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Full title: Diverse roles for chloroplast stromal and thylakoid-bound ascorbate peroxidases in plant stress responses

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SUMMARY

Photosynthetic light reactions comprise a significant source of hydrogen peroxide (H_2O_2) in illuminated leaves. Ascorbate peroxidases (APXs) reduce H_2O_2 to water, and play an important role in the antioxidant system of plants. We addressed the significance of chloroplast APXs in stress tolerance and signaling in *Arabidopsis thaliana*. To this end, T-DNA insertion mutants *tapx*, *sapx* and *tapx sapx*, lacking the thylakoid-bound APX (tAPX), stromal APX (sAPX) or both, respectively, were characterized. Photo-oxidative stress during germination lead to bleaching of chloroplasts in *sapx* 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 two-week acclimation period under high light or under low temperature, none of the mutants exhibited enhanced stress symptoms. Immunoblot analysis revealed that high-light-stress-acclimated *tapx sapx* double mutants compensated the absence of tAPX and sAPX by increasing the level of 2-cys peroxiredoxin. Furthermore, the absence of tAPX and sAPX induced alterations in the transcriptomic profile of *tapx sapx* double mutant plants already under quite optimal growth conditions. We conclude that sAPX is particularly important for photoprotection during the early greening process. In mature leaves, tAPX and sAPX are functionally redundant, and crucial upon sudden onset of oxidative stress. Moreover, chloroplast APXs contribute to chloroplast retrograde signaling pathways upon slight fluctuations in the accumulation of H_2O_2 in chloroplasts.

INTRODUCTION

Generation of reactive oxygen species (ROS) is characteristic of a number of metabolic reactions that take place in different compartments of plant cells. Under favorable 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-signaling 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 superoxide dismutases (SODs), ascorbate peroxidases (APXs), peroxiredoxins (PRXs), glutaredoxins, glutathione peroxidases and catalases. In addition, several nonenzymatic antioxidants like 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 Photosystem I (PSI) leads to formation of superoxide (O_2^-), which in turn becomes rapidly dismutated to hydrogen peroxide (H_2O_2), either spontaneously or enzymatically via SOD activity [3, 4]. Chloroplasts possess a multilayer antioxidative system to insure efficient detoxification of H_2O_2 . The enzymatic detoxification systems for H_2O_2 include the APX-dependent ascorbate-glutathione cycle [3] and the PRX-dependent scavenging system [5, 6]. Recently, the importance of chloroplastic glutaredoxin and glutathione peroxidase activities in the scavenging of H_2O_2 were also demonstrated [7, 8].

APXs are heme-binding enzymes that reduce H_2O_2 to water using ascorbate as an electron donor [3]. Regeneration of ascorbate utilizes a complex set of reactions linked to the glutathione and NADPH metabolism [9]. Plants contain several APX isoenzymes, three of which reside in chloroplasts. A 38 kDa thylakoid-bound APX (tAPX), a 33 kDa stromal APX (sAPX), 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 H₂O₂ detoxifying enzymes, peroxiredoxins, are 17-22 kDa enzymes that possess N-terminal cystein residue(s) responsible for peroxidase activity. In *Arabidopsis*, four PRXs are targeted to chloroplasts: 2-cystein peroxiredoxins A and B, peroxiredoxin Q and peroxiredoxin II E [5]. Re-reduction of chloroplastic PRXs occurs via the action of various thioredoxins and thioredoxin-like proteins [5, 18].

Ascorbate peroxidases have long been considered as key enzymes in the detoxification of H₂O₂ 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, 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 over-expressing 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 signaling events conferring resistance to heat stress in *Arabidopsis* [23].

In this study, we have characterized *Arabidopsis thaliana* T-DNA insertion mutants deficient in the thylakoid-bound and stromal forms of APX. Special emphasis was paid on (i) the significance of tAPX and sAPX in oxidative stress tolerance under various developmental stages and on (ii) signaling 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 signaling pathways upon slight fluctuations in the accumulation of H₂O₂ in chloroplasts. On the contrary, in the course of long-term acclimation to various stress conditions, the chloroplast APXs can be compensated by other components of the chloroplast antioxidative system.

EXPERIMENTAL

Material and control growth conditions

Homozygous T-DNA insertion mutants for chloroplast thylakoid-bound ascorbate peroxidase (*tapx*; At1g77490; SALK_027804) or stromal ascorbate peroxidase (*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 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 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ /20°C with 60% humidity at an 8-hour light period. We chose short day conditions to avoid general upregulation 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 Murashige and Skoog basal salt mixture (MS) (Sigma-Aldrich; <http://www.sigmaaldrich.com>), and kept at 4°C in darkness for two 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 $\mu\text{mol photons m}^{-2}\text{s}^{-1}/20^{\circ}\text{C}$ at 8-hour light period for seven days.

Stress treatments

Sensitivity of expanded, four-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 $\mu\text{mol photons m}^{-2}\text{s}^{-1}/20^{\circ}\text{C}$, plants were illuminated under 130 or 1000 $\mu\text{mol photons m}^{-2}\text{s}^{-1}/20^{\circ}\text{C}$ for 6 or 24 hours. Control samples were sprayed with water and illuminated under normal growth conditions (130 $\mu\text{mol photons m}^{-2}\text{s}^{-1}/20^{\circ}\text{C}$). After the treatment, rosettes were excised and incubated in 30 ml of ion-exchanged water at 4°C in darkness over night. The extent of cell death and membrane disruption was measured as ion leakage to the solution with a conductivity meter (Radiometer, Copenhagen, Denmark). Finally, the results were normalized to the value of ion leakage obtained after boiling the samples for five minutes.

For short term light treatments, four-week-old plants, grown under 130 $\mu\text{mol photons m}^{-2}\text{s}^{-1}/20^{\circ}\text{C}$, were shifted at the end of the dark period to light intensities of 130, 300, 450, 600, 1000 and 1300 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ at 20°C for two or six hours. Contribution of PSII repair cycle to the light sensitivity was assessed by incubating the plants under 300 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ for two hours on Petri dishes in the presence of 1 mM lincomycin.

For long term stress treatments, plants were first grown for two weeks under the control growth conditions (130 $\mu\text{mol photons m}^{-2}\text{s}^{-1}/20^{\circ}\text{C}$, 8-hour light period), and thereafter shifted for two weeks to moderately high light intensity (600 $\mu\text{mol photons m}^{-2}\text{s}^{-1}/20^{\circ}\text{C}$), to high light stress (1300 $\mu\text{mol photons m}^{-2}\text{s}^{-1}/28^{\circ}\text{C}$), to low temperature (130 $\mu\text{mol photons m}^{-2}\text{s}^{-1}/10^{\circ}\text{C}$) or maintained at control growth conditions. For biochemical analysis, samples were collected from four-week-old plants, six hours after the lights were turned on.

Laser scanning confocal microscopy

Confocal microscope images of seven-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 over night at 4°C in darkness, and the chlorophyll content was determined spectrophotometrically according to (Inskeep and Bloom, 1985).

The photoinhibition state of PSII was monitored as a ratio of variable to maximal fluorescence, F_v/F_{max} (F_v is a difference between maximal, F_{max} , and initial fluorescence, F_o), measured from intact leaves with a Hansatech PEA fluorometer (King's Lynn, UK) after a 30-min dark incubation.

