Photosynthetic light reactions comprise a significant source of hydrogen peroxide (H$_2$O$_2$) in illuminated leaves. APXs (ascorbate peroxidases) reduce H$_2$O$_2$ to water and play an important role in the antioxidant system of plants. In the present study we addressed the significance of chloroplast APXs in stress tolerance and signalling in Arabidopsis thaliana. To this end, T-DNA (transfer DNA) insertion mutants tapx, sapx and tapx sapx, lacking the tAPX (thylakoid-bound APX), sAPX (stromal APX) or both respectively, were characterized. Photo-oxidative stress during germination led to bleaching of chloroplasts in respectively, were characterized. Photo-oxidative stress during germination led to bleaching of chloroplasts in single-mutant and particularly in the tapx sapx double-mutant plants, whereas the greening process of wild-type and tapx plants was only partially impaired. Mature leaves of tapx sapx double mutants were also susceptible to short-term photo-oxidative stress induced by high light or methyl viologen treatments. After a 2-week acclimation period under high light or under low temperature, none of the mutants exhibited enhanced stress symptoms.

**INTRODUCTION**

Generation of ROS (reactive oxygen species) is characteristic of a number of metabolic reactions that take place in different compartments of plant cells. Under favourable conditions, plants generally maintain ROS at low levels. Under adverse environmental conditions, however, imbalances in metabolic processes may lead to increased accumulation of ROS, forming a potential threat of oxidative damage to cellular components. On the other hand, transient increases in ROS levels also have a vital role in stress signalling and thereby in the survival of plants under adverse environmental conditions [1]. In plants, ROS levels are controlled via a versatile antioxidant network. Components of the antioxidant system reside in various cellular locations and include a range of enzymatic scavengers, such as SODs (superoxide dismutases), APXs (ascorbate peroxidases), PRXs (peroxiredoxins), glutaredoxins, glutathione peroxidases and catalases. In addition, several non-enzymatic antioxidants such as ascorbate, glutathione, tocopherols, carotenoids and phenolic compounds also contribute to the cellular redox balance [2].

The water–water cycle of chloroplasts comprises a significant source of ROS in light-exposed green tissues [3]. Photoreduction of molecular oxygen via PSI (Photosystem I) leads to formation of superoxide (O$_2^-$), which in turn becomes rapidly dismutated to hydrogen peroxide (H$_2$O$_2$), either spontaneously or enzymatically via SOD activity [3,4]. Chloroplasts possess a multilayer antioxidative system to insure efficient detoxification of H$_2$O$_2$. The enzymatic detoxification systems for H$_2$O$_2$ include the APX-dependent ascorbate–glutathione cycle [3] and the PRX-dependent scavenging system [5,6]. The importance of chloroplastic glutaredoxin and glutathione peroxidase activities in the scavenging of H$_2$O$_2$ have also been demonstrated [7,8]. APXs are haem-binding enzymes that reduce H$_2$O$_2$ to water using ascorbate as an electron donor [3]. Regeneration of ascorbate utilizes a complex set of reactions linked to glutathione and NADPH metabolism [9]. Plants contain several APX isoenzymes, three of which reside in chloroplasts. A 38 kDa tAPX (thylakoid-bound APX), a 33 kDa sAPX (stromal APX), which is dual-targeted to chloroplasts and mitochondria, and a putative luminal APX are each encoded by a single nuclear gene in Arabidopsis [3,10–12]. Outside chloroplasts, 25 kDa APX1 and APX2 have been found in the cytoplasm [13], and a 31 kDa APX3 is located in peroxisomes and in oilseed glyoxisomes [14–17]. Members of the second group of H$_2$O$_2$-detoxifying enzymes, PRXs, are 17–22 kDa enzymes that possess N-terminal cysteine residue(s) responsible for peroxidase activity. In Arabidopsis, four PRXs are targeted to chloroplasts: 2-cysteine (2-Cys) PRXs A and B, PRX Q and PRX II E [5]. Re-reduction of chloroplastic PRXs occurs via the action of various thioredoxins and thioredoxin-like proteins [5,18].

APXs have long been considered as key enzymes in the detoxification of H$_2$O$_2$ in chloroplasts [3]. However, the functional specificities of tAPX and sAPX in various plant species and stress conditions, and particularly their functional overlaps with...
other cellular antioxidant agents, remain poorly understood. In a wheat mutant line, a 40% reduction in tAPX activity resulted in lowered photosynthetic carbon assimilation as well as reduced growth rate and seed production [19]. Moreover, unsuccessful attempts to create antisense tapx lines from tobacco suggested that suppression of tAPX in tobacco may be lethal [20]. On the other hand, transgenic tobacco and Arabidopsis plants overexpressing tAPX showed enhanced tolerance to methyl-viologen-induced photo-oxidative stress [20,21]. Inconsistent with their role in photo-oxidative stress tolerance, chloroplastic APXs are known to become inactivated by H₂O₂ if ascorbic acid is present at low levels [9,22]. Indeed, tAPX was recently connected with signalling events conferring resistance to heat stress in Arabidopsis [23].

In the present study, we have characterized Arabidopsis thaliana T-DNA (transfer DNA) insertion mutants deficient in tAPX and sAPX. Special emphasis was put on (i) the significance of tAPX and sAPX in oxidative stress tolerance at various developmental stages, and (ii) signalling effects that the absence of tAPX and sAPX provokes in the mutant plants. We demonstrate that sAPX is particularly important for photoprotection during the early greening process. In mature leaves, tAPX and sAPX appear to be functionally redundant, and exhibit a key role in the maintenance of chloroplast functionality upon sudden onset of oxidative stress. Moreover, chloroplast APXs are required for accurate fine-tuning of chloroplast signalling pathways upon slight fluctuations in the accumulation of H₂O₂ in chloroplasts. In contrast, in the course of long-term acclimation to various stress conditions, the chloroplast APXs can be compensated for by other components of the chloroplast antioxidative system.

EXPERIMENTAL

Material and control growth conditions

Homozygous T-DNA insertion mutants for chloroplast tAPX (tapx; At1g77490; SALK_027804) or sAPX (sapx; At4g08390; SALK_083737) were identified from the SALK Institute’s collection by PCR analysis of genomic DNA according to the Institute’s protocols (http://signal.salk.edu/cgi-bin/tdnaexpress) [24]. The tapx and sapx lines were back-crossed to wild-type Columbia, selfed and re-identified to remove additional T-DNA inserts from the genome. A tapx sapx double mutant was created by crossing the tapx and sapx single mutants, and was identified from the F2 generation by PCR analysis of genomic DNA using the same set of primers that were used to screen for the single mutants.

