Running Title:
2-Cys peroxiredoxin function

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2-Cys peroxiredoxins and thylakoid ascorbate peroxidase create a water-water cycle that is essential to protect the photosynthetic apparatus under high light stress conditions

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2-Cys peroxiredoxins and thylakoid ascorbate peroxidase act together to protect plants against high light damage by creating a water-water cycle and by restricting light-induced redox signaling.
Footnotes

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ABSTRACT
Different peroxidases, including 2-Cys peroxiredoxins (2-Cys PRX) and thylakoid ascorbate peroxidase (tAPX), have been proposed to be involved in the water-water cycle and H$_2$O$_2$-mediated signaling in plastids. We generated an Arabidopsis thaliana double mutant line deficient in the two plastid 2-Cys PRX (2cpa 2cpb) and a triple mutant deficient in 2-Cys PRX and tAPX (2cpa 2cpb tapx). In contrast to wild type and tapx single knockout plants, 2cpa 2cpb double knock-out plants showed an impairment of photosynthetic efficiency and became photobleached under high light growth conditions. In addition, double mutant plants also generated elevated levels of superoxide anion radicals, hydrogen peroxide and carbonylated proteins but lacked anthocyanin accumulation under high light stress conditions. Under high light conditions, 2-Cys PRX appear to be essential in maintaining the water-water cycle while tAPX is dispensable. By comparison, this high light sensitive phenotype was more severe in 2cpa 2cpb tapx triple mutant plants indicating that tAPX partially compensates for the loss of functional 2-Cys PRX by mutation or inactivation by over-oxidation. In response to high light, H$_2$O$_2$- and photooxidative stress-responsive marker genes were found to be dramatically up-regulated in 2cpa 2cpb tapx but not in 2cpa 2cpb mutant plants suggesting that high light-induced plastid to nucleus retrograde photooxidative stress signaling takes place after loss or inactivation of both water-water cycle enzymes, 2-Cys PRX and tAPX.
INTRODUCTION

Plants are frequently exposed to different abiotic stresses including high light, UV irradiation, heat, cold and drought. A component common to these stresses is the rapid formation of reactive oxygen species (ROS) as the result of metabolic dysbalances. A major ROS produced under moderate light and, in particular, under high light photooxidative stress conditions was shown to be singlet oxygen, $^1\text{O}_2$, that is produced in illuminated chloroplasts predominantly at the photosystem II (PSII) (Triantaphylides et al., 2008). Most of the singlet oxygen is quenched by carotenoids and tocopherols or reacts with galactolipids in thylakoid membranes, yielding galactolipid hydroperoxides (Zoeller et al., 2012; Farmer and Mueller, 2013). In addition, superoxide radicals, $\text{O}_2^-$, are produced predominantly at the PSI and rapidly dismutate to hydrogen peroxide ($\text{H}_2\text{O}_2$) either spontaneously or catalyzed by superoxide dismutase (SOD). Hence, lipid peroxides and hydrogen peroxide are produced close to the photosystems and may damage thylakoid proteins. In this context, 2-Cys PRX enzymes have been implicated in the reductive detoxification of lipid peroxides and hydrogen peroxide (Konig et al., 2002).

During photosynthesis, light energy absorbed by PSII is used to split water molecules and the electrons are channeled from PSII via PSI to ferredoxin (Fd). As a result, electrons flow from water to Fd. The main electron sink reaction is the Fd-NADP oxidoreductase (FNR) catalyzed production of NADPH that functions as electron donor to reduce CO$_2$ to sugars. Under high light conditions, excessive excitation energy is dissipated into heat, as indicated by non-photochemical quenching of chlorophyll fluorescence. In addition, excessive photosynthetic electrons can be donated from PSI to O$_2$ yielding O$_2^-$ (Miyake, 2010). This process, the Mehler reaction, creates an alternative electron sink and electron flow. Superoxide anion radicals, O$_2^-$, can be dismutated to O$_2$ and H$_2$O$_2$ by a thylakoid-attached copper/zinc superoxide dismutase (Cu/ZnSOD) (Rizhsky et al., 2003). H$_2$O$_2$ can then be reduced to water by peroxidases. As a result, O$_2$ molecules originating from the water splitting process at PSII are reduced to water by electrons originating from PSI. This process is termed the water-water cycle (WWC) that is thought to protect the photosynthetic apparatus from excessive light and to alleviate photoinhibition.