Gas exchange of intact Arabidopsis plants was measured with CIRAS-1 Combined infrared gas analysis system (PP Systems, Hitchin, UK) equipped with an Arabidopsis pot chamber (PP Systems, Hitchin, UK; <http://www.ppsystems.com/>). The response of net photosynthesis (A_n) to the reference CO_2 was measured under photosynthetically active photon flux density (PPDF) that was saturating for net photosynthesis ($500 \mu mol m^{-2} s^{-1}/20^\circ C$). The parameters for maximal carboxylation rate of Rubisco (V_{cmax} , $\mu mol CO_2 m^{-2} s^{-1}$), maximal electron transport rate (J_{max} , $\mu mol m^{-2} s^{-1}$) and rate of mitochondrial respiration in light (R_d , $\mu mol m^{-2} s^{-1}$) were obtained by modeling the response of net CO_2 assimilation to increasing extracellular CO_2 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, 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 (330 mM sucrose, 25 mM Hepes-KOH, pH 7.4, 10 mM MgCl₂, 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 with the Biorad assay kit.

SDS-PAGE and Western blotting

Thylakoid polypeptides corresponding to 1 µg 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, <http://www.millipore.com>). After blocking with 5 % BSA (fatty acid free, Sigma-Aldrich), the polypeptides were immunodetected with protein-specific antibodies using a PHOTOTOPETM-Star Detection Kit (New England Biolabs, Beverly, USA; <http://www.neb.com/>). Thylakoid protein phosphorylation in isolated thylakoids was explored by using a phosphothreonine-specific antibody (New England Biolabs, Beverly, USA; <http://www.neb.com/>) [29]. An Arabidopsis ascorbate peroxidase-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-COOH). An additional cysteine was added to the C-terminus of the peptide to facilitate conjugation to bovine serum albumin. The peptide synthesis, conjugation to BSA and

antibody production was performed at Innovagen (<http://www.innovagen.se/>) according to the commercial standard package offered for custom peptide antibody production. Other protein-specific antibodies were purchased from Research Genetics (D1; <http://www.resgen.com/about/index.php3>), Agrisera (Lhcb1, Lhca2; <http://www.agrisera.se/>), or were kindly provided by Professor H. Scheller (psaD) and Professor K.-J. Dietz (2-cysteine peroxiredoxin, peroxiredoxin 2E and peroxiredoxin Q).

In vivo detection of H₂O₂

Accumulation of H₂O₂ in the leaves was detected by using diaminobenzidine (DAB, Sigma-Aldrich; <http://www.sigmaaldrich.com>) as a substrate [30]. Briefly, rosettes were excised at the end of the light period, and incubated on Petri dishes supplied with a 0,1 mg/ml solution of DAB (pH 3.8) over night in darkness. At the onset of the light period, the dishes were transferred to growth light (130 $\mu\text{mol photons m}^{-2}\text{s}^{-1}/20^{\circ}\text{C}$) for two hours, or incubated under high light (1000 $\mu\text{mol photons m}^{-2}\text{s}^{-1}/20^{\circ}\text{C}$) for 30 minutes. Thereafter the rosettes were incubated in 96% (v/v) ethanol until chlorophyll bleached, and finally photographed under a Zeiss Lumar V12 stereomicroscope (<http://www.zeiss.com>).

Microarray analysis

Global changes in gene expression were explored with spotted Arabidopsis 24k oligonucleotide arrays (MWG Biotech; <http://www.mwg-biotech.com>; ArrayExpress database accession number A-ATMX-2; <http://www.ebi.ac.uk/arrayexpress>). Twenty three-week-old rosettes of wild type and *tapx sapx* double mutant plants were collected from normal growth conditions (130 $\mu\text{mol photons m}^{-2}\text{s}^{-1}/20^{\circ}\text{C}$) two hours after the onset of the light period. For analysis of high-light-induced modulations in gene expression, the plants were illuminated under 1000 $\mu\text{mol photons m}^{-2}\text{s}^{-1}/20^{\circ}\text{C}$ for 30 minutes. Total RNA was isolated with Trizol as described previously [31]. Subsequently, DNA was removed with Qiagen RNeasy Mini Kit, and cDNA synthesis was performed in the presence of 0,2 mM aminoallyl-dUTP using anchored poly-dT

primer (Oligomer, Finland) and the reverse transcriptase SuperScript III (Invitrogen). The aminoallyl-labeled cDNA was purified with QIAquick PCR purification kit (Qiagen), and stained with CYTM Post-labeling Reactive Dye Pack (Amersham). cDNA corresponding to 15 pg of each dye was hybridized to the arrays in 50 % formamide, 5 x SSC, 0,1% SDS, 0,1 mg/ml herring sperm, 5 x Denhart's at 42°C over night.

The arrays were scanned with Agilent scanner (<http://agilent.com>) and the spot intensities were quantified with ScanArray Express Microarray Analysis system 2.0 (Perkin-Elmer Life Sciences, <http://las.perkinelmer.com/>). The data from three biological replicates was analyzed with GeneSpring 7.2. Genes with Student's t-test P value below 0,05 were chosen for further analysis. Gene annotation was derived from 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) (Fig. 1a). To confirm the absence of tAPX and sAPX in 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 1:2000 dilution was tested by performing an anti-APX immunoblot with increasing amounts of total foliar protein (Fig. 1b, left panel). Based on the results, loading of 10 µg of total protein was selected for further experimentation. sAPX and tAPX could be identified after biochemical separation of the chloroplast thylakoid and soluble fractions, as well as by a 5 kDa difference in molecular masses (Fig. 1b, central panel). The cytoplasmic APXs were identified on the basis of their presence in the soluble leaf extract and a molecular weight of 25 kDa. The antibody also recognized a 31-kDa protein, which copurified with thylakoid membranes, and may represent the putative luminal APX4 or the peroxisomal APX3

(Fig. 1b, central panel). Clearly, no accumulation of sAPX could be observed in leaf extracts isolated from *sapx* plants, nor could tAPX be observed in leaf extracts isolated from *tapx* plants (Fig. 1b, right panel). The *tapx sapx* double mutants were devoid of both tAPX and sAPX (Fig. 1b, right panel).

Photo-oxidative 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 photo-oxidative stress conditions. To this end, seedlings were germinated in the presence of 1.5 μ M methyl viologen under standard growth conditions (130 μ mol photons $\text{m}^{-2}\text{s}^{-1}/20^{\circ}\text{C}$). Methyl viologen extracts electrons from Photosystem I, and then reacts with molecular oxygen to produce $\text{O}_2^{\cdot-}$ and subsequently H_2O_2 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 (Fig. 2a). Interestingly, *sapx* plants exhibited an intermediate phenotype in the presence of methyl viologen, and both white and pale green seedlings developed (Fig. 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 Murashige and Skoog (MS) medium (Fig. 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 (Fig. 2a). On the contrary, 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 (data 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 (Figs 2a and b).

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 had lead to almost complete bleaching of mesophyll cell chloroplasts in the *sapx* single mutant and the *tapx sapx* double mutant plants (Fig. 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 (data 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 (Fig. 2b). Interestingly, despite of 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 (Fig. 2b). These results obtained with the *sapx* and *tapx sapx* seedlings imply that the guard cell and bundle sheath cell chloroplasts are more tolerant against photo-oxidative stress than the mesophyll cell chloroplasts (Figs 2a and b).

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

Next we examined whether expanded, four-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 μ M methyl viologen, incubated for 30 min under 50 μ mol photons $\text{m}^{-2}\text{s}^{-1}/20^{\circ}\text{C}$, and thereafter illuminated under 130 or 1000 μ mol photons $\text{m}^{-2}\text{s}^{-1}/20^{\circ}\text{C}$ for six hours. Illumination under growth light induced an ion leakage of similar extent for the wild type, *tapx* and *sapx* plants (Fig. 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 to wild type plants (Fig. 2c). On the contrary, *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 (Fig. 2c).