Arabidopsis thaliana ecotype Columbia wild-type and the mutant plants were grown under control conditions of 130 μmol of photons·m⁻²·s⁻¹ at 20°C with 60% humidity and an 8 h light period. We chose short-day conditions to avoid general up-regulation of antioxidative capacity, which was recently shown to be typical of plants grown under long-day conditions [25].

Germination tests

For germination tests, seeds were sown on filter paper on Petri dishes containing half-strength MS medium (Murashige and Skoog basal salt mixture; Sigma–Aldrich), and kept at 4°C in darkness for 2 days. Subsequently, the sensitivity of germination to photo-oxidative stress was explored by supplementing half-strength MS medium with 1.5 μM methyl viologen (Sigma–Aldrich) and following greening at 130 μmol of photons·m⁻²·s⁻¹ at 20°C and an 8 h light period for 7 days.

Stress treatments

The sensitivity of expanded 4-week-old rosettes to methyl-viologen-induced photo-oxidative stress was explored by gently spraying the plants with 50 μM methyl viologen at the end of the dark period. After 30 min incubation under 50 μmol of photons·m⁻²·s⁻¹ at 20°C, plants were illuminated under 130 or 1000 μmol of photons·m⁻²·s⁻¹ at 20°C for 6 or 24 h. Control samples were sprayed with water and illuminated under normal growth conditions (130 μmol of photons·m⁻²·s⁻¹ at 20°C). After the treatment, rosettes were excised and incubated in 30 ml of ion-exchanged water at 4°C in darkness overnight. The extent of cell death and membrane disruption was measured as ion leakage to the solution with a conductivity meter (Radiometer). Finally, the results were normalized to the value of ion leakage obtained after boiling the samples for 5 min.

For short-term light treatments, 4-week-old plants, grown under 130 μmol of photons·m⁻²·s⁻¹ at 20°C, were shifted at the end of the dark period to light intensities of 130, 300, 450, 600, 1000 and 1300 μmol of photons·m⁻²·s⁻¹ at 20°C for 2 or 6 h. Contribution of the PSII (Photosystem II) repair cycle to the light sensitivity was assessed by incubating the plants under 300 μmol of photons·m⁻²·s⁻¹ for 2 h on Petri dishes in the presence of 1 mM lincomycin.

For long-term stress treatments, plants were first grown for 2 weeks under control growth conditions (130 μmol of photons·m⁻²·s⁻¹ at 20°C, 8-h light period), and thereafter shifted for 2 weeks to moderately high-light intensity (600 μmol of photons·m⁻²·s⁻¹ at 20°C), to high-light stress (1300 μmol of photons·m⁻²·s⁻¹ at 28°C), to low temperature (130 μmol of photons·m⁻²·s⁻¹ at 10°C) or maintained at control growth conditions. For biochemical analysis, samples were collected from 4-week-old plants, 6 h after the lights were turned on.

Laser-scanning confocal microscopy

Confocal microscope images of 7-day-old seedlings were obtained with an inverted confocal laser-scanning microscope (Zeiss LSM510 META; http://www.zeiss.com) using a 20×/0.50 water objective. Chlorophyll fluorescence was excited at 488 nm with an argon diode laser, and detected with a 650–710 nm passing emission filter. Maximal projections of the sequential confocal images were created with the Zeiss LSM Image Browser Version 3.5,0,376 available through http://www.zeiss.com.

Measurement of chlorophyll content, chlorophyll fluorescence and gas exchange

To determine the leaf chlorophyll content, two leaf discs, 3 mm in diameter, were incubated in 1 ml of dimethylformamide overnight at 4°C in darkness, and the chlorophyll content was determined spectrophotometrically according to [25a].

The photoinhibition state of PSII was monitored as a ratio of variable to maximal fluorescence, Fv/Fm (Fv is a difference between maximal, Fmax, and initial, Fo, fluorescence), measured from intact leaves with a Hansatech PEA fluorometer after a 30 min dark incubation.

Gas exchange of intact Arabidopsis plants was measured with CIRAS-1 combined IR gas analysis system (PP Systems) equipped with an Arabidopsis pot chamber (PP Systems). The response of net photosynthesis (Aᵣ) to the reference CO₂ was measured under PPFD (photosynthetically active photon flux density) that was saturating for net photosynthesis (500 μmol·m⁻²·s⁻¹ at 20°C). The parameters for maximal carboxylation rate of Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase; EC 4.1.1.39) (Vcmax, μmol of CO₂·m⁻²·s⁻¹), maximal electron transfer rate of PSII...
transport rate (Jmax, μmol · m⁻² · s⁻¹) and rate of mitochondrial respiration in light (RD, μmol · m⁻² · s⁻¹) were obtained by modelling the response of net CO₂ assimilation to increasing extracellular CO₂ concentration according to [26].

Isolation of thylakoid membranes, soluble extract and total leaf extract

For isolation of thylakoids and soluble leaf extract, leaves were harvested and immediately frozen in liquid nitrogen. After homogenization in ice-cold shock buffer [25 mM Hepes/KOH (pH 7.4), 10 mM MgCl₂, and 10 mM NaF] and filtration through Miracloth, the suspension was centrifuged at 6000 g for 5 min at 4°C. The thylakoid pellet was resuspended in isolation buffer, and the supernatant was further centrifuged at 12 000 g for 15 min at 4°C to collect the soluble extract. For isolation of total leaf extract, six 12.56 mm² leaf discs from three individual rosettes were punched and immediately frozen in liquid nitrogen. The leaf discs were completely and carefully homogenized in 150 μl of ice-cold isolation buffer [350 mM sucrose, 25 mM Hepes/KOH (pH 7.4), 10 mM MgCl₂, and 10 mM NaF] in a dark cold room. All samples were frozen in liquid nitrogen and stored at −80°C. The chlorophyll content of isolated thylakoids was determined according to [27], and the protein content of the soluble and total leaf extracts was determined using a Bio-Rad assay kit.

