoxide dismutase (SOD EC 1.15.1.1) were higher in the overexpressing lines than in the wild type. The transgenic plants showed increased tolerance to oxidative stress caused by high light. These results suggest that SsCHLAPX plays an important role in scavenging ROS in chloroplasts under stress conditions such as high light.

Introduction

Under stresses caused by drought, chilling, high salinity, heat shock and high light, the production of reactive oxygen species (ROS) including the superoxide radical (O₂⁻), hydrogen peroxide (H₂O₂), the hydroxyl radical (OH•) and singlet oxygen (¹O₂) is dramatically increased especially in chloroplasts and especially when high light is combined with other environmental stresses. H₂O₂, a more stable ROS, can diffuse across biological membranes (Sairam and Srivastava 2002, Takahashi and

Abbreviations – APX, ascorbate peroxidase; AsA, ascorbic acid; AsA–GSH cycle, ascorbate–glutathione cycle; CAM, crassulacean acid metabolism; cAPX, cytosolic APX; CAT, catalase; CHLAPX, chloroplastic ascorbate peroxidase; DAB, 3, 3-diaminobenzidine; DAsAR, dehydroascorbate reductase; EDTA, ethylenediaminetetraacetic acid; FW, fresh weight; GFP, green fluorescent protein; GR, glutathione reductase; GSH, glutathione; H₂O₂, hydrogen peroxide; MDA, malondialdehyde; MDAsAR, monodehydroascorbate reductase; MV, methyl violoten; O₂⁻, superoxide radical; ¹O₂, singlet oxygen; OH•, hydroxyl radical; ORF, open reading frame; PCD, programmed cell death; ROS, reactive oxygen species; SsCHLAPX, CHLAPX of Suaeda salsa; sAPX, stromal APX; Ss.sAPX, stromal APX of Suaeda salsa; Ss.tAPX, thylakoid APX of Suaeda salsa; SOD, superoxide dismutase; tAPX, thylakoid APX; TBA, 2-thiobarbituric acid; TCA, trichloroacetic acid.
Asada 1983) and cause oxidative protein modifications at areas distal from its production site (Scandalios et al. 1997). Increasing evidence indicates that H₂O₂ functions as a signaling molecule and is involved in multiple plant-resistant reactions against environmental stress (Mittler et al. 2004, Neill et al. 2002a, Pastori and Foyer 2002). Evidence also indicates that H₂O₂ regulates plant development and programmed cell death (PCD) (Apel and Hirt 2004). Indeed, the dual function of H₂O₂ depends on its cellular concentration (Dat et al. 2000).

Higher plants have developed a complex antioxidant system to cope with the oxidative stress induced by H₂O₂ and to maintain equilibrium between the production and scavenging of H₂O₂. Catalase (CAT) and the ascorbate–glutathione (AsA–GSH) cycle are important components of the H₂O₂ scavenging system in plants. CAT catalyzes the dissipation of H₂O₂ to H₂O and O₂ without requiring reducing power (Mittler 2002). CAT has been found predominantly in leaf peroxisomes. Compared with APX, CAT has an extremely high reaction rate and poor affinity for H₂O₂. In plants, the AsA–GSH cycle operates in the cytosol, mitochondria, plastids and peroxisomes (Jiménez et al. 1998, Meyer 2008). The members of this pathway involve the antioxidants ascorbic acid (AsA) and GSH and antioxidant enzymes such as APX, monodehydroascorbate reductase (MDAsAR), dehydroascorbate reductase (DAsAR) and glutathione reductase (GR EC 1.8.1.7). APX is the key enzyme in the AsA–GSH cycle.

APX, which reduces H₂O₂ to H₂O with AsA as the specific electron donor, is one of the most important components of the H₂O₂ scavenging system. In many higher plants, APX comprises a family of isoenzymes located in the chloroplasts, mitochondria, peroxisomes and the cytosol (Shigeoka et al. 2002). There are two kinds of APXs in chloroplasts: stromal APX (sAPX) and thylakoid APX (tAPX). tAPX spans the stroma-exposed thylakoid membranes via the hydrophobic domain at the C-terminal (Jespersen et al. 1997, Shigeoka et al. 2002).