In the classical WWC, the Mehler-ascorbate peroxidase (MAP) pathway, ascorbate peroxidases (APX) have been considered as key enzymes in the reductive detoxification of H$_2$O$_2$ in
chloroplasts (Kangasjarvi et al., 2008). Ascorbate peroxidases reduce H$_2$O$_2$ to water and oxidize ascorbate to monodehydroascorbate radicals (MDA). NADPH functions as an electron donor to regenerate ascorbate by MDA reductase. There are two functional APX homologs in plastids: a 33 kDa stromal APX (sAPX) and a 38 kDa tAPX (thylakoid-bound APX). The latter tAPX is thought to reside close to the site of H$_2$O$_2$ generation at PSI. Surprisingly, KO-tAPX mutants as well as double mutants lacking both the tAPX and the stromal sAPX exhibited no visible symptoms of stress after long-term (1 to 14d) high light (1.000 µmol photons m$^{-2}$s$^{-1}$) exposure (Giacomelli et al., 2007; Kangasjarvi et al., 2008; Maruta et al., 2010). Moreover, the photosynthetic efficiency of PSII (as judged by the F$_v$/F$_m$ ratios), H$_2$O$_2$ production, antioxidant levels (ascorbate, glutathione and tocopherols), protein oxidation and anthocyanin accumulation were similar between light-stressed mutant and wild type plants. Hence, other H$_2$O$_2$ detoxification mechanisms can efficiently compensated for the lack of the stromal and thylakoid APX detoxification system.

In addition to APX, glutathione peroxidases (GPX) and peroxiredoxins (PRX) may reduce H$_2$O$_2$ to H$_2$O. It has been postulated that in the chloroplast two highly homologous thylakoid-associated 2-Cys PRX A and B can create an ‘alternative’ ascorbate-independent WWC (Dietz et al., 2006). In support of this concept, high light stress-acclimated tapx sapx double-mutant plants showed increased levels of 2-Cys PRX as compared to wild type plants (Kangasjarvi et al., 2008). Since the two plastidial 2-Cys PRX A and B (2-CPA and 2-CPB) dynamically interact with the stromal side of thylakoid membranes and are capable of reducing peroxides, 2-Cys PRX enzymes may be involved in both H$_2$O$_2$ detoxification and reduction of lipid peroxides in thylakoids (Konig et al., 2002).

The reaction mechanism of 2-Cys PRX is highly conserved and involves a cysteine residue which becomes transiently oxidized to sulphenic acid (termed the peroxidatic Cys residue) thereby reducing H$_2$O$_2$ to water. The sulphenic acid is subsequently attacked by a second cysteine residue, termed resolving Cys residue, yielding an intermolecular disulfide bridge and water (Dietz, 2011).

At high peroxide concentrations, the peroxidase function of 2-Cys PRX becomes inactivated through overoxidation, and excess H$_2$O$_2$ may function as redox signal (Puerto-Galan et al., 2013).
It has been postulated that 2-Cys PRX function as a floodgate that allows H$_2$O$_2$ signaling only under oxidative stress conditions (Wood et al., 2003; Dietz, 2011; Puerto-Galan et al., 2013). In addition to its function as peroxidases, 2-Cys PRX may also serve as proximity-based thiol oxidases and chaperones (Konig et al., 2013).