SDS/PAGE and Western blotting

Thylakoid polypeptides corresponding to 1 μg of chlorophyll and the soluble and total leaf extracts corresponding to 10 μg of protein were solubilized and separated by SDS/PAGE [28], using 15% acrylamide and 6 M urea in the separation gel, and subsequently electroblotted to a PVDF membrane (Millipore). After blocking with 5% BSA (fatty-acid free, Sigma–Aldrich), the polypeptides were immunodetected with protein-specific antibodies using a PHOTOTOPE™-Star Detection Kit (New England Biolabs). Thylakoid protein phosphorylation in isolated thylakoids was explored by using a phosphothreonine-specific antibody (New England Biolabs) [29]. An Arabidopsis APX-specific antibody was raised against a highly conserved region among TAPX, sAPX and the cytoplasmic APXs, corresponding to amino acids 204–215 of tAPX (EEGRLPDAGPPS). An additional cysteine residue was added to the C-terminus of the peptide to mimic the 5’ untranslated region of the tAPX gene (At1g77490), and in the first exon of the SAPX gene (At4g08390) (Figure 1a). To confirm the absense of tAPX and sAPX from the tapx and sapx lines respectively, we designed an antibody that could be used to explore both the chloroplastic and cytoplasmic forms of Arabidopsis APXs. The linear range with a 1:2000 dilution was 0.15 M NaCl/0.015 M sodium citrate, 0.1% SDS, 0.1 mg/ml herring sperm and 5% poly-dT primer (Oligomer) and reverse transcriptase SuperScript III (Invitrogen). The aminoallyl-labelled cDNA was purified with the QiAquick PCR purification kit (Qiagen), and stained with CY™ Post-Labeling Reactive Dye Pack (Amersham). cDNA corresponding to 15 pg of each dye was hybridized to the arrays in 50% formamide, 5 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate), 0.1% SDS, 0.1 mg/ml herring sperm and 5 × Denhardt’s at 42°C overnight.

The arrays were scanned with an Agilent scanner, and the spot intensities were quantified with ScanArray Express Microarray Analysis system 2.0 (PerkinElmer Life Sciences). The data from three biological replicates were analysed with GeneSpring 7.2. Genes with a Student’s t test P value below 0.05 were chosen for further analysis. Gene annotation was derived from The Arabidopsis Information Resource (http://www.arabidopsis.org) and was based on TAIR7.

RESULTS

Identification of tapx and sapx single mutants and a tapx sapx double mutant

Sequencing of the T-DNA insertion sites of homozygous tapx and sapx single mutant lines revealed a T-DNA insertion in the 5‘ untranslated region of the TAPX gene (At1g77490), and in the first exon of the SAPX gene (At4g08390) (Figure 1a). To confirm the absense of tAPX and sAPX from the tapx and sapx lines respectively, we designed an antibody that could be used to explore both the chloroplastic and cytoplasmic forms of Arabidopsis APXs. The linear range with a 1:2000 dilution was tested by performing an anti-APX immunoblot with increasing amounts of total foliar protein (Figure 1b, left-hand panel). Based on the results, loading of 10 μg of total protein was selected for further experimentation. sAPX and tAPX could be identified after further experimentation. sAPX could be observed in leaf extracts isolated from sapx plants, nor could tAPX be observed in leaf extracts isolated from tapx plants (Figure 1b, right-hand panel). The tapx sapx double mutants were devoid of both tAPX and sAPX (Figure 1b, right-hand panel).

In vivo detection of H₂O₂

Accumulation of H₂O₂ in the leaves was detected using DAB (diaminobenzidine; Sigma–Aldrich) as a substrate [30]. Briefly, rosettes were excised at the end of the light period, and incubated on Petri dishes containing a 0.1 mg/ml solution of DAB (pH 3.8) overnight in darkness. At the onset of the light period, the dishes were transferred to growth light (130 μmol of photons · m⁻² · s⁻¹ at 20°C) for 2 h, or incubated under high light (1000 μmol of photons · m⁻² · s⁻¹ at 20°C) for 30 min. Thereafter the rosettes were incubated in 96% (v/v) ethanol until chlorophyll was bleached, and finally photographed under a Zeiss Lumar V12 stereomicroscope.

Microarray analysis

Global changes in gene expression were explored with spotted Arabidopsis 24k oligonucleotide arrays (MWG Biotech; ArrayExpress database accession number A-ATMX-2). Twenty rosettes, 3 weeks of age, of wild-type and tapx sapx double-mutant plants were collected under normal growth conditions (130 μmol of photons · m⁻² · s⁻¹ at 20°C) 2 h after the onset of the light period. For analysis of high-light-induced modulations in gene expression, the plants were illuminated under 1000 μmol of photons · m⁻² · s⁻¹ at 20°C for 30 min. Total RNA was isolated with TRIzol® as described previously [31]. Subsequently, DNA was removed with the Qiagen RNeasy Mini Kit, and cDNA synthesis was performed in the presence of 0.2 mM aminomethyl-dUTP using anchored poly-dT primer (Oligomer) and reverse transcriptase SuperScript III (Invitrogen). The aminomethyl-labelled cDNA was purified with the QIAquick PCR purification kit (Qiagen), and stained with CY™ Post-Labeling Reactive Dye Pack (Amersham). cDNA corresponding to 15 pg of each dye was hybridized to the arrays in 50% formamide, 5 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate), 0.1% SDS, 0.1 mg/ml herring sperm and 5 × Denhardt’s at 42°C overnight.

The arrays were scanned with an Agilent scanner, and the spot intensities were quantified with ScanArray Express. This analysis system 2.0 (PerkinElmer Life Sciences). The data from three biological replicates were analysed with GeneSpring 7.2. Genes with a Student’s t test P value below 0.05 were chosen for further analysis. Gene annotation was derived from The Arabidopsis Information Resource (http://www.arabidopsis.org) and was based on TAIR7.

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complete lack of pigmentation was observed for the greening of wild-type and plastid. The presence of methyl viologen somewhat hindered sapx in the mutant and the and black and grey boxes represent exons and untranslated regions respectively. (blots with an Arabidopsis anti-APX antibody. The left-hand panel shows the linear range of the soluble leaf extract. The right-hand panel confirms the absence of tAPX, sAPX or both in double-mutant plants when germinated under photoinhibition (Figure 2d). Thus the vulnerability of the wild-type, and the single mutants compared with wild-type plants (Figure 2c). In contrast, tapx double mutants showed higher methyl-viologen-induced cell death than wild-type or the single apx mutant plants even under high-light conditions, and this effect became drastically pronounced under high-light intensity (Figure 2c).