The high light sensitivity of four-week-old *tapx*, *sapx* 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 $\mu\text{mol photons m}^{-2}\text{s}^{-1}/20^{\circ}\text{C}$) for six hours. A gradual PSII photoinhibition of similar extent, measured as F_v/F_{max} after a 30 minute dark incubation, was recorded for the wild type and the single mutants *tapx* and *sapx* with increasing irradiance (Fig. 2d). In the *tapx sapx* double mutant, however, a significant decrease in the F_v/F_{max} ratio was observed under 1000 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ (Fig. 2d). However, when the plants were illuminated under 300 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ in the presence of lincomycin, which prevents the repair of PSII by inhibiting translation in chloroplasts, the wild type, *tapx*, *sapx* and *tapx sapx* double mutant plants were all equally sensitive to photoinhibition (Fig. 2d). Thus, the vulnerability of the *tapx sapx* double mutant plants to photoinhibition under 1000 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ in the absence of lincomycin presumably resulted from disturbances in the repair process of PSII.

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 two weeks under the control growth conditions of 130 $\mu\text{mol photons m}^{-2}\text{s}^{-1}/20^{\circ}\text{C}$, and thereafter shifted for two weeks to moderately high light (600 $\mu\text{mol photons m}^{-2}\text{s}^{-1}/20^{\circ}\text{C}$), to high light stress (1300 $\mu\text{mol photons m}^{-2}\text{s}^{-1}/28^{\circ}\text{C}$) or to low temperature (130 $\mu\text{mol photons m}^{-2}\text{s}^{-1}/10^{\circ}\text{C}$). 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 (Fig. 3a, Supplemental table 1). Indeed, the mutant and wild type plants showed similar morphological adaptations (Fig. 3a), and similar modulations in the content of chlorophyll (Supplemental table 1). Also the photochemical efficiency of PSII, expressed as F_v/F_{max} , was after two

weeks stress treatment almost the same as the control value of the wild type plants (Supplemental table 1.).

The similar long-term stress tolerance of WT, *tapx*, *sapx* and the *tapx sapx* double mutant plants (Fig. 3a, Supplemental table 1) raised a question 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 μ M methyl viologen, and the extent of cell death was measured after 24-hour treatment under standard growth conditions (130 μ mol photons $\text{m}^{-2}\text{s}^{-1}/20^{\circ}\text{C}$, 8/16 h light/dark period) (Fig. 3b). Clearly, all the lines grown under normal growth conditions wilted during the treatment (Figs. 3b and c). 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 (Fig. 3b). In sharp contrast to 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-hour illumination period (Figs. 3b and c).

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

Besides ascorbate peroxidases, chloroplast peroxiredoxins 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 (Fig. 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 two weeks under the control growth conditions of 130 μ mol photons $\text{m}^{-2}\text{s}^{-1}/20^{\circ}\text{C}$, and thereafter shifted for two weeks to moderately high light intensity (600 μ mol photons $\text{m}^{-2}\text{s}^{-1}$

$1/20^{\circ}\text{C}$), to high light stress ($1300\ \mu\text{mol photons m}^{-2}\text{s}^{-1}/28^{\circ}\text{C}$) or to low temperature ($130\ \mu\text{mol photons m}^{-2}\text{s}^{-1}/10^{\circ}\text{C}$). Then comparisons in the levels of H_2O_2 -detoxifying enzymes between the wild type, the *tapx* and *sapx* single mutants and the *tapx sapx* double mutant plants were made. These experiments also confirmed the absence of tAPX and/or sAPX in the respective mutants under all the experimental conditions explored (Figs. 4a and 4b).

Acclimation to increasing light intensities induced an increase in the steady-state level of the cytoplasmic APX in all the lines (Figs 4a and b). Notably, the high-light-stress-acclimated *tapx sapx* double mutant plants also showed an increased level of 2-cys peroxiredoxin as compared to the wild type plants (Fig. 4b). No distinct light-intensity-dependent modulations were observed for tAPX or sAPX (Figs 4a and b). On the contrary, in low-temperature-acclimated wild type and *tapx* plants, a strict up-regulation of sAPX occurred. Under these conditions, the amounts of peroxiredoxin IIE and also of 2-cys peroxiredoxin rather decreased (Fig. 4a). Low-temperature-acclimated *tapx* and *sapx* single mutants, however, maintained somewhat higher levels of these enzymes as compared to wild type plants (Fig. 4a). The 31 kDa polypeptide (depicted in Fig. 1) showed no distinct modulations under any of the conditions studied (data not shown). In conclusion, the absence of tAPX and/or sAPX induced no drastic compensatory increases in the steady-state levels of other ascorbate peroxidase isoforms. Moreover, only under high light stress, the absence of both tAPX and sAPX induced apparent compensatory increase in the steady-state level of 2-cys peroxiredoxin in the *tapx sapx* double mutant plants (Figs. 4a and b).

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 Lchb2 as well as the psaD and D1 proteins, representatives of the PSI and PSII core complexes, respectively, under normal growth conditions (Fig. 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, Lchb2 and psaD (Fig. 4c). The D1 protein, on the other hand, showed no consistent changes after two weeks light acclimation in either of the lines (Fig. 4c). Low temperature

induced no significant modulations in any of the polypeptides in either the wild type or the *tapx sapx* double mutant plants (Fig. 4c). Moreover, the contents of Rubisco large subunit remained quite invariable under all conditions explored (Fig. 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 $\mu\text{mol photons m}^{-2}\text{s}^{-1}/20^{\circ}\text{C}$) did not drastically differ from each other (data not shown). Modeling of photosynthetic parameters revealed quite similar values for maximal carboxylation rate of Rubisco (V_{cmax}) and for maximal photosynthetic electron transport rate (J_{max}) in the wild type and *tapx sapx* double mutant plants (Supplemental Table 2). Interestingly, however, the calculated parameters indicated reduced rate of mitochondrial respiration (R_{d}) in *tapx sapx* double mutant plants as compared to wild type plants (Supplemental Table 2).

Redox regulation of LHCII protein phosphorylation in *tapx sapx* double mutant plants

The redox conditions of chloroplasts are known to modulate signaling 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 signaling, 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 $\mu\text{mol photons m}^{-2}\text{s}^{-1}/20^{\circ}\text{C}$), the LHCII showed no drastic differences in the phosphorylation level between wild type and the *tapx sapx* double mutant plants (Fig. 4d). When the plants were shifted to higher light intensity (300 $\mu\text{mol photons m}^{-2}\text{s}^{-1}/20^{\circ}\text{C}$) for two hours, LHCII protein phosphorylation became down-regulated in the wild type plants. However, the LHCII of *tapx sapx* double mutant plants remained slightly more phosphorylated as compared wild type plants (Fig. 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 (Fig. 4d).

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

The altered regulation of LHCII protein phosphorylation in the *tapx sapx* double mutant plants (Fig. 4d) implied a role for chloroplast APXs in the fine-tuning of signaling 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 $\mu\text{mol photons m}^{-2}\text{s}^{-1}/20^{\circ}\text{C}$), and after a short 30 minute illumination under high light intensity (1000 $\mu\text{mol photons m}^{-2}\text{s}^{-1}/20^{\circ}\text{C}$). 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 (Supplemental Fig. 1). Exposure of the rosettes to a 30-minute high-light illumination period under 1000 $\mu\text{mol photons m}^{-2}\text{s}^{-1}/20^{\circ}\text{C}$ enhanced the accumulation of H₂O₂ to some extent in the wild type plants (Supplemental Fig. 1). In the leaves of *tapx sapx* double mutant plants, the accumulation of H₂O₂ was slightly more pronounced as compared to the wild type plants (Supplemental Fig. 1).