The genome of A. thaliana contains two 2-Cys PRX (2CP) genes. In order to study 2-Cys PRX function, transgenic plants with reduced 2-Cys PRX levels were generated by antisense suppression (Baier et al., 2000) as well as by crossing of T-DNA insertion mutants (Pulido et al., 2010). The T-DNA insertion double mutant was shown to contain less than 5% of the wild type content of 2CPA and no 2CPB. Hence, full knock out lines lacking both 2-Cys PRX have not yet been established. Under standard growth conditions, 2-Cys PRX double mutants (similar to plastid APX-deficient plants) did also not show a photooxidative stress phenotype that might be due to compensation by alternative H$_2$O$_2$ reduction systems (Pulido et al., 2010). Due to the lack of a clear phenotype of the 2-Cys PRX double knock-down mutant under moderate light conditions, the physiological function of 2-Cys PRX A and B remains to be elucidated.

The main aim of this study was to identify the physiological function of 2-Cys PRX A and B under high light stress conditions when the WWC is of particular importance in protecting the photosynthetic apparatus from photooxidative damage. We investigated mutants completely deficient in 2-Cys PRX (2cpa 2cpb) or tAPX (tapx) and, in addition, 2cpa 2cpb tapx triple knock-out plants to study the extent of the functional overlap between these enzymes. Results suggest that 2-Cys PRX are involved in a 2-Cys PRX-dependent WWC that appears to be more important in protecting the photosynthetic apparatus than the tAPX-dependent WWC, the MAP cycle.
RESULTS

High light exposure of 2cpa 2cpb double and 2cpa 2cpb tapx triple knockout mutants causes photosynthetic impairment and photobleaching

To clarify the specific functional role of 2-Cys PRX in comparison to tAPX in protecting the photosynthetic tissue from photooxidative damage, we generated a double knockout mutant 2cpa 2cpb of Arabidopsis thaliana, completely lacking the plastidial 2-Cys PRX homologues A and B (Supplemental Fig. S1), and compared its high light stress responses with those of a mutant line (tapx) which does not express tAPX (Maruta et al., 2010). In addition, a triple knockout mutant line, 2cpa 2cpb tapx, lacking both 2-Cys PRXs and tAPX, was generated to study the effect of the loss of both membrane-associated peroxide-detoxification systems.

The 2cpa 2cpb double and the 2cpa 2cpb tapx triple mutants were selected on Murashige and Skoog (MS) medium containing 1% (w/v) sucrose. Unlike wild type plants as well as single 2cpa and 2cpb mutant lines, cotyledons of the 2cpa 2cpb double and 2cpa 2cpb tapx triple mutants bleached and seedlings remained small during early development under these growth conditions. Only four plants of 201 individuals of a segregating 2cpa 2cpb progeny survived. Germination and growth of the double and triple mutants on MS medium without sugar or supplemented with 3% (w/v) sorbitol was similar to the wild type and the tapx mutant line (Supplemental Fig. S2). It is therefore likely that growth on sucrose rather than osmotic stress is responsible for this phenotype.

Mutant plants completely deficient in 2-Cys PRX (2cpa 2cpb) or in both 2-Cys PRX and tAPX (2cpa 2cpb tapx) grown on soil showed growth retardation while the tapx mutant grew like wild type (Fig. 1A). In 2cpa 2cpb plants, growth retardation was more pronounced under long day compared to short day conditions (Supplemental Fig. S3). Under standard growth conditions, 2cpa 2cpb mutant plants displayed a pale-green leaf phenotype with reduced chlorophyll content, the latter of which was also previously noted in the 2-Cys PRX double knock-down mutant (Pulido et al., 2010). Compared to the wild type, the ratio of chlorophyll a to chlorophyll b was not significantly different in the 2-Cys PRX double mutant (Supplemental Figure S3).
Under high light (HL, 900 µmol m\(^{-2}\) s\(^{-1}\)) conditions, leaves of wild type and tapx plants showed no photooxidative damage while extensive photobleached lesions at the edges of older leaves were observed already after 1 day of high light treatment in the 2cpa 2cpb tapx triple mutant and in 2cpa 2cpb double mutant leaves after 2 days of high light exposure (Fig. 1A).