The high-light sensitivity of 4-week-old tapx and tapx sapx double-mutant plants was explored by exposing the mutant and wild-type plants to increasing light intensities (130, 300, 450, 600, 1000 and 1300 μmol of photons · m⁻² · s⁻¹ at 20°C) for 6 h. A gradual PSII photoinhibition of similar extent, measured as the photosynthetic photon flux density (Fv/Fmax) after a 30 min dark incubation, was recorded for the wild-type, tapx sapx double-mutant plants as well as for tapx sapx single-mutant plants as compared with wild-type plants (Figure 2c). In contrast, tapx sapx double-mutant plants showed higher methyl-viologen-induced cell death than wild-type or the single apx mutant plants even under high-light conditions, and this effect became drastically pronounced under high-light intensity (Figure 2c).

The high-light sensitivity of 4-week-old tapx, sapx and tapx sapx double-mutant plants to photooxidative stress in germinating tapx, sapx and tapx sapx double-mutant plants

To evaluate the role of chloroplast APXs in photoprotection during early stages of seedling development, wild-type, tapx, sapx and tapx sapx double-mutant plants were germinated under photooxidative stress conditions. To this end, seedlings were germinated in the presence of 1.5 μM methyl viologen under standard growth conditions (130 μmol of photons · m⁻² · s⁻¹ at 20°C). Methyl viologen extracts electrons from PSI, and then reacts with molecular oxygen to produce O₂⁻ and subsequently H₂O₂ in chloroplasts. The presence of methyl viologen somewhat hindered the greening of wild-type and tapx plants, whereas almost complete lack of pigmentation was observed for tapx sapx double-mutant plants (Figure 2a). Interestingly, sapx plants exhibited an intermediate phenotype in the presence of methyl viologen, and both white and pale-green seedlings developed (Figure 2a).

Imaging of chlorophyll fluorescence by confocal microscopy revealed no structural differences in the cotyledons of wild-type, tapx, sapx and tapx sapx double-mutant plants when germinated on control plates containing half-strength MS medium (Figure 2a). In the presence of methyl viologen, cotyledons of the wild-type and tapx plants also showed accumulation of chloroplasts which, however, appeared to be smaller than those observed in the control plants (Figure 2a). In contrast, hardly any chlorophyll fluorescence could be detected when cotyledons of the sapx single mutants or the tapx sapx double mutants were imaged with the same microscope settings that were used to examine the wild-type and tapx plants (results not shown). By enhancing the sensitivity of detection by increasing the detector gain, however, accumulation of a drastically reduced number of chloroplasts in the cotyledons of sapx and the tapx sapx double mutant could be documented (Figures 2a and 2b).

The severity of chloroplast photo-oxidation in the sapx single-mutant and the tapx sapx double-mutant plants varied to some extent. Typically, the methyl-viologen-induced oxidative stress led to almost complete bleaching of mesophyll cell chloroplasts in the sapx single-mutant and the tapx sapx double-mutant plants (Figure 2a). In some of the cotyledons, however, chlorophyll autofluorescence could still be observed from the guard cell chloroplasts of the abaxial side of the leaves, as demonstrated in Figure 2a. For the wild-type and tapx cotyledons, imaging of chlorophyll fluorescence from guard cells failed due to interference by the strong chlorophyll fluorescence that originated from the mesophyll cell chloroplasts (results not shown). Apart from the almost completely bleached cotyledons, some sapx and tapx sapx seedlings accumulated up to 25% of the amount of chloroplasts that were observed in the methyl-viologen-treated wild-type plants (Figure 2b). Interestingly, despite the apparent photo-oxidation of mesophyll cell chloroplasts, distinct chlorophyll fluorescence could be observed from the emerging veinal regions of the methyl-viologen-treated tapx sapx double-mutant cotyledons (Figure 2b). These results obtained with the tapx and tapx sapx seedlings imply that the guard cell and bundle sheath cell chloroplasts are more tolerant to photo-oxidative stress than the mesophyll cell chloroplasts (Figures 2a and 2b).

Sensitivity of tapx, sapx and tapx sapx double-mutant plants to short-term stress treatments

Next we examined whether expanded, 4-week-old tapx, sapx or tapx sapx double-mutant plants are sensitive to methyl-viologen-induced stress. At the end of the dark period, plants were sprayed with 50 μM methyl viologen, incubated for 30 min under 50 μmol of photons · m⁻² · s⁻¹ at 20°C, and thereafter illuminated under 130 or 1000 μmol of photons · m⁻² · s⁻¹ at 20°C for 6 h. Illumination under growth light induced an ion leakage of a similar extent for the wild-type, tapx and sapx plants (Figure 2c), and under high-light intensity only a slight increase in ion leakage became evident in the tapx and sapx single-mutant plants as compared with wild-type plants (Figure 2c). In contrast, tapx sapx double mutants showed higher methyl-viologen-induced cell death than wild-type or the single apx mutant plants even under growth light conditions, and this effect became drastically pronounced under high-light intensity (Figure 2c).

Long-term acclimation of tapx, sapx and tapx sapx double-mutant plants to environmental stress

To study the capability of tapx, sapx and tapx sapx lines for long-term acclimation to stressful conditions, plants were grown for 2 weeks under control growth conditions of 130 μmol of photons · m⁻² · s⁻¹ at 20°C, and thereafter shifted for 2 weeks to moderately high light (600 μmol of photons · m⁻² · s⁻¹ at 20°C), to high-light stress (1300 μmol of photons · m⁻² · s⁻¹ at 28°C) or
Figure 2  Sensitivity of wild-type, tapx, sapx and tapx sapx double-mutant plants to short-term photo-oxidative stress