When the gene expression was analyzed under control growth conditions, only one gene, At1g26170 encoding an importin beta-2 subunit family protein, showed statistically significant (t-test p-value < 0,05) up-regulation in the *tapx sapx* double mutant as compared to wild type plants. On the contrary, distinctly lower transcript levels for 67 genes were observed in the *tapx sapx* double mutant as compared to wild type plants (Supplemental Fig. 1, Supplemental Table 3). Twelve of these genes, including *TAPX* and *SAPX*, were found to encode for chloroplast proteins (Supplemental Table 3). 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 to wild type plants (Supplemental table 3). Among these transcriptional regulators, At1g04400, which encodes the blue light photoreceptor cryptochrome 2 (CRY2), was down-regulated in the *tapx sapx* double mutant plants

(Supplemental table 3). 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 start to flower one or two days earlier than the wild type plants, whereas the *tapx sapx* double mutant plants show floral induction simultaneously with the wild type plants (data not shown).

The short 30-minute high-light illumination period induced further adjustments in the pattern of gene expression in the *tapx sapx* plants as compared to wild type plants (Supplemental Fig. 1, Supplemental Table 3; Sheet 2). The up-regulated genes included At5g55910, a chloroplastic member of the thus far poorly characterized Arabidopsis AGC family protein kinases (Supplemental Table 3; sheet 2). 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 SIMILAR TO RCD1 2 (SRO2). RCD1 and the SRO family members have crucial functions in the regulation of stress-responsive genes in Arabidopsis [35, 36]. Notably, despite of 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 to wild type plants (Supplemental Fig. 1, Supplemental Table 3). Interestingly, the down-regulated genes included a dynamin superfamily GTPase, At1g03160, which has been assigned crucial functions in the biogenesis of chloroplasts [37] (Supplemental Table 3, Sheet 2).

DISCUSSION

Chloroplast ascorbate peroxidases have been assigned a distinct role in scavenging reactive oxygen species 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 hydrogen peroxide (H_2O_2) by a thylakoid-associated Cu/Zn superoxide dismutase (Cu/Zn-SOD) [3, 4]. H_2O_2 in turn becomes enzymatically reduced to water by two distinct groups of water-water cycle enzymes, ascorbate peroxidases (APXs) and peroxiredoxins (PRXs), both of which comprise several isoforms [9]. Here we attempted to dissect the roles of the thylakoid-bound (tAPX) and the stromal (sAPX) ascorbate peroxidases in the antioxidative network of *Arabidopsis thaliana* as influenced by the different developmental stage and by the exposure of plants to stress conditions.

Stromal APX has a key role in oxidative stress tolerance during early greening phases

Arabidopsis seedlings lacking the stromal APX (sAPX) were sensitive to mild photo-oxidative stress, and produced pale green seedlings when germinated in the presence of methyl viologen in low concentration (Fig 2a). The single mutants lacking the thylakoid-bound APX (tAPX), on the contrary, developed like wild type plants (Fig. 2a). Nevertheless, the *tapx sapx* double mutant exhibited even more severe failure in the greening process than that observed for the *sapx* single mutant (Fig 2a), demonstrating the contribution of both tAPX and sAPX to the antioxidant defence in young seedlings. Thus, chloroplast APXs, and particularly the stromal APX, 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 alteration 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 (Fig. 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 (Supplemental Table 3.; [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 for 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 cotyledons of the *sapx* single mutants and the *tapx sapx* double mutants were less sensitive to oxidative stress than the mesophyll cell chloroplasts (Figs. 2a and b). Similarly, high-light illumination of a *tapx sapx vtc2* triple mutant deficient in chloroplast ascorbate peroxidases 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 sheath cell chloroplasts are the primary site for accumulation of hydrogen peroxide during high-light-illumination of leaves [44](see also Supplemental Fig. 1), and several genes related to plant antioxidant network seem to be preferentially expressed in the veinal regions in Arabidopsis leaves. These gene products include the cytoplasmic ascorbate peroxidase 2 [44], a microRNA (miR398) that targets Cu/Zn superoxide dismutase [45] and a chloroplastic glutaredoxin [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 (Figs. 2a and 2b; [42]).

Chloroplast APXs are crucial for photoprotection and signaling

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 (Fig 3; [42]) or under low temperature (Fig. 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 (Fig. 2d). An abrupt increase in excitation energy lead 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 (Fig. 2d). This corroborates with results obtained for transgenic tobacco plants over-expressing tAPX, which were capable of maintaining photosynthetic activity under a combination of short-term high light and low temperature stress [20]. These data 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 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, while 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 (Fig. 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 (Fig. 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 (Fig. 4d; [29]). Such inhibition can, however, be restored by hydrogen peroxide, which serves as an oxidant reactivates the LHCII kinase [48]. Thus, it is likely that minor accumulation of H_2O_2 in the chloroplasts of the *tapx sapx* double mutant plants upon a shift to higher light intensity maintained the LHCII kinase at more active state as compared to the wild type plants (Fig. 4d; [29]).

It is noticeable that both the chloroplast translation and the activity of the LHCII kinase have been connected to retrograde signaling, 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 to wild type plants in the level of H_2O_2 are reflected in the regulation of nuclear genes (Supplemental Fig. 1). Apparently due to only a low level of H_2O_2 production, no preferential up-regulation of genes generally regarded as hallmarks for oxidative stress responses [49, 50] was observed for the double mutant despite of the absence of both tAPX and sAPX in chloroplasts (Supplemental Table 3.). Rather, as deduced from the altered regulation of LHCII protein phosphorylation (Fig. 4d), the moderate accumulation of H_2O_2 in the *tapx sapx* double mutant plants modulates the thiol-redox- state of chloroplasts, which in turn is likely to modulate the retrograde signaling pathways from chloroplasts to the nucleus.

Functional redundancy between thylakoid-bound and stromal APXs

Among four-week-old plants, predominantly only the *tapx sapx* double mutant plants showed both the acceleration of high-light-induced decline in PSII activity (Fig. 2d) and the enhanced methyl viologen-induced cell death (Fig. 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 ascorbate peroxidases, H_2O_2 can be detoxified in chloroplasts also by peroxiredoxins, glutaredoxins, and glutathione peroxidases as well as by the low-molecular-weight antioxidants ascorbate and glutathione, both of which are present in millimolar concentrations in chloroplasts [2]. Indeed, the stress-acclimation and cross-tolerance of plants involve multiple H_2O_2

detoxification systems that efficiently prevent oxidative damage by controlling the level of H_2O_2 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 (Figs 3b and c) (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 MV-treatment was exceeded, contrary to that of the wild type plants and single mutants (Fig 3c). It is conceivable that the thylakoid-bound and stromal APXs operate in concert during the initial detoxification of H_2O_2 , and are capable of poising the H_2O_2 level unless the production of H_2O_2 suddenly exceeds the threshold level (Fig. 2). This interpretation is consistent with the observation that chloroplastic APXs become rapidly inactivated by high concentrations of H_2O_2 , 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 (Fig. 3a). Recently, Giacomelli et al. [42] showed that this is not due to compensatory accumulation of the low-molecular-weight ROS scavengers ascorbate, glutathione or alpha-tocopherol.

Our 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 peroxiredoxin (Fig. 4b), which exhibits over-lapping 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 for 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 (Fig. 4). These results suggest that upon prolonged high light exposure of plants,

both the chloroplastic peroxiredoxins and the cytoplasmic APXs have a crucial role in the removal of H_2O_2 that originates from photosynthetic processes [54].