To assess HL-induced photoinhibition of PS II, the maximal quantum yield after dark acclimation \((F_v/F_m)\) was determined using chlorophyll fluorescence measurements in the dark with a pulse-amplitude modulation (PAM) fluorometer by implementing the saturation pulse method (Schreiber, 2004). In plants grown under moderate light (160 µmol m\(^{-2}\) s\(^{-1}\), ML), the \(F_v/F_m\) ratio (0.77) did not differ between the wild type and all mutant lines. After 1 d exposure to high light (900 µmol m\(^{-2}\) s\(^{-1}\), HL), the \(F_v/F_m\) ratio of the wild type and the tapx mutant declined to 0.60 while the \(F_v/F_m\) ratio of the 2cpa 2cpb and the 2cpa 2cpb tapx mutant decreased to 0.44 and 0.40, respectively. The maximal quantum yield of PSII did not further decrease in the wild type and the tapx mutant after 2 d of high light. In contrast, the \(F_v/F_m\) ratio of the 2cpa 2cpb and the 2cpa 2cpb tapx mutant further decreased to 0.36 and 0.06, respectively, after 2 d of high light treatment (Fig. 1B), indicating that the PS II of the 2cpa 2cpb double and the 2cpa 2cpb tapx triple mutants is more sensitive to high light stress than that of the wild type.

Ultimately, under steady-state conditions of illumination, most electrons are delivered to the carbon fixating Calvin cycle or photorespiration through the linear electron flow from PSII via PSI and Fd. After continuous moderate light or 1 d of high light illumination, CO\(_2\) fixation rates of the wild type and the 2cpa 2cpb double mutant were similar suggesting that the activity of the Calvin cycle is not compromised in the 2cpa 2cpb double mutant (Fig. 1C). However, after 2 d of high light treatment, CO\(_2\) fixation was reduced in the double mutant relative to wild type, suggesting a late impairment of the Calvin cycle when leaves become photobleached.

**Superoxide anion accumulation, oxidative stress and protein oxidation in the 2cpa 2cpb and 2cpa 2cpb tapx mutants.**

Alternative electron flows are of particular importance under high light conditions. A potential alternative electron flow could be the Mehler reaction, i.e. the transfer of electrons to molecular oxygen thereby producing superoxide at PSI. By performing nitroblue tetrazolium (NBT)
staining, indicative of $O_2^-$ production, the $2cpa~2cpb$ double and $2cpa~2cpb~tapx$ triple mutants were found to produce more $O_2^-$ than wild type and $tapx$ mutant plants already under ML (Fig. 2). Under high light illumination, increased $O_2^-$ formation could be detected in the wild type and $tapx$ mutant plants. Highest $O_2^-$ generation could be detected in the $2cpa~2cpb$ and $2cpa~2cpb~tapx$ mutant plants. Compared to moderate light conditions, however, little or no further increase of the staining could be measured possibly due to signal saturation (Fig. 2).

Light-induced over-accumulation of $O_2^-$ in the $2cpa~2cpb$ double and $2cpa~2cpb~tapx$ triple mutants may contribute to photobleaching of leaf tissues observed after 1 – 2 days of high light exposure (Fig. 1). To further estimate the extent of oxidative stress we determined leaf autoluminescence as a marker of oxidative stress and lipid peroxidation (Havaux et al., 2006). Autoluminescence of wild type and mutant plants exposed to moderate or high light was detected with a CCD camera. Unlike the wild type and $tapx$ mutant plants, $2cpa~2cpb$ double and $2cpa~2cpb~tapx$ triple mutant plants exhibited strong autoluminescence under high light conditions (Fig. 3A).

Oxidative stress-induced increase in autoluminescence has been correlated with lipid peroxidation, although oxidized proteins can also participate in the signal (Birtic et al., 2011). Therefore, protein carbonylation as an indicator of oxidative protein damage (Suzuki et al., 2010) was determined using a highly sensitive chemiluminescence immunoassay that allows to detect basal protein carbonylation even in unstressed wild type Arabidopsis leaves. To avoid signal saturation in samples from the mutants, chemoluminescent immunoblots were only briefly exposed to the detection film. In agreement with the autoluminescence data, we observed no apparent chemiluminescence signals under moderate light conditions in all genotypes. Under high light conditions, protein carbonylation was detectable in $2cpa~2cpb$ and, even more pronounced, in $2cpa~2cpb~tapx$ mutants while there was comparable little protein modification in wild type and $tapx$ plants (Fig. 3B). Collectively, these data suggest that complete loss of 2-Cys PRX activity results in increased formation of $O_2^-$, oxidative stress and protein oxidation.