The molecular and enzymatic properties of chloroplast APX isoenzymes have been identified in many higher plants. Interestingly, the N-terminal amino acids of sAPX are identical with those of tAPX, whereas the C-terminal amino acids differ. This finding has been reported for Spinacia oleracea (Yoshimura et al. 1999), pumpkin (Mano et al. 1997), Mesembryanthemum crystallinum (Shigeoka et al. 2002) and tobacco (Shigeoka et al. 2002). In these plants, both sAPX and tAPX are encoded by the same gene, and their mRNAs are generated by alternative splicing. sAPX and tAPX share the transit peptide and catalytic domain. In Arabidopsis thaliana, however, two different isoforms of chloroplastic APX are encoded by two different genes (Jespersen et al. 1997).

The role of APX in protecting against oxidative stress induced by abiotic stress has been examined with inconsistent results. Overexpression of A. thaliana tAPX and pea cytosolic APX (cAPX) in tobacco chloroplasts reduced the oxidative stress induced by paraquat treatment (Murgia et al. 2004, Yabuta et al. 2002). However, Torsethaugen et al. (1997) reported that overproduction of APX in tobacco chloroplasts did not protect against ozone-induced stress. An increase in tAPX activity was detected in wheat subjected to water deficit (Gherri et al. 2000), but oxidative stress does not affect the level of chloroplastic APX transcripts (Panchuk et al. 2002, Shigeoka et al. 2002). Therefore, the role of APX as an antioxidant in chloroplasts remains controversial.

Suaeda salsa, a C₃ eualophyte belonging to the Chenopodiaceae, is native to saline soil and is adapted to the high salinity region in northern China (Wang et al. 2001). Treatment of S. salsa with 200 mM l⁻¹ NaCl significantly increased its growth and net photosynthetic rate but did not significantly affect the Fv/Fm of its leaves (Pang et al. 2005, Zhang et al. 2005). These results indicated that photoinhibition did not occur in S. salsa under 200 mM l⁻¹ NaCl condition and suggested that S. salsa chloroplasts under environmental stress have an efficient ROS-scavenging system. Our earlier results showed that chloroplastic ascorbate peroxidase (CHLAPX) activities of S. salsa increased markedly under salt stress (Pang et al. 2005). To clarify the function of CHLAPX in plant protection against oxidative stress, we cloned the CHLAPX of S. salsa (SsCHLAPX) and produced A. thaliana lines overexpressing SsCHLAPX. The specific objective was to determine whether CHLAPX plays a key role in protection against oxidative stress induced by high light.

Materials and methods

Plant materials

Seeds of S. salsa were grown in pots containing sand and were watered daily with Hoagland nutrient solution according to Pang et al. (2005). When seedlings were 3 weeks old, they were exposed to one of the several NaCl treatments. In one treatment, they were watered with 400 mM l⁻¹ NaCl, and shoots and roots were harvested 24 or 48 h later. In other NaCl treatments, the seedlings were watered with 200 mM l⁻¹ NaCl and shoot and roots were harvested 48 h later. For controls, shoot and roots were harvested from similar seedlings that were not exposed to NaCl. RNA was extracted from the shoots and roots as described later.
A. thaliana (Columbia, col) and transgenic line (T3) seeds were germinated and grown on Murashige and Skoog nutrient medium with 3% (w/v) sucrose and 0.7% agar in glass Petri plates. After the seeds were added to the plates, the plates were sealed, incubated for 1–3 days in the dark at 4°C, and then transferred to an artificially lit growth room at 23°C for germination. After 1 week, the seedlings were transferred to 340-cm³ pots filled with a mixture of peat/forest soil and vermiculite (3:1) and kept in a greenhouse at 22°C and with 70% relative humidity, a photoperiod of 12 h and a light intensity of 90 μmol m⁻² s⁻¹.

**SsCHLAPX isolation and sequence analysis**

Total RNAs of S. salsa were isolated with TRIzol® Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instruction. The cDNA was synthesized with the SMART™ RACE cDNA Amplification Kit (Clontech, Palo Alto, CA).