The expression of genes encoding for different chloroplast PRXs has been shown to respond specifically to various environmental conditions and developmental stages, presumably due to complex regulation via overlapping signaling pathways [53, 55]. These regulatory pathways seem to involve highly specific chloroplast retrograde signaling systems, as the accumulation of 2-cys peroxiredoxin became specifically enhanced in high-light-stress-acclimated *tapx sapx* double mutant plants (Fig. 4b). The retrograde signaling 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 activities, signaling components and cellular regulators that ultimately determine the metabolic status of the cells [23, 54]. In this entirety, the chloroplast APXs seem to be indispensable upon the onset of environmental stresses, whereas various compensatory systems may take over during the long-term acclimation to contrasting environmental cues.

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REFERENCES

- 1 Dat, J., Vandenabeele, S., Vranova, E., Van Montagu, M., Inze, D. and Van Breusegem, F. (2000) Dual action of the active oxygen species during plant stress responses. *Cell Mol.Life Sci.* **57**, 779-795
- 2 Mittler, R., Vanderauwera, S., Gollery, M. and Van Breusegem, F. (2004) Reactive oxygen gene network of plants. *Trends Plant Sci.* **9**, 490-498
- 3 Asada, K. (1999) The water-water cycle in chloroplasts: Scavenging of active oxygens and dissipation of excess photons. *Annu.Rev.Plant Physiol.Plant Mol.Biol.* **50**, 601-639
- 4 Foyer, C. H., Lopez-Delgado, H., Dat, J. F. and Scott, I. M. (1997) Hydrogen peroxide- and glutathione-associated mechanisms of acclimatory stress tolerance and signalling. *Physiol.Pantarum.* **100**, 241-254
- 5 Dietz, K. J., Jacob, S., Oelze, M. L., Laxa, M., Tognetti, V., de Miranda, S. M., Baier, M. and Finkemeier, I. (2006) The function of peroxiredoxins in plant organelle redox metabolism. *J. Exp. Bot.* **57**, 1697-1709

- 6 Rouhier, N. and Jacquot, J. P. (2002) Plant peroxiredoxins: Alternative hydroperoxide scavenging enzymes. *Photosynth Res.* **74**, 259-268
- 7 Cheng, N., Liu, J., Brock, A., Nelson, R. S. and Hirschi, K. D. (2006) AtGRXcp, an arabidopsis chloroplastic glutaredoxin, is critical for protection against protein oxidative damage. *J. Biol. Chem.* **281**, 26280-26288
- 8 Milla, M. A. R., Maurer, A., Huete, A. R. and Gustafson, J. P. (2003) Glutathione peroxidase genes in arabidopsis are ubiquitous and regulated by abiotic stresses through diverse signaling pathways. *Plant J.* **36**, 602-615
- 9 Shigeoka, S., Ishikawa, T., Tamoi, M., Miyagawa, Y., Takeda, T., Yabuta, Y. and Yoshimura, K. (2002) Regulation and function of ascorbate peroxidase isoenzymes. *J. Exp. Bot.* **53**, 1305-1319
- 10 Chew, O., Whelan, J. and Millar, A. H. (2003) Molecular definition of the ascorbate-glutathione cycle in arabidopsis mitochondria reveals dual targeting of antioxidant defenses in plants. *J. Biol. Chem.* **278**, 46869-46877
- 11 Kieselbach, T., Bystedt, M., Hynds, P., Robinson, C. and Schroder, W. P. (2000) A peroxidase homologue and novel plastocyanin located by proteomics to the arabidopsis chloroplast thylakoid lumen. *FEBS Lett.* **480**, 271-276
- 12 Yoshimura, K., Yabuta, Y., Ishikawa, T. and Shigeoka, S. (2002) Identification of a cis element for tissue-specific alternative splicing of chloroplast ascorbate peroxidase pre-mRNA in higher plants. *J. Biol. Chem.* **277**, 40623-40632
- 13 Santos, M., Gousseau, H., Lister, C., Foyer, C., Creissen, G. and Mullineaux, P. (1996) Cytosolic ascorbate peroxidase from arabidopsis thaliana L. is encoded by a small multigene family. *Planta.* **198**, 64-69
- 14 Narendra, S., Venkataramani, S., Shen, G., Wang, J., Pasapula, V., Lin, Y., Korniyev, D., Holaday, A. S. and Zhang, H. (2006) The arabidopsis ascorbate peroxidase 3 is a peroxisomal membrane-bound antioxidant enzyme and is dispensable for arabidopsis growth and development. *J. Exp. Bot.* **57**, 3033-3042

- 15 Shi, W. M., Muramoto, Y., Ueda, A. and Takabe, T. (2001) Cloning of peroxisomal ascorbate peroxidase gene from barley and enhanced thermotolerance by overexpressing in *arabidopsis thaliana*. *Gene*. **273**, 23-27
- 16 Bunkelmann, J. R. and Trelease, R. N. (1996) Ascorbate peroxidase. A prominent membrane protein in oilseed glyoxysomes. *Plant Physiol.* **110**, 589-598
- 17 Jespersen, H. M., Kjaersgård, I. V., Ostergaard, L. and 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. *Biochem. J.* **326**, 305-310
- 18 Broin, M., Cuine, S., Eymery, F. and Rey, P. (2002) The plastidic 2-cysteine peroxiredoxin is a target for a thioredoxin involved in the protection of the photosynthetic apparatus against oxidative damage. *Plant Cell*. **14**, 1417-1432
- 19 Danna, C. H., Bartoli, C. G., Sacco, F., Ingala, L. R., Santa-Maria, G. E., Guamet, J. J. and Ugalde, R. A. (2003) Thylakoid-bound ascorbate peroxidase mutant exhibits impaired electron transport and photosynthetic activity. *Plant Physiol.* **132**, 2116-2125
- 20 Yabuta, Y., Motoki, T., Yoshimura, K., Takeda, T., Ishikawa, T. and Shigeoka, S. (2002) Thylakoid membrane-bound ascorbate peroxidase is a limiting factor of antioxidative systems under photo-oxidative stress. *Plant J.* **32**, 915-925
- 21 Murgia, I., Tarantino, D., Vannini, C., Bracale, M., Carravieri, S. and Soave, C. (2004) *Arabidopsis thaliana* plants overexpressing thylakoidal ascorbate peroxidase show increased resistance to paraquat-induced photooxidative stress and to nitric oxide-induced cell death. *Plant J.* **38**, 940-953
- 22 Miyake, C., Shinzaki, Y., Nishioka, M., Horiguchi, S. and Tomizawa, K. (2006) Photoinactivation of ascorbate peroxidase in isolated tobacco chloroplasts: *Galdieria partita* APX maintains the electron flux through the water-water cycle in transplastomic tobacco plants. *Plant Cell Physiol.* **47**, 200-210