(a) Susceptibility of seedlings to photo-oxidative stress during germination. Seedlings were germinated in the presence of 1.5 μM methyl viologen under 130 μmol of photons ©m−2·s−1 at 20°C. The confocal images are projections of chlorophyll fluorescence from 30 μm stacks of sequential images starting from the abaxial side of the cotyledons. Scale bars=100 μm. (b) Confocal images of chlorophyll fluorescence demonstrating cell-type-specific effects of photo-oxidative stress on chloroplast development in germinating tapx sapx double-mutant plants. Seedlings were germinated in the presence of 1.5 μM methyl viologen under 130 μmol of photons ©m−2·s−1 at 20°C. The confocal images are projections of 50 μm stacks of sequential images starting from the abaxial side of the cotyledons. Scale bars=100 μm. (c) Methyl-viologen-induced photo-oxidative stress in 4-week-old plants. Plants were sprayed with 50 μM methyl viologen and illuminated under 130 or 1000 μmol of photons ©m−2·s−1 at 20°C for 6 h prior to measurement of ion leakage from the leaves. Control samples were sprayed with water and illuminated under normal growth conditions. The values are means ± S.D. for four independent experiments. **P < 0.05 and ***P < 0.005 measured using Student’s t test when compared with wild-type. MV, methyl viologen. (d) Photoinhibition of PSII in 4-week-old plants under various irradiance levels. Plants were exposed to increasing light intensities for 6 h, and the photochemical efficiency of PSII was measured as Fv/Fmax after a 30 min dark incubation. The light and temperature conditions are indicated in the Figure. The lincomycin-treated plants were illuminated under 300 μmol of photons ©m−2·s−1 for 2 h. The values are means ± S.D. for at least three independent experiments. **P < 0.05 and ***P < 0.005 measured using Student’s t test when compared with wild-type. WT, wild-type; LM, lincomycin.
The similar long-term stress tolerance of wild-type, tapx, sapx and the tapx sapx double-mutant plants (Figure 3a and Supplementary Table S1) raised a question as to whether the acclimation to contrasting environmental conditions had also induced cross-tolerance against methyl-viologen-induced abrupt oxidative stress. Thus the differentially stress-acclimated plants were sprayed at the end of the dark period with 50 μM methyl viologen, and the extent of cell death was measured after 24 h treatment under standard growth conditions (130 μmol of photons · m⁻² · s⁻¹ at 20°C, 8 h light/16 h dark period) (Figure 3b). Clearly, all the lines grown under normal growth conditions wilted during the treatment (Figures 3b and 3c). In contrast, acclimation to high light or to low temperature induced tolerance against subsequent methyl-viologen-treatment in the wild-type and the tapx and sapx single-mutant plants (Figure 3b). In sharp contrast with the wild-type and the single mutants, the differentially stress-acclimated tapx sapx double-mutant plants acquired only partial tolerance against methyl-viologen-induced photo-oxidative stress, and showed cell death, bleaching of chlorophyll and wilting of leaves after the 24 h illumination period (Figures 3b and 3c).

Steady-state levels of APXs and chloroplast PRXs upon long-term acclimation to stress

Besides APXs, chloroplast PRXs have been associated with the water–water cycle, and thus with the protection of chloroplasts against oxidative damage [5]. It is therefore conceivable that different environmental stresses promote differential adjustments in these antioxidant enzymes in plants. Moreover, increases in the levels of tAPX and/or sAPX would explain why the differentially stress-acclimated wild-type and the tapx and sapx single-mutant plants, but not the tapx sapx double-mutant plants, acquired tolerance against methyl-viologen-induced oxidative damage (Figure 3b). Thus we examined the levels of chloroplastic and cytoplasmic APXs and chloroplast PRXs in the differentially stress-acclimated plants by Western blotting. Again, plants were grown for 2 weeks under the control growth conditions of 130 μmol of photons · m⁻² · s⁻¹ at 20°C, and thereafter shifted for 2 weeks to moderately high-light intensity (600 μmol of photons · m⁻² · s⁻¹ at 20°C for 24 h prior to measurement of ion leakage from the leaves). The values are means ± S.D. for four independent experiments. **P < 0.005 measured using Student's t test when compared with wild-type. (f) Photograph depicting the tolerance of high-light-acclimated wild-type plants and the sensitivity of tapx sapx double-mutant plants to methyl-viologen-induced cell death. WT, wild-type; MV, methyl viologen.

to low temperature (130 μmol of photons · m⁻² · s⁻¹ at 10°C). Control plants were maintained at initial growth conditions. After the acclimation period, neither the tapx and sapx single mutants nor the tapx sapx double mutants exhibited any enhanced stress symptoms or any kind of visual phenotype under any of the stress conditions studied (Figure 3a and Supplementary Table S1 at http://www.BiochemJ.org/bj/412/bj4120275add.htm). Indeed, the mutant and wild-type plants showed similar morphological adaptations (Figure 3a), and similar modulations in the content of chlorophyll (Supplementary Table S1). Also the photochemical efficiency of PSII, expressed as Fv/Fm max was, after 2 weeks of stress treatment, almost the same as the control value of the wild-type plants (Supplementary Table S1).

Figure 3 Stress tolerance of wild-type, tapx, sapx and tapx sapx double-mutant plants upon long-term acclimation to adverse environmental conditions

(a) Phenotypes after a 2-week acclimation period under high light, high-light stress or low temperature. Plants were grown for 2 weeks under normal growth conditions, and thereafter shifted for another 2 weeks to various light and temperature conditions as indicated in the Figure. (b) Methyl-viologen-induced photo-oxidative stress in differentially stress-acclimated plants. Plants were sprayed with 50 μM methyl viologen and illuminated under 130 μmol of photons · m⁻² · s⁻¹ at 20°C for 24 h prior to measurement of ion leakage from the leaves. The values are means ± S.D. for four independent experiments. **P < 0.005 measured using Student’s t test when compared with wild-type. (c) Photograph depicting the tolerance of high-light-acclimated wild-type plants and the sensitivity of tapx sapx double-mutant plants to methyl-viologen-induced cell death. WT, wild-type; MV, methyl viologen.
Stromal and thylakoid-bound APXs in stress responses in chloroplasts

Figure 4  Modulation in the steady-state levels of H₂O₂-detoxifying enzymes, photosynthetic proteins and LHCII protein phosphorylation under contrasting environmental cues

(a) Immunoblots depicting the levels of H₂O₂-detoxifying enzymes in the tapx and sapx single mutants and wild-type plants. (b) Immunoblots depicting the levels of H₂O₂-detoxifying enzymes in the tapx sapx double mutants and wild-type plants. (c) Immunoblots depicting the levels of photosynthetic proteins in the tapx sapx double mutants and wild-type plants. (d) Phosphothreonine-specific immunoblot showing thylakoid protein phosphorylation in the tapx sapx double mutants and wild-type plants. For experiments shown in (a)–(c), the plants were grown for 2 weeks under normal growth conditions, and thereafter shifted for another 2 weeks to various stress conditions as indicated above the lines. For immunoblot analysis, 10 μg of total protein was loaded in the wells. Each horizontal line represents bands excised from the same immunoblot with equal handling of the image. For analysis of thylakoid protein phosphorylation shown in (d), the plants were grown under normal growth conditions, and shifted to a higher light intensity for 2 h. DM, tapx sapx double mutant; cAPX, cytoplasmic APX; 2-CP, chloroplastic 2-cysteine PRX A and B; D1 and psaD, representatives of PSII and PSI core complexes; Lhcb2 and Lhca1, representatives of light-harvesting antenna proteins; RbcL, Rubisco large subunit; CP43-P, D2-P, D1-P and LHCII-P, phosphorylated forms of CP43, D2, D1 and LHCII respectively; WT, wild-type.