A PCR product of SsCHLAPX that was about 280 bp was amplified with degenerate primers from the template S. salsa cDNA: 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′ ends and 3′ ends of the SsCHLAPX sequence were obtained by using 5′-RACE and 3′-RACE amplification with the SMART™ RACE cDNA Amplification Kit. Then, two kinds of SsCHLAPX [including stromal APX of Suaeda salsa (Ss.sAPX) and thylakoid APX of S(Ss.tAPX)] full-length sequences were obtained by PCR with primer pair 5′-CCATATCATCAGCTAGTCAA ATCAGC-3′ and 5′-TGTCCTCATAG AATCCGACAG C. Both strands of all RT-PCR products were sequenced.

Sequences were analyzed with DNASIS software, and databanks were searched with the BLAST program. Sequences were aligned and phylogenetic trees were constructed with CLUSTALX and DNAMAN software.

**Northern blot analysis**

Total RNA of S. salsa was isolated by guanidinium thiocyanate extraction methods (Chomczynski and Sacchi 1987). RNAs of A. thaliana were isolated with TRIzol Reagent. RNAs were subjected to electrophoresis on 1.2% (w/v) agarose denaturing formaldehyde gels and were transferred to a nylon membrane. Northern blot hybridization was performed as described by Sambrook et al. (1989). For high stringency in the presence of 50% (w/v) formamide, ³²P-labeled DNA probes were prepared using a random primer labeled kit (Random Primers Systems, TaKaRa, Japan). Hybridization and washes were carried out at 65°C. After the blots were dried, autoradiography of the filters was obtained on Kodak Xar-5-ray films with an intensifying screen at −70°C.

The probe for analysis of SsCHLAPX transcript levels in S. salsa was amplified with the following primers: SsCHLAPX-f (5′-GTCGTCACACCCCAACCACTCCTC-3′) and SsCHLAPX-r (5′-ACTCTTTGCGGATGAAATCTTG-3′).

The probe for analysis of Ss.sAPX/Ss.tAPX transcript levels in transgenic A. thaliana was amplified with the following primers:

- Ss.sAPX/Ss.tAPX-f (5′-GTCGTCACACCCCAACCACTCCTC-3′)
- Ss.sAPX/Ss.tAPX-r (5′-TGCCCTCATAG AATCCGACAG CTCAC-3′).

**Vector construction and transformation of A. thaliana**

For the construction of transgenic vectors, the full-length sAPX and tAPX cDNA from S. salsa were ligated into the pCAMBIA1300 and the GATEWAY vector (produced for C-terminal protein fusions with green fluorescent protein, GFP) under the control of the CaMV35S promoter. The recombinant plasmids were introduced into Agrobacterium tumefaciens strain GV3101 and were then introduced into A. thaliana by the floral-dip method (Clough and Bent 1998).

**Detection of the transgene by PCR**

Genomic DNA samples were isolated from wild-type and transgenic A. thaliana plants according to the methods of Yabuta et al. (2002). About 100 ng of genomic DNA was used to amplify the tAPX and sAPX transgene by PCR with the following primers:

- Ss.sAPX/Ss.tAPX-f (5′-GTCGTCACACCCCAACCACTCCTC-3′)
- Ss.sAPX/Ss.tAPX-r (5′-TGCCCTCATAG AATCCGACAG CTCAC-3′).

**Preparation of crude enzyme extracts**

Crude enzyme was extracted from leaves of wild-type and transgenic A. thaliana plants according to the method of Rao et al. (1997). One gram of fresh leaves was homogenized at 4°C in 2 ml of medium: 50 mM l⁻¹ K-phosphate buffer (pH 7.8) containing 5 mM l⁻¹ ethylenediaminetetraacetic acid (EDTA) and 2 mM l⁻¹ AsA. The homogenate was centrifuged at 15 000 g (Eppendorf Centrifuge 5417R) for 15 min at 4°C. The supernatant was used for determination of protein content and CAT, APX and superoxide dismutase (SOD) activities.
High light treatment

Detached leaves of both wild-type (Columbia, col) and transgenic *A. thaliana* were subjected to high light (1000 μmol m$^{-2}$ s$^{-1}$) for 2 h to induce oxidative stress. The high light was generated with a halogen lamp. A flowing water layer between the lamp and the leaf samples filtered out the far-red light and thereby prevented leaf heating. Detached leaves were put in Petri dishes containing water (Tallon and Quiles 2007). Control detached leaves (CK) were not subjected to a high light intensity and were kept under a light intensity of 90 μmol m$^{-2}$ s$^{-1}$ for 2 h.