- 23 Miller, G., Suzuki, N., Rizhsky, L., Hegie, A., Koussevitzky, S. and Mittler, R. (2007) Double mutants deficient in cytosolic and thylakoid ascorbate peroxidase reveal a complex mode of interaction between reactive oxygen species, plant development, and response to abiotic stresses. *Plant Physiol.* **144**, 1777-1785
- 24 Alonso, J. M., Stepanova, A. N., Leisse, T. J., Kim, C. J., Chen, H., Shinn, P., Stevenson, D. K., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C. C., Parker, H., Prednis, L., Ansari, Y., Choy, N., Deen, H., Geralt, M., Hazari, N., Hom, E., Karnes, M., Mulholland, C., Ndubaku, R., Schmidt, I., Guzman, P., Aguilar-Henonin, L., Schmid, M., Weigel, D., Carter, D. E., Marchand, T., Risseuw, E., Brogden, D., Zeko, A., Crosby, W. L., Berry, C. C. and Ecker, J. R. (2003) Genome-wide insertional mutagenesis of *arabidopsis thaliana*. *Science.* **301**, 653-657
- 25 Becker, B., Holtgreffe, S., Jung, S., Wunrau, C., Kandlbinder, A., Baier, M., Dietz, K. J., Backhausen, J. E. and Scheibe, R. (2006) Influence of the photoperiod on redox regulation and stress responses in *arabidopsis thaliana* L. (heynh.) plants under long- and short-day conditions. *Planta.* **224**, 380-393
- 26 Farquhar, G. D., von Caemmerer, S. and Berry, J. A. (1980) A biochemical model of photosynthetic CO₂ assimilation in leaves of C₃ species. *Planta.* **149**, 78-90
- 27 Porra, R. J., Thompson, W. A. and Kriedemann, P. E. (1989) Determination of accurate extinction coefficients and simultaneous-equations for assaying chlorophyll-a and chlorophyll-b extracted with 4 different solvents – verification of the concentration of chlorophyll standards by atomic-absorption spectroscopy. *Biochim.Biophys.Acta.* **975**, 384-394
- 28 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227**, 680-685
- 29 Rintamäki, E., Martinsuo, P., Pursiheimo, S. and Aro, E. M. (2000) Cooperative regulation of light-harvesting complex II phosphorylation via the

plastoquinol and ferredoxin-thioredoxin system in chloroplasts.

Proc.Natl.Acad.Sci.U.S.A. **97**, 11644-11649

- 30 Thordal-Christensen, H., Zhang, Z., Wei, Y. and Collinge, D. B. (1997) Subcellular localization of H₂O₂ in plants. H₂O₂ accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. *Plant J.* **11**, 1187-1194
- 31 Piippo, M., Allahverdiyeva, Y., Paakkanen, V., Suoranta, U. M., Battchikova, N. and Aro, E. M. (2006) Chloroplast-mediated regulation of nuclear genes in arabidopsis thaliana in the absence of light stress. *Physiol.Genomics.* **25**, 142-152
- 32 Nott, A., Jung, H., Koussevitzky, S. and Chory, J. (2006) PLASTID-TO-NUCLEUS RETROGRADE SIGNALING. *Annu.Rev.Plant.Biol.* **57**, 739-759
- 33 Endo, M., Mochizuki, N., Suzuki, T. and Nagatani, A. (2007) CRYPTOCHROME2 in vascular bundles regulates flowering in arabidopsis. *Plant Cell.* **19**, 84-93
- 34 Bögre, L., Ökrész, L., Henriques, R. and Anthony, R. G. (2003) Growth signalling pathways in arabidopsis and the AGC protein kinases. *Trends Plant Sci.* **8**, 424-431
- 35 Ahlfors, R., Lang, S., Overmyer, K., Jaspers, P., Brosche, M., Tauriainen, A., Kollist, H., Tuominen, H., Belles-Boix, E., Piippo, M., Inze, D., Palva, E. T. and Kangasjarvi, J. (2004) Arabidopsis RADICAL-INDUCED CELL DEATH1 belongs to the WWE protein-protein interaction domain protein family and modulates abscisic acid, ethylene, and methyl jasmonate responses. *Plant Cell.* **16**, 1925-1937
- 36 Borsani, O., Zhu, J., Verslues, P. E., Sunkar, R. and Zhu, J. (2005) Endogenous siRNAs derived from a pair of natural cis-antisense transcripts regulate salt tolerance in arabidopsis. *Cell.* **123**, 1279-1291

- 37 Gao, H., Sage, T. L. and Osteryoung, K. W. (2006) FZL, an FZO-like protein in plants, is a determinant of thylakoid and chloroplast morphology. *Proc.Natl.Acad.Sci.U.S.A.* **103**, 6759-6764
- 38 Lee, S. H., Ahsan, N., Lee, K. W., Kim, D. H., Lee, D. G., Kwak, S. S., Kwon, S. Y., Kim, T. H. and Lee, B. H. (2007) Simultaneous overexpression of both CuZn superoxide dismutase and ascorbate peroxidase in transgenic tall fescue plants confers increased tolerance to a wide range of abiotic stresses. *J.Plant Physiol.* **164**, 1626-1638.
- 39 Yu, F., Fu, A., Alutu, M., Park, S., Xu, Y., Liu, H., Liu, X., Foudree, A., Nambogga, M. and Rodermeel, S. (2007) Variegation mutants and mechanisms of chloroplast biogenesis. *Plant, Cell Environ.* **30**, 350-365
- 40 Kanervo, E., Singh, M., Suorsa, M., Paakkarinen, V., Aro, E., Battchikova, N. and Aro, E. -. (2008) Expression of protein complexes and individual proteins upon transition of etioplasts to chloroplasts in pea (*pisum sativum*). *Plant Cell Physiol.* **in Press**
- 41 Pena-Ahumada, A., Kahmann, U., Dietz, K. J. and Baier, M. (2006) Regulation of peroxiredoxin expression versus expression of halliwell-asada-cycle enzymes during early seedling development of *arabidopsis thaliana*. *Photosynth Res.* **89**, 99-112
- 42 Giacomelli, L., Masi, A., Ripoll, D. R., Lee, M. J. and van Wijk, Klaas J. (2007) *Arabidopsis thaliana* deficient in two chloroplast ascorbate peroxidases shows accelerated light-induced necrosis when levels of cellular ascorbate are low. *Plant Mol.Biol.* **65**, 627-644
- 43 Mullineaux, P. M., Karpinski, S. and Baker, N. R. (2006) Spatial dependence for hydrogen peroxide-directed signaling in light-stressed plants. *Plant Physiol.* **141**, 346-350
- 44 Fryer, M. J., Ball, L., Oxborough, K., Karpinski, S., Mullineaux, P. M. and Baker, N. R. (2003) Control of ascorbate peroxidase 2 expression by hydrogen

- peroxide and leaf water status during excess light stress reveals a functional organisation of arabidopsis leaves. *Plant J.* **33**, 691-705
- 45 Sunkar, R., Kapoor, A. and Zhu, J. K. (2006) Posttranscriptional induction of two Cu/Zn superoxide dismutase genes in arabidopsis is mediated by downregulation of miR398 and important for oxidative stress tolerance. *Plant Cell.* **18**, 2051-2065
- 46 Nishiyama, Y., Allakhverdiev, S. I. and Murata, N. (2006) A new paradigm for the action of reactive oxygen species in the photoinhibition of photosystem II. *Biochim.Biophys.Acta.* **1757**, 742-749
- 47 Nishiyama, Y., Yamamoto, H., Allakhverdiev, S. I., Inaba, M., Yokota, A. and Murata, N. (2001) Oxidative stress inhibits the repair of photodamage to the photosynthetic machinery. *EMBO J.* **20**, 5587-5594
- 48 Martinsuo, P., Pursiheimo, S., Aro, E. M. and Rintamäki, E. (2003) Dithiol oxidant and disulfide reductant dynamically regulate the phosphorylation of light-harvesting complex II proteins in thylakoid membranes. *Plant Physiol.* **133**, 37-46
- 49 Gadjev, I., Vanderauwera, S., Gechev, T. S., Laloi, C., Minkov, I. N., Shulaev, V., Apel, K., Inze, D., Mittler, R. and Van Breusegem, F. (2006) Transcriptomic footprints disclose specificity of reactive oxygen species signaling in arabidopsis. *Plant Physiol.* **141**, 436-445
- 50 Ma, S. and Bohnert, H. J. (2007) Integration of arabidopsis thaliana stress-related transcript profiles, promoter structures, and cell-specific expression. *Genome Biol.* **8**, R49
- 51 Mano, J., Ohno, C., Domae, Y. and Asada, K. (2001) Chloroplastic ascorbate peroxidase is the primary target of methylviologen-induced photooxidative stress in spinach leaves: Its relevance to monodehydroascorbate radical detected with in vivo ESR. *Biochim.Biophys.Acta.* **1504**, 275-287