Wild type-like lipid peroxidation in the $2cpa~2cpb$ mutant in response to high light stress.
Induction of autoluminescence under high light conditions in 2cpa 2cpb and 2cpa 2cpb tapx mutant plants suggested that lipid peroxidation products may accumulate in 2cpa 2cpb mutant plants in response to high light stress. In vitro, 2-Cys PRX have previously been shown to reduce not only H$_2$O$_2$ but also synthetic alkyl peroxides (Konig et al., 2002). The predominant plastid lipid peroxides in vivo are monogalactosyldiacylglycerols (MGDGs) with the acyl chain combination 18:3,18:3 and 18:3,16:3 in which one acyl chain is peroxidized (Zoeller et al., 2012). In order to assess the capacity of 2-Cys PRXs to reduce naturally occurring MGDG peroxides (MGDG-OOH) to their corresponding hydroxides (MGDG-OH), we incubated recombinant 2-Cys PRX A with different peroxide substrates and dithiothreitol as reductant in vitro. Enzyme assays revealed that the enzyme has a strong preference for H$_2$O$_2$. The activity of the enzyme towards the artificial peroxide tert-butyl hydroperoxide (t-BOOH) was less than 30% compared to H$_2$O$_2$, while the activity towards endogenously occurring 13-hydroperoxy octadecatrienoic acid (18:3-OOH) and peroxidized MGDG (MGDG-OOH) was very low (Fig. 4A). We also determined the levels of the two major MGDG-OOH, (18:3, 18:3-OOH)MGDG and (18:3, 16:3-OOH)MGDG, as well as their reduced metabolites, (18:3, 18:3-OH)MGDG and (18:3, 16:3-OH)MGDG, relative to their non-oxidized precursors, (18:3,18:3)MGDG and (18:3, 16:3)MGDG in vivo (Fig. 4B). Under moderate and high light conditions, we detected comparable levels of oxidized MGDGs in wild type and 2cpa 2cpb mutant leaves indicating wild type-like thylakoid lipid oxidation in the 2cpa 2cpb mutant (Fig. 4B). Moreover, the percentage of peroxidized MGDG (MGDG-OOH) relative to total oxidized MGDGs (MGDG-OOH + MGDG-OH) was higher than 90% in both genotypes under moderate and high light conditions (Fig. 4C). Therefore, MGDG peroxides are to the most part not reduced in vivo and appear not to be major substrates of 2-Cys PRX.

**Light-induced H$_2$O$_2$ over-accumulation in 2cpa 2cpb and 2cpa 2cpb tapx mutant plants**

Compared to wild type and tapx leaves, 2cpa 2cpb and 2cpa 2cpb tapx mutant leaves are expected to accumulate more H$_2$O$_2$ in the absence of the H$_2$O$_2$ detoxifying enzymes 2-Cys PRX and tAPX. To determine light-induced H$_2$O$_2$ levels in wild type, tapx, 2cpa 2cpb and 2cpa 2cpb tapx leaves, whole leaf H$_2$O$_2$ levels were quantitated with the homovanillic acid fluorescence
assay (Creissen et al., 1999). In wild type and tapx leaves, H₂O₂ levels were low under dark conditions, 1.6 – 1.8 µmol g⁻¹ fresh weight (FW), and displayed higher H₂O₂ levels under moderate and high light conditions (4.7 – 5 µmol g⁻¹ FW after 6 h). Compared to wild type plants, 2cpa 2cpb mutant plants displayed slightly elevated H₂O₂ levels (2.5 µmol g⁻¹ FW) already in the dark (Fig. 5). Levels of H₂O₂ further increased to 7.2 and 8.8 µmol g⁻¹ FW under moderate and high light conditions (6 h), respectively. In the 2cpa 2cpb tapx mutant