Antioxidant enzyme assay

Activities of CAT and SOD were measured following the method described by Rao et al. (1997) and Giannopolitis and Ries (1977), respectively. APX was determined according to the method of Jiménez et al. (1997).

Protein content was measured following the method of Bradford (1976), with bovine serum albumin as a standard.

Analysis of lipid peroxidation

Lipid peroxidation in the leaves of *A. thaliana* plants exposed or not exposed to high light was determined by measuring malondialdehyde (MDA); MDA was measured using the 2-thiobarbituric acid (TBA) assay procedure of Gueta-Dahan et al. (1997) with some modifications. Briefly, 0.1 g of leaves (fresh weight, FW) was extracted in a solution containing 1.5 ml of 0.1% trichloroacetic acid (TCA) and 1.5 ml of 0.5% TBA. The extracts were boiled for 10 min, cooled in tap water and then centrifuged at 1400 g for 15 min. The MDA concentration was calculated based on absorbance at A532, absorbance at A600 and its molar extinction coefficient (156 mM$^{-1}$ cm$^{-1}$) as follows:

\[
\text{MDA content (μmol g$^{-1}$ FW)} = \frac{(A532 - A600) \times 10^3 \times \text{extract volume (ml)}}{1.56 \times 10^3 \times \text{sample fresh weight (g)}}
\]

**H$_2$O$_2$ staining**

In situ H$_2$O$_2$ production in *A. thaliana* leaves was detected by an endogenous peroxidase-dependent staining procedure using DAB (3,3-diaminobenzidine) (Thordal-Christensen et al. 1997). Leaves were detached and placed in a solution of 1 mg ml$^{-1}$ DAB (pH 3.8) for 8 h under light. Subsequently, the leaves were immersed in boiling 96% (v/v) ethanol for 10 min and then stored in 96% (v/v) ethanol. H$_2$O$_2$ production was visualized as a reddish-brown coloration.

Chlorophyll content

Chlorophyll content in *A. thaliana* leaves was measured according to Lichtenthaler (1987) with some modifications. Fresh leaves (0.1 g) were briefly rinsed in deionized water and then extracted in 80% acetone (v/v). Total chlorophyll content, expressed as μg g$^{-1}$ FW, was determined spectrophotometrically by measuring the absorbance at 663 and 645 nm.

Chlorophyll fluorescence

The change in chlorophyll fluorescence in *A. thaliana* leaves was measured with a PAM 2000 chlorophyll fluorometer (Walz, Germany). The maximal quantum yield of PSII (Fv/Fm) was determined from the following equation: Fv/Fm= (Fm – Fo)/Fm, where Fo is the initial minimal fluorescence on dark-adapted leaves for 15 min and Fm is maximal dark-adapted fluorescence.

Confocal microscopy

Transgenic *Arabidopsis* seeds containing sAPX/tAPX + GFP were transferred to agar plates. Seedlings that were 4 days old were examined microscopically. For observing GFP localization, whole *A. thaliana* seedlings were mounted in water under glass cover slips, and GFP fluorescence was visualized with a confocal microscope (Eclipse E-800 C1; Nikon Instrument Inc, NY) equipped with a krypton/argon laser.