- 52 Shikanai, T., Takeda, T., Yamauchi, H., Sano, S., Tomizawa, K. I., Yokota, A. and Shigeoka, S. (1998) Inhibition of ascorbate peroxidase under oxidative stress in tobacco having bacterial catalase in chloroplasts. *FEBS Lett.* **428**, 47-51
- 53 Baier, M., Noctor, G., Foyer, C. H. and Dietz, K. J. (2000) Antisense suppression of 2-cysteine peroxiredoxin in arabidopsis specifically enhances the activities and expression of enzymes associated with ascorbate metabolism but not glutathione metabolism. *Plant Physiol.* **124**, 823-832
- 54 Davletova, S., Rizhsky, L., Liang, H., Shengqiang, Z., Oliver, D. J., Coutu, J., Shulaev, V., Schlauch, K. and Mittler, R. (2005) Cytosolic ascorbate peroxidase 1 is a central component of the reactive oxygen gene network of arabidopsis. *Plant Cell.* **17**, 268-281
- 55 Horling, F., Lamkemeyer, P., Konig, J., Finkemeier, I., Kandlbinder, A., Baier, M. and Dietz, K. J. (2003) Divergent light-, ascorbate-, and oxidative stress-dependent regulation of expression of the peroxiredoxin gene family in arabidopsis. *Plant Physiol.* **131**, 317-325
- 56 Koussevitzky, S., Nott, A., Mockler, T. C., Hong, F., Sachetto-Martins, G., Surpin, M., Lim, J., Mittler, R. and Chory, J. (2007) Signals from chloroplasts converge to regulate nuclear gene expression. *Science.* **316**, 715-719

FIGURE LEGENDS

Figure 1. Characteristics of *tapx* and *sapx* single mutant and *tapx sapx* double mutant lines.

- (a) Molecular structures and T-DNA insertion sites of *TAPX* (At1g77490; SALK_027804) and *SAPX* (At4g08390; SALK_083737) genes. T-DNA insertion sites are indicated with triangles, and black and gray boxes represent exons and untranslated regions, respectively.
- (b) Immunoblots with Arabidopsis anti-APX antibody. The left panel demonstrates the linear range of the Arabidopsis APX antibody. The middle panel depicts the APX content of isolated thylakoids and the soluble leaf extract. The right-hand panel confirms the absence of tAPX, sAPX or both in total leaf extracts isolated from *tapx*, *sapx* and the *tapx sapx* double mutant. DM, *tapx sapx* double mutant; WT, wild type; tAPX, thylakoid bound APX; sAPX, stromal APX; cAPX, cytoplasmic APX.

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 $\mu\text{mol photons m}^{-2}\text{s}^{-1}/20^{\circ}\text{C}$. The confocal images are projections of chlorophyll fluorescence from 30 μm stacks of sequential images starting from the abaxial side of the cotyledons. The scale bars correspond to 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 $\mu\text{mol photons m}^{-2}\text{s}^{-1}/20^{\circ}\text{C}$. The confocal images are projections of 50 μm stacks of sequential images starting from the abaxial side of the cotyledons. The scale bars correspond to 100 μm .
- (c) Methyl viologen -induced photo-oxidative stress in four-week old plants. Plants were sprayed with 50 μM methyl viologen and illuminated under 130 or 1000 $\mu\text{mol photons m}^{-2}\text{s}^{-1}/20^{\circ}\text{C}$ for six hours 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 \pm SD of four independent experiments. **, $P < 0,05$; ***, $P < 0,005$ in Student's T-test.

(d) Photoinhibition of PSII in four-week-old plants under various irradiance levels. Plants were exposed to increasing light intensities for six hours, and the photochemical efficiency of PSII was measured as F_v/F_{max} after a 30 minute dark incubation. The light and temperature conditions are indicated in the figure. The lincomycin-treated plants were illuminated under $300 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ for two hours. The values are means \pm SD of at least three independent experiments. **, $P < 0,05$; ***, $P < 0,005$ in Student's T-test. WT, wild type; MV, methyl viologen; LM, lincomycin.

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 two-week acclimation period under high light, high light stress or low temperature. Plants were grown for two weeks under normal growth conditions, and thereafter shifted for another two 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 $50 \mu\text{M}$ methyl viologen and illuminated under $130 \mu\text{mol photons m}^{-2}\text{s}^{-1}/20^\circ\text{C}$ for 24 hours prior to measurement of ion leakage from the leaves. The values are means \pm SD of four independent experiments. ***, $P < 0,005$ in Student's T-test.
- (c) Photograph depicting the tolerance of high-light-acclimated wild type plants and sensitivity of *tapx sapx* double mutant plants to methyl viologen-induced cell death.

Figure 4. Modulations in the steady-state levels of H_2O_2 -detoxifying enzymes, photosynthetic proteins and LHCII protein phosphorylation under contrasting environmental cues.

- (a) Immunoblots depicting the levels of H_2O_2 -detoxifying enzymes in the *tapx* and *sapx* single mutants and wild type plants.
- (b) Immunoblots depicting the levels of H_2O_2 -detoxifying enzymes in the *tapx sapx* double mutants (DM) and wild type plants.

(c) Immunoblots depicting the levels of photosynthetic proteins in the *tapx sapx* double mutants (DM) and wild type plants.

(d) Phosphothreonine-specific immunoblot showing thylakoid protein phosphorylation in proteins in the *tapx sapx* double mutants (DM) and wild type plants.

For experiments shown in (a-c), the plants were grown for two weeks under normal growth conditions, and thereafter shifted for another two 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 higher light intensity for two hours. DM, *tapx sapx* double mutant; tAPX, thylakoid APX; sAPX, stromal APX; cAPX, cytoplasmic APX; 2-CP, chloroplastic 2-cysteine peroxiredoxins A and B; PrxE, peroxiredoxin IIE; PrxQ, peroxiredoxin Q; D1 and psaD, representatives of PS II and PS I 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.