In conclusion, the absence of tAPX and/or sAPX induced no drastic compensatory increases in the steady-state levels of other APX isoforms. Moreover, only under high-light stress, the absence of both tAPX and sAPX induced an apparent compensatory increase in the steady-state level of 2-Cys PRX in the tapx sapx double-mutant plants (Figures 4a and 4b).

Photosynthetic adjustments in tapx sapx double-mutant plants

The wild-type and tapx sapx double-mutant plants showed similar accumulation of the light-harvesting antenna I and II polypeptides, Lhca1 and Lhcb2, as well as the psaD and D1 proteins, representatives of the PSI and PSII core complexes respectively, under normal growth conditions (Figure 4c). Likewise, acclimation of the wild-type and tapx sapx double-mutant plants to high-light intensity induced similar decreases in the levels of Lhca1, Lhcb2 and psaD (Figure 4c). The D1 protein, on the other hand, showed no consistent changes after 2 weeks of light acclimation in either of the lines (Figure 4c). Low temperature induced no significant modulations in any of the polypeptides in either the wild-type or the tapx sapx double-mutant plants (Figure 4c). Moreover, the contents of the Rubisco large subunit remained quite invariable under all conditions explored (Figure 4c). Consistently, the response of net CO₂ assimilation to increasing atmospheric CO₂ concentrations in wild-type and tapx sapx double mutants grown under control growth conditions (130 μmol of photons • m⁻² • s⁻¹ at 20°C) did not drastically differ from each other (results not shown). Modelling of photosynthetic parameters revealed quite similar values for the maximal carboxylation rate of Rubisco (Vcmax) and for the maximal photosynthetic electron transport rate (Jmax) in the wild-type and tapx sapx double-mutant plants (Supplementary Table S2 at http://www.BiochemJ.org/bj/412/bj4120275add.htm). Interestingly, however, the calculated parameters indicated a reduced rate of mitochondrial respiration (Rd) in tapx sapx double-mutant plants as compared with wild-type plants (Supplementary Table S2).

Redox regulation of LHCII (light-harvesting complex II) protein phosphorylation in tapx sapx double-mutant plants

The redox conditions of chloroplasts are known to modulate signalling events that dynamically regulate photosynthetic processes [32]. In the thylakoid membrane, the reversible phosphorylation of the PSII light-harvesting antenna polypeptides Lhcb1 and Lhcb2 on stroma-exposed threonine residues is particularly sensitive to modulations in the thiol redox state of chloroplast stroma [29]. To explore whether tAPX and sAPX contribute to chloroplast redox signalling, we studied the light-dependent adjustments in the level of LHCII protein phosphorylation in wild-type and the tapx sapx double-mutant plants. Under normal growth light conditions (130 μmol of photons • m⁻² • s⁻¹ at 20°C), the LHCII showed no drastic differences in the phosphorylation...
level between wild-type and the tapx sapx double-mutant plants (Figure 4d). When the plants were shifted to higher light intensity (300 μmol of photons · m⁻² · s⁻¹ at 20°C) for 2 h, LHClII protein phosphorylation became down-regulated in the wild-type plants. However, the LHClII of tapx sapx double-mutant plants remained slightly more phosphorylated as compared with wild-type plants (Figure 4d). Under these experimental conditions, no distinct differences were observed in the phosphorylation of the PSII core proteins D1, D2 and CP43 between wild-type and the tapx sapx double-mutant plants (Figure 4d).

Accumulation of H₂O₂ and adjustments in gene expression in tapx sapx double-mutant plants

The altered regulation of LHClII protein phosphorylation in the tapx sapx double-mutant plants (Figure 4d) implied a role for chloroplast APXs in the fine-tuning of signalling components in chloroplasts. Therefore we explored the accumulation of H₂O₂ and the pattern of gene expression in the tapx sapx double-mutant and wild-type plants grown under normal growth conditions (130 μmol of photons · m⁻² · s⁻¹ at 20°C), and after a short 30 min illumination under high-light intensity (1000 μmol of photons · m⁻² · s⁻¹ at 20°C). Under normal growth light conditions, the wild-type plants showed hardly any accumulation of H₂O₂, and only slightly higher accumulation of H₂O₂ was observed as an appearance of brown precipitate in the leaves of the tapx sapx double-mutant plants (Supplementary Figure S1 at http://www.BiochemJ.org/bj/412/bj4120275add.htm). Exposure of the rosettes to a 30-min high-light illumination period under 1000 μmol of photons · m⁻² · s⁻¹ at 20°C enhanced the accumulation of H₂O₂ to some extent in the wild-type plants (Supplementary Figure S1). In the leaves of tapx sapx double-mutant plants, the accumulation of H₂O₂ was slightly more pronounced as compared with the wild-type plants (Supplementary Figure S1).

When the gene expression was analysed under control growth conditions, only one gene, At1g26170, encoding an importin β-2 subunit family protein, showed statistically significant (t test P value < 0.05) up-regulation in the tapx sapx double mutants as compared with wild-type plants. In contrast, distinctly lower transcript levels for 67 genes were observed in the tapx sapx double mutant as compared with wild-type plants (Supplementary Figure S1 and Supplementary Table S3 at http://www.BiochemJ.org/bj/412/bj4120275add.htm). Twelve of these genes, including TAPX and SAPX, were found to encode chloroplast proteins (Supplementary Table S3). Furthermore, distinct down-regulation of six transcription factors that control gene expression in the nucleus occurred in the tapx sapx double-mutant plants as compared with wild-type plants (Supplementary Table S3).

Among these transcriptional regulators, At1g04400, which encodes the blue light photoreceptor CRY2 (cryptochrome 2), was down-regulated in the tapx sapx double-mutant plants (Supplementary Table S3). CRY2 was recently shown to function as a positive regulator of flowering in the vascular tissue of Arabidopsis leaves [33]. Recently, tapx single-mutant plants were reported to induce flowering a few days earlier than wild-type plants [23]. Under our control growth conditions, both tapx and sapx single-mutant plants also started to flower 1 or 2 days earlier than the wild-type plants, whereas the tapx sapx double-mutant plants show floral induction simultaneously with the wild-type plants (results not shown).