Statistical analysis

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

**Results**

**Isolation and sequencing of CHLAPX from *S. salsa***

Based on sequence homologies of the CHLAPX genes from *M. crystallinum*, *Nicotiana tabacum*, *S. oleracea* and *A. thaliana*, reverse transcriptase (RT)-PCR amplification and 5′- and 3′-RACE amplification were used to obtain the full-length transcript sequences of *SsCHLAPX*. By alternative processing of the 3′-terminal region, two forms of mature mRNA were produced: one form encodes thylakoid-bound APX (*Ss.tAPX*, ACCESSION GU228492) and the other encodes stroma APX.
The Ss.tAPX cDNA complement sequence consists of 1561 nucleotides including a 1284-bp open reading frame (ORF). The deduced protein contains 427 amino acids with a predicted molecular mass of 46.3 kDa. The Ss.sAPX cDNA complement sequence consists of 1726 nucleotides including a 1137-bp ORF. The deduced protein contains 378 amino acids with a predicted molecular mass of 40.96 kDa. Interestingly, the N-terminal 378 amino acids of Ss.sAPX are identical with those of Ss.tAPX, whereas the C-terminal 49 amino acids differ. SsCHLAPX isoenzymes have a transit peptide consisting of approximately 64 residues in their N-terminus. According to the program (http://www.cbs.dtu.dk/services/TargetP), we predict that the protein Ss.tAPX has one transmembrane domain in the C-terminal (SELSDSMRQKIRA EYESFGGSPDKPLPTNYFLNIMIVGVLAILSYLAGN) that is responsible for spanning to the stroma-exposed thylakoid membranes in chloroplasts.

**Homology analysis of amino acid sequence of SsCHLAPX**

To detect similarities and differences in individual amino acid sequence positions, we aligned the deduced amino acid sequences of CHLAPXs from different plant species. Analysis of amino acid sequence alignment indicated that the sequence of SsCHLAPX was similar to the sequence of CHLAPX in the halophyte *M. crystallinum* (Fig. 1). *M. crystallinum* is a facultative halophyte that responds to drought- or high salinity-induced water stress by switching from C3 photosynthesis to crassulacean acid metabolism (CAM), a physiological adaptation that increases water conservation. This species contains specialized epidermal bladder cells that rapidly expand under salt stress.

**Localization of SsCHLAPX proteins**

Sequence analysis of the SsCHLAPX proteins suggested that they have a signal peptide in the N-terminal. To clarify the subcellular sites of SsCHLAPX, we generated stable transgenic plants expressing the GFP: Ss.tAPX + GFP and Ss.sAPX + GFP fusion protein. As shown in Fig. 2G, K, the fusion proteins are indeed localized exclusively in the chloroplast.

**Changes in mRNA levels for SsCHLAPX in response to NaCl treatment**

The CHLAPX gene is expressed even in non-photosynthetic tissues such as roots (Shigeoka et al. 2002). To investigate transcript induction of CHLAPX in *S. salsa* during NaCl treatment, Northern blot hybridization was carried out with total RNA isolated from the shoots and roots of *S. salsa* plants treated with 400 mM l−1 NaCl for 0 (CK), 24 or 48 h and with 200 mM l−1 NaCl for 0 (CK) or 48 h. Northern blot analysis indicated that the transcription of chloroplast APX was upregulated by treatment with 400 mM l−1 NaCl for 24 or 48 h (Fig. 3A). SsCHLAPX transcript levels in shoots of plants treated with 400 mM l−1 NaCl increased for up to 24 h and then decreased. At 48 h, however, the mRNA levels of SsCHLAPX in the shoots of the 400-mM NaCl treatment were still higher than those of the control. In the roots, the transcripts level of SsCHLAPX increased with treatment time (Fig. 3A). In the comparison of 200 and 400 mM l−1 NaCl (48-h exposure), SsCHLAPX transcript levels in the shoots were higher with 200 mM l−1 NaCl than with 400 mM l−1 NaCl (Fig. 3B); in the roots, however, SsCHLAPX mRNA levels were higher with 400 mM l−1 NaCl than with 200 mM l−1 NaCl (Fig. 3B).

**Identification of A. thaliana transgenic lines**

The transgenic *A. thaliana* plants overexpressing Ss.sAPX and Ss.tAPX under the control of the CaMV 35S promoter were generated and verified by Northern blot analysis (Fig. 4). Among the transgenic lines homozygous for the single insertion site, the Ss.sAPX-7/15(s-7, s-15) and the Ss.tAPX-11/16(t-11, t-16) lines showed increased mRNA accumulation relative to the WT (col). Therefore, these four lines (T3 generation) were isolated and propagated for further analysis. No difference was observed in phenotype and growth between transgenic plants and...
wild-type plants grown under the normal conditions (data not shown).