Fig. 1

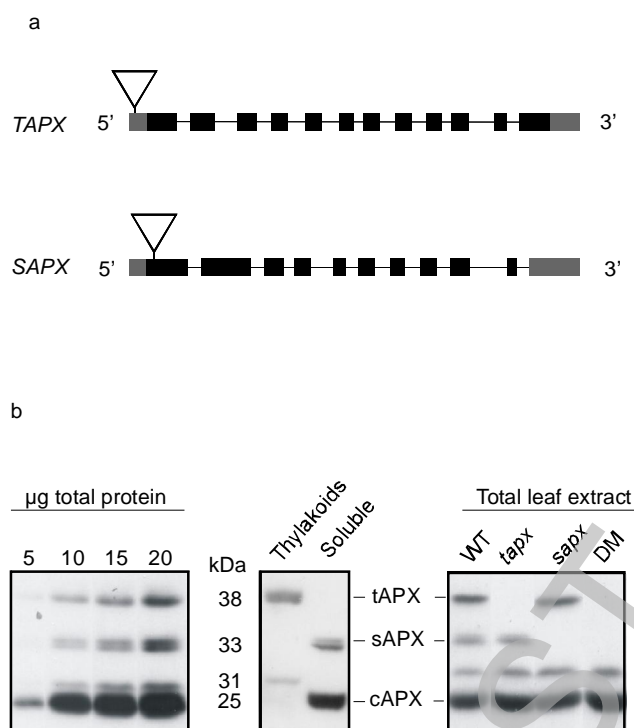


Fig. 2

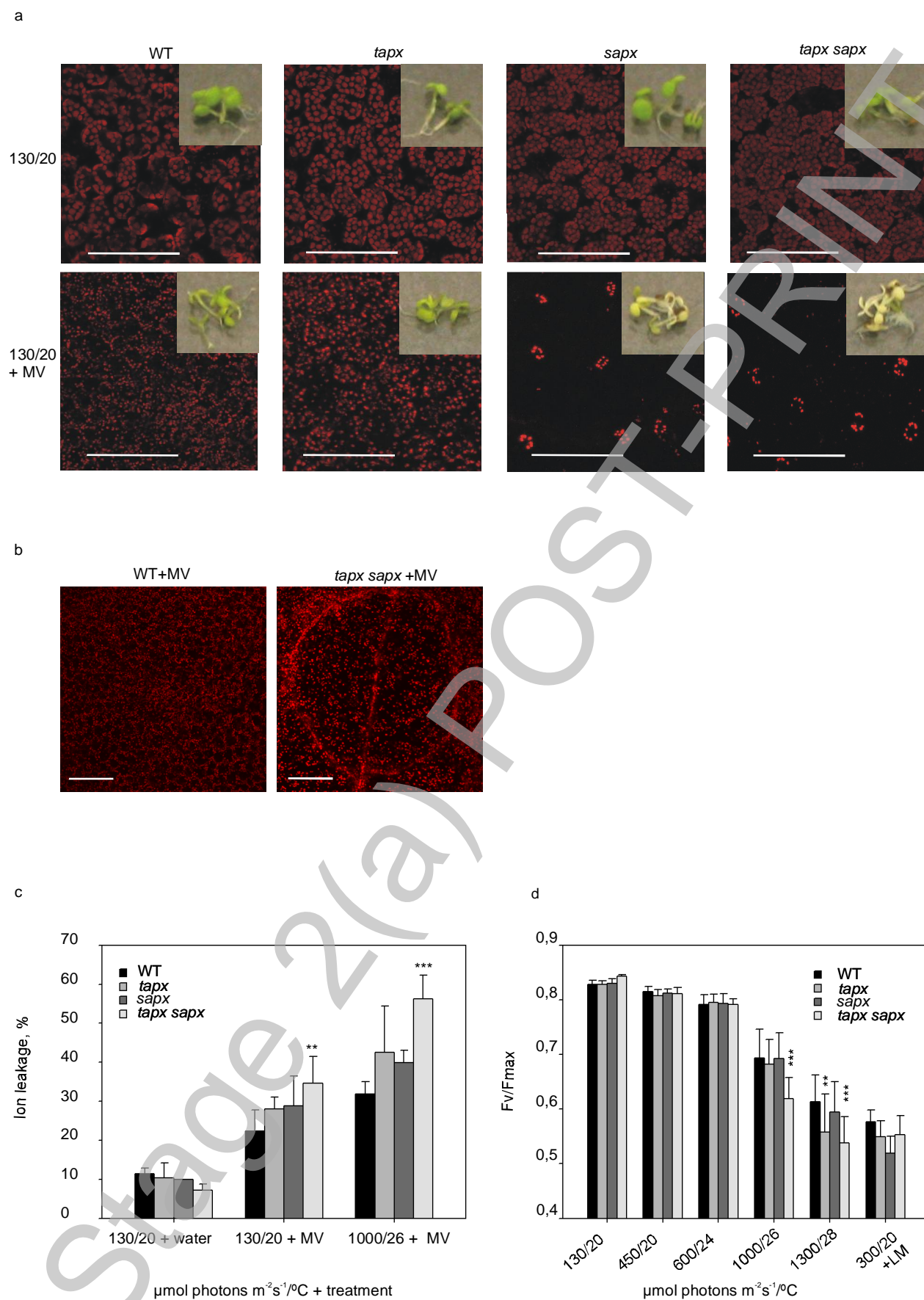


Fig. 3

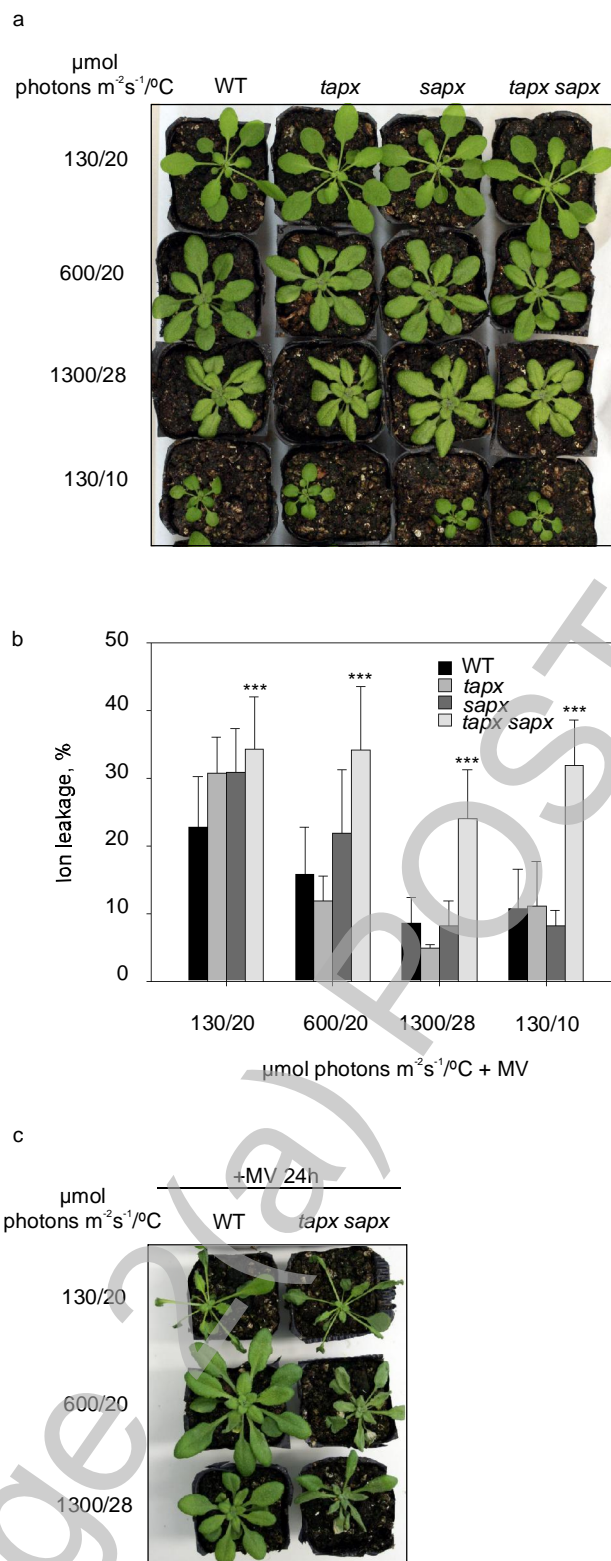


Fig. 4