The short 30-min high-light illumination period induced further adjustments in the pattern of gene expression in the tapx sapx plants as compared with wild-type plants (Supplementary Figure S1 and Supplementary Table S3B). The up-regulated genes included At1g55910, a chloroplastic member of the thus far poorly characterized Arabidopsis AGC family protein kinases (Supplementary Table S3B). Members of the AGC family protein kinases mediate signals from key secondary messengers, including cAMP, cGMP and calcium, and act as crucial regulators of basic cellular metabolism [34]. Another intriguing observation in the tapx sapx double-mutant plants was a high-light-induced up-regulation of At1g23550, which encodes for SRO2 [SIMILAR TO RCD1 (RADICAL-INDUCED CELL DEATH 1)]. RCD1 and the SRO family members have crucial functions in the regulation of stress-responsive genes in Arabidopsis [35,36]. Notably, despite the absence of both tAPX and sAPX, no compensatory up-regulation of genes related to the antioxidant system was observed. The high-light treatment further strengthened the down-regulation of several genes in tapx sapx double-mutant plants as compared with wild-type plants (Supplementary Figure S1 and Supplementary Table S3). Interestingly, the down-regulated genes included a dynamin superfamily GTPase, At1g03160, which has been assigned crucial functions in the biogenesis of chloroplasts (Supplementary Table S3B) [37].

**DISCUSSION**

Chloroplast APXs have been assigned a distinct role in scavenging ROS produced by photosynthetic electron transfer reactions in chloroplasts [3,19,20,38]. In the water–water cycle, electron transfer from PSI to molecular oxygen leads to generation of superoxide on the stromal side of the thylakoid membrane. Superoxide is highly reactive and becomes rapidly dismutated to H₂O₂ by a thylakoid-associated Cu/Zn SOD [3,4]. H₂O₂ in turn becomes enzymatically reduced to water by two distinct groups of water–water cycle enzymes, APXs and PRXs, both of which comprise several isoforms [9]. In the present study, we attempted to dissect the roles of the tAPX and the sAPX in the antioxidative network of Arabidopsis thaliana as influenced by the different developmental stage and by the exposure of plants to stress conditions.

**sAPX has a key role in oxidative stress tolerance during early greening phases**

Arabidopsis seedlings lacking the sAPX were sensitive to mild photo-oxidative stress, and produced pale green seedlings when germinated in the presence of methyl viologen at a low concentration (Figure 2a). In contrast, the single mutants lacking the tAPX developed like wild-type plants (Figure 2a). Nevertheless, the tapx sapx double mutant exhibited even more severe failure in the greening process than that observed for the sapx single mutant (Figure 2a), demonstrating the contribution of both tAPX and sAPX to antioxidant defence in young seedlings. Thus chloroplast APXs, and particularly the sAPX, are of primary importance for photoprotection during the initial greening process. Light absorption by the newly accumulating chlorophylls in developing chloroplasts poses a threat of photo-oxidative damage, especially if the metabolic balance between the absorption and utilization of light energy has not yet been attained. Such photo-oxidative disturbances during chloroplast development have been suggested also to cause the formation of white plastids in the class of variegated mutants, which are characterized by alternation of white and green sectors in leaves [39]. In accordance with the crucial importance for tAPX and sAPX in the maintenance of optimal chloroplast development (Figure 2a), the high-light-treated tapx sapx double-mutant plants showed down-regulation of a recently identified dynamin superfamily GTPase, which is required for proper biogenesis of chloroplasts (Supplementary Table S3) [37]. Moreover, both sAPX and tAPX have been shown...
to accumulate in high amounts in pea etioplasts in darkness, and then to decrease during the progress of the greening process upon illumination [40]. Likewise, the genes encoding chloroplast PRXs and APXs are highly expressed during the first days after germination in Arabidopsis [41].

Another intriguing phenomenon was that the chloroplasts located in the guard cells or along the emerging veins in the cotyle-
dons of the sapx single mutants and the tapx sapx double mutants were less sensitive to oxidative stress than the mesophyll cell chloroplasts (Figures 2a and 2b). Similarly, high-light illumin-
ation of a tapx sapx vc2 triple mutant deficient in chloroplast APXs and also of ascorbate resulted in bleaching of interveinal leaf tissues [42]. Indeed, there is increasing evidence indicating that the different cell types possess specialized roles in the regulation of leaf development in response to environmental cues [39,43]. The bundle shear cell chloroplasts are the primary site for accumulation of H₂O₂ during high-light-illumination of leaf tissues [44] (see also Supplementary Figure S1), and several genes related to the plant antioxidant network seem to be preferentially expressed in the veinial regions in Arabidopsis leaves. These gene products include the cytoplasmic APX 2 [44], a microRNA (miR398) that targets Cu/Zn SOD [45] and a chloroplastic glutara-
redoxin [7]. It is conceivable that cell-type-specific expression of antioxidant genes also contributed to the differential oxidative-stress tolerance of various cell types in the tapx sapx double-mutant and wild-type plants (Figures 2a and 2b) [42].

Chloroplast APXs are crucial for photoprotection and signalling

The tapx and sapx single mutants and the tapx sapx double-
mutant plants exhibited no visible stress symptoms after long-term growth under high light (Figure 3) [42] or under low temperature (Figure 3). However, the importance of chloroplast APXs in the maintenance of photosynthetic activity in mature leaves became evident when plants were suddenly exposed to high-light intensity (Figure 2d). An abrupt increase in excitation energy led to an enhanced photoinhibition of PSII in tapx sapx double-mutant plants, which could be recorded as a pronounced decline in the photochemical efficiency of PSII (Figure 2d). This corroborates with results obtained for transgenic tobacco plants overexpressing tAPX, which were capable of maintaining photosynthetic activity under a combination of short-term high-light and low-temperature stress [20]. These results indicate that chloroplastic APX activity is crucial for photoprotection, yet the mechanism of protection is not fully understood.