*A. thaliana* plants overexpressing *S. Salsa* CHLAPX are more resistant to high light intensity

**Effect of high light on MDA content**

Lipid peroxidation is a useful indicator of cellular oxidative damage, and the changes in lipid peroxidation induced by high light were measured by determining MDA content. Under normal conditions, leaf MDA content did not differ between the wild-type and transgenic plants (Fig. 5). Under high light conditions, however, the MDA content was significantly lower in transgenic plants than in wild-type plants ($P < 0.05$).

**Effect of high light on Fv/Fm and chlorophyll content of *A. thaliana* leaves**

To further investigate the role of SsCHLAPX in protecting against the oxidative stress induced by high light, we determined maximal photochemical efficiency (Fv/Fm) and chlorophyll content of transgenic and wild-type *A. thaliana*. Under normal growth conditions, Fv/Fm and chlorophyll content did not differ between the transgenic lines and the wild type (Fig. 6). Under high light conditions, however, Fv/Fm of both SsCHLAPX-overexpression lines and the wild type declined (Fig. 6), although Fv/Fm were still significantly higher for SsCHLAPX-overexpression lines than for the wild type ($P < 0.05$). The pattern for chlorophyll content was similar to that for Fv/Fm (Table 1). Moreover, under the stress of high light, the contents of chla, chla+b and the ratio chla/b were higher in t11, t16 and s15 than in the wild type ($P < 0.05$) (Table 1).

**Effect of high light on the SOD, CAT and APX activity in *A. thaliana* leaves**

Under normal light conditions, SOD and CAT activities of transgenic lines were similar to those of the wild type (col) (Fig. 7A, B, D and E) but APX activity was higher in the overexpressing lines than in the wild type (Fig. 7C and F). After treatment with high light, SOD, CAT and APX activities of both wild-type and transgenic plants were significantly reduced but the enzyme activities were significantly higher in the transgenic plants than in the wild-type plants. In particular, APX activities were substantially greater in both Ss.sAPX-overexpression and Ss.tAPX-overexpression lines than in the wild type.
Northern blot analysis of SsCHLAPX transcript levels in S. salsa. (A) Transcript levels of SsCHLAPX after treatment of S. salsa with 400 mM l^-1 NaCl for 0 (CK), 24 or 48 h; 30 μg of total RNA was loaded per lane. (B) Transcript levels of SsCHLAPX after treatment with 0 (CK), 200 or 400 mM l^-1 NaCl for 48 h; 15 μg of total RNA was loaded per lane. In the lower panel, equal loading of RNA was verified by ethidium bromide staining of the agarose gel.

Interestingly, insertion of one antioxidant enzyme gene led to a coordinated upregulation of antioxidant enzyme activities under stress conditions.

Effect of high light on H_2O_2 levels in leaves

Upon treatment with high light, inhibition of the activity of antioxidative enzymes was accompanied by a rise in H_2O_2 levels in all the tested lines, as indicated by DAB staining (Fig. 8). However, DAB staining was less intense in the overexpressing lines, indicating that H_2O_2 accumulation caused by exposure to high light was lower in the SsCHLAPX-overexpression lines than in the wild type (col).

Discussion

When plants are exposed to light, the oxygen pressure in chloroplasts is much higher than in other organelles. Therefore, the chloroplast is considered a major producer of O_2^- and H_2O_2 (Davletova et al. 2005). APX is the primary H_2O_2 scavenging enzyme in chloroplasts (Asada 1992). In some plants, the level of chloroplastic APX does not change in response to environment stress (Panchuk et al. 2002, Shigeoka et al. 2002). In spinach leaves (Yoshimura et al. 2000), for example, the transcript level of sAPX and tAPX did not change in response to high light, methyl viologen (MV), drought, salinity or abscisic acid. In the leaves of rice seedlings, however, the expression of stromal APX was increased by salt stress while transcripts of the thylakoid-bound APX genes were down-regulated (Kim et al. 2007).

To further clarify whether S. salsa chloroplast APXs have a protective role under stresses such as high light, we cloned the euhalophyte S. salsa CHLAPXs and overexpressed Ss.tAPX or Ss.sAPX in A. thaliana. As shown in Fig. 2, the fusion proteins (Ss.tAPX + GFP and Ss.sAPX + GFP fusion protein) are exclusively localized in the chloroplasts. Moreover, northern blot analysis indicated that the overexpressed Ss.tAPX or Ss.sAPX in A. thaliana lines had markedly increased mRNA accumulation relative to the WT (Fig. 4) and the APX activities were higher in leaf extracts of the transgenic plants than wild-type plants (Fig. 7). These results indicate that Ss.CHLAPXs were expressed and localized in the chloroplasts of the transgenic A. thaliana.
Interestingly, the N-terminal 378 amino acids of Ss.sAPX are identical with those of Ss.tAPX, whereas the C-terminal 49 amino acids are different. This suggests that one gene encodes chloroplastic APXs in S. salsa. Alternative processing of the 3\' -terminal region as a result of alternative polyadenylation and splicing produces two forms of mature mRNAs. SsCHLAPX isoenzymes have a transit peptide consisting of approximately 64 residues in their N-terminus. Ss.tAPX has one major hydrophobic domain in the C-terminal that is responsible for spanning to the stroma-exposed thylakoid membranes in chloroplasts. In higher plants, alternative splicing has been documented in the spinach ribulose bisphosphate carboxylase/oxygenase (Rubisco) activase and pumpkin hydroxypyruvate reductase (Hayashi et al. 1996, Werneke et al. 1989). Alternative splicing is a major contributor to genome complexity and protein diversity. It modulates all aspects of enzyme activity and regulates the sublocalization of a protein within an organelle (Guo et al. 2007). Therefore, it is possible that the H2O2 level in the chloroplast is modulated by alternative processing, which generates different amounts of Ss.tAPX and Ss.sAPX under stress conditions.

Fig. 5. Effect of high light on the MDA content of leaves of wild-type and transgenic Arabidopsis thaliana plants. Values for transgenic A. thaliana plants overexpressing Ss.sAPX and Ss.tAPX are in the left and right figure, respectively. Detached leaves were subjected to high light (1000 \( \mu \text{mol} \ \text{m}^{-2} \ \text{s}^{-1} \)) for 2 h. Control detached leaves (CK) were not subjected to a high light intensity and were kept under a light intensity of 90 \( \mu \text{mol} \ \text{m}^{-2} \ \text{s}^{-1} \). Values are means \( \pm \) SD (n = 3), and a significant difference between the high light treatment and control (CK) is indicated by * (P < 0.05).

Fig. 6. Effect of high light on the Fv/Fm of leaves of wild-type and transgenic Arabidopsis thaliana plants. Values for transgenic A. thaliana plants overexpressing Ss.sAPX and Ss.tAPX are in the left and right figure, respectively. Detached leaves were subjected to high light (1000 \( \mu \text{mol} \ \text{m}^{-2} \ \text{s}^{-1} \)) for 2 h. Control detached leaves (CK) were not subjected to a high light intensity and were kept under a light intensity of 90 \( \mu \text{mol} \ \text{m}^{-2} \ \text{s}^{-1} \). Values are means \( \pm \) SD (n = 20), and a significant difference between the high light treatment and control (CK) is indicated by * (P < 0.05).
Table 1. Effect of high light on the contents of Chla, b, a+b and the ratio Chla/Chlb in leaves of wild-type (col) and transgenic (s7, s15, t11 and t16) Arabidopsis thaliana plants. Detached leaves were subjected to high light (1000 μmol m\(^{-2}\) s\(^{-1}\)) for 2 h. Control detached leaves were not subjected to a high light intensity and were kept under a light intensity of 90 μmol m\(^{-2}\) s\(^{-1}\). Values are means ± SD (n = 3), and a significant difference between the high light treatment and control in a column is indicated by * (P < 0.05) and by ** (P < 0.01).