On one hand, the water–water cycle may attenuate over-
excitation of the photosynthetic electron transfer chain by utilizing molecular oxygen as an alternative electron acceptor, and thereby diminishing ROS-induced photodamage to PSII [3]. On the other hand, detoxification of H₂O₂ is linked to the maintenance of the Calvin cycle [20] and translational activity [46] in chloroplasts. Indeed, in cyanobacterial cells, the accumulation of H₂O₂ led to enhanced photoinhibition due to impairment of the PSII repair cycle, whereas the rate of photodamage to PSII was not affected [47]. This was also the case for the tapx sapx double-mutant plants, which were as sensitive as the wild-type plants to light-
induced inactivation of PSII when the repair of damaged PSII complexes during illumination was inhibited by the chloroplast translation inhibitor lincomycin (Figure 2d). This observation strongly suggests that the enhanced susceptibility of the tapx sapx double mutant to photoinhibition resulted from translational perturbations thereby hampering the repair cycle of PSII.

The tapx sapx double-mutant plants also showed slightly altered regulation of LHCII protein phosphorylation upon a shift of plants to higher light intensity (Figure 4d). In wild-type plants, LHCII protein phosphorylation becomes strongly down-regulated under high light intensities upon inhibition of the LHCII kinase by a thiol reductant (Figure 4d) [29]. Such inhibition can, however, be restored by H₂O₂, which serves as an oxidant and reactivates the LHCII kinase [48]. Thus it is likely that minor accumulation of H₂O₂ in the chloroplasts of the tapx sapx double-mutant plants upon a shift to higher light intensity maintained the LHCII kinase at a more active state as compared with the wild-type plants (Figure 4d) [29].

It is noticeable that both the chloroplast translation and the activity of the LHCII kinase have been connected to retrograde signalling, which initiates in the plastids to regulate gene expression in the nucleus [32]. It was therefore of interest to explore whether the minor differences in the tapx sapx double mutant as compared with wild-type plants in the level of H₂O₂ are reflected in the regulation of nuclear genes (Supplementary Figure S1). Apparently owing to only a low level of H₂O₂ production, no preferential up-regulation of genes generally regarded as hallmarks for oxidative stress responses [49,50] was observed for the double mutant despite the absence of both tAPX and sAPX in chloroplasts (Supplementary Table S3). Rather, as deduced from the altered regulation of LHCII protein phosphorylation (Figure 4d), the moderate accumulation of H₂O₂ in the tapx sapx double-mutant plants modulates the thiol-redox state of chloroplasts, which in turn is likely to modulate the retrograde signalling pathways from chloroplasts to the nucleus.

Functional redundancy between tAPX and sAPXs

Among 4-week-old plants, predominantly only the tapx sapx double-mutant plants showed both the acceleration of high-light-induced decline in PSII activity (Figure 2d) and the enhanced methyl-viologen-induced cell death (Figure 2c), whereas the single mutants tapx and sapx showed less severe stress symptoms. Thus it is clear that tAPX and sAPX are functionally redundant with respect to photo-oxidative stress in mature Arabidopsis leaves. Besides scavenging by the two types of APXs, H₂O₂ can also be detoxified in chloroplasts by PRXs, glutaredoxins and glutathione peroxidases as well as by the low-molecular-mass antioxidants ascorbate and glutathione, both of which are present at millimolar concentrations in chloroplasts [2]. Indeed, the stress-acclimation and cross-tolerance of plants involve multiple H₂O₂ detoxification systems that efficiently prevent oxidative damage by controlling the level of H₂O₂ in chloroplasts. Accordingly, the differentially stress-acclimated wild-type plants as well as the tapx and sapx single mutants had acquired tolerance against sudden attack by methyl-viologen-induced accumulation of ROS (Figures 3b and 3c) (see below), whereas the stress-acclimated tapx sapx double-mutant plants exhibited only partial tolerance. This indicates that the capacity of the double mutant to efficiently scavenge the ROS induced by a sudden methyl-viologen-treat-
ment was exceeded, compared with that of the wild-type plants and single mutants (Figure 3c). It is conceivable that the tAPX and sAPX operate in concert during the initial detoxification of H₂O₂, and are capable of controlling the H₂O₂ level unless the production of H₂O₂ suddenly exceeds the threshold level (Figure 2). This interpretation is consistent with the observation that chloroplastic APXs become rapidly inactivated by high concentrations of H₂O₂, especially if ascorbic acid is present at suboptimal levels [51,52].

Plants deficient in chloroplast APXs acquire compensatory mechanisms to tolerate long-term stress

Considering the distinct susceptibility of the tapx sapx double-mutant plants to short-term photo-oxidative stress, it was
intriguing that no stress symptoms appeared under any of the long-term stress conditions studied (Figure 3a). Recently, Giacomelli et al. [42] showed that this is not due to compensatory accumulation of the low-molecular-mass ROS scavengers ascorbate, glutathione or α-tocopherol.

The present study revealed that the deficiency of tAPX and sAPX in the high-light-stress-acclimated tapx sapx double-mutant plants resulted in accumulation of chloroplast 2-Cys PRX (Figure 4b), which exhibits overlapping activities in ROS scavenging with the chloroplast APXs [5]. Conversely, transgenic Arabidopsis plants with reduced amounts of 2-Cys PRX were shown to up-regulate genes encoding chloroplast APXs [53]. Furthermore, a distinctly enhanced accumulation of cytoplasmic APX occurred in all high-light-acclimated plants, the wild-type, tapx, sapx and tapx sapx double-mutant plants (Figure 4). These results suggest that upon prolonged high-light exposure of plants, both the chloroplastic PRXs and the cytoplasmic APXs have a crucial role in the removal of H₂O₂ that originates from photosynthetic processes [54].

The expression of genes encoding different chloroplast PRXs has been shown to respond specifically to various environmental conditions and developmental stages, presumably due to complex regulation via overlapping signalling pathways [53,55]. These regulatory pathways seem to involve highly specific chloroplast retrograde signalling systems, as the accumulation of 2-Cys PRX became specifically enhanced in high-light-stress-acclimated tapx sapx double-mutant plants (Figure 4b). The retrograde signalling mechanisms require functional chloroplasts with intact metabolic pathways [56], and are capable of sensing even slight alterations in the chloroplast redox state to induce specific responses and scavenging systems according to the prevailing environmental challenges. Altogether, stress tolerance in plants is acquired via a complex network of antioxidant defenses in plants. J. Biol. Chem. 278, 3033–3042

We thank Minna Lintala, Mika Keranen and Colin Ruprecht for excellent technical assistance, and Dr Paula Mulo for critical reading of the manuscript. Jouko Sandholm at the National Science Foundation, is acknowledged for providing the sequence-indexed Arabidopsis T-DNA insertion mutants. This work was financially supported by the Academy of Finland (project numbers 107039 and 204521).

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