<table>
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<th>Plant material</th>
<th>Light intensity (μmol m(^{-2}) s(^{-1}))</th>
<th>Chla (μg g(^{-1}) FW)</th>
<th>Chlb (μg g(^{-1}) FW)</th>
<th>Chla+b (μg g(^{-1}) FW)</th>
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Fig. 7. Effect of high light on the SOD, CAT and APX activity in leaves of wild-type and transgenic Arabidopsis thaliana plants (values for Ss.sAPX-overexpression lines are in the top row and values for Ss.tAPX-overexpression lines are in the bottom row). Detached leaves were subjected to high light (1000 μmol m\(^{-2}\) s\(^{-1}\)) for 2 h. Control detached leaves (CK) were not subjected to a high light intensity and were kept under a light intensity of 90 μmol m\(^{-2}\) s\(^{-1}\). Values are means ± SD (n = 3), and a significant difference between the high light treatment and CK is indicated by * (P < 0.05) and ** (P < 0.01).
Homology analysis of amino acid sequence of SsCHLAPX showed that the sequence of SsCHLAPX was similar to the sequence of CHLAPX in a facultative halophyte M. crystallinum (Fig. 1). M. crystallinum is a halophyte which has salt bladders on the leaves and stems under salt stress. In particular, the photosynthesis will be switched from C3 photosynthesis to CAM under environmental stresses such as salinity and drought, which makes M. crystallinum survive high salinity and drought. These results suggest that SsCHLAPXs of the halophytes maybe have more efficiency in removal of \( \text{H}_2\text{O}_2 \) than those of the non-halophytes under stress conditions such as high salinity, but comprehensive analysis including enzyme characteristics, pattern of gene expression and their relations to maintaining ROS balance needs to be investigated in greater detail.

In this study we examined the hypothesis that oxidative damage induced by high light could be alleviated in A. thaliana plants overexpressing SsCHLAPXs in the chloroplasts. Under high light, the transgenic plants experienced less oxidative damage than the wild type. The MDA content was lower and \( F_v/F_m \) and chlorophyll contents were higher in the transgenic plants than in the wild-type under high light (Figs 5 and 6 and Table 1). \( \text{H}_2\text{O}_2 \) accumulation upon high light treatment was also lower in the overexpressing lines than in the wild type (Fig. 8). Analysis of the antioxidant enzymes indicates that SOD and CAT activities of transgenic lines were similar to those of the wild type and only APX activity was significantly higher in the overexpressing lines than in the wild type under normal light conditions. Under high light, the activities of the antioxidant enzymes APX, SOD and CAT were all reduced, but their activities were still significantly higher in the transgenic plants than in the wild-type plants (Fig. 7). Together, these results suggest that SsCHLAPX-overexpression A. thaliana lines enhanced the protection against oxidative damage induced by high light mainly due to the coordinately higher activities of the antioxidant enzymes.

Insertion of one antioxidant enzyme gene led to a series of changes in antioxidant enzyme activity. This suggests that these antioxidant enzymes are coordinately upregulated during oxidative stress. What are the possible reasons for the coordinated upregulation of enzyme activity? Under high light conditions, oxygen pressure in chloroplasts is much higher than that in other organelles, and as noted earlier, the chloroplast is consequently considered a major producer of \( \text{H}_2\text{O}_2 \) (Davletova et al. 2005). SsCHLAPX could maintain \( \text{H}_2\text{O}_2 \) at a relatively low level. At the same time, \( \text{H}_2\text{O}_2 \) is involved in oxidative stress signaling (Neill et al. 2002b), leading to the induction of antioxidant enzyme genes and regulating the activity of the other antioxidant enzymes such as SOD and CAT.

In this study, overexpression of SsCHLAPX and the coordinated upregulation of CAT, SOD and APX enhanced the tolerance of transgenic A. thaliana to high light. This finding is in general agreement with other reports. Overexpression of tAPX in tobacco significantly increased tolerance to oxidative stress caused by the application of MV and by chilling temperature combined with high light intensity (Yabuta et al. 2002). The expression of pea cAPX in chloroplasts increased the tolerance to oxidative damage caused by MV treatment (Allen et al. 1997). Mutants of TAPX are thought to be lethal (Yabuta et al. 2002). However, A. thaliana lines overexpressing tAPX showed an increased resistance to the herbicide paraquat but no increased resistance to high light (Murgia et al. 2004). Overexpression of APX in tobacco chloroplasts enhances the tolerance to salt.
stress and water deficit mainly via increasing APX activity (Badawi et al. 2004).

In summary, these results provide strong evidence that overexpression of chloroplastic APX from the euhalophyte S. salsa in transgenic A. thaliana confers protection against oxidative stress induced by high light via coordinated upregulation of antioxidant enzymes. The reduction in oxidative damage was similar for S.sAPX-overexpression and Ss.tAPX-overexpression A. thaliana plants.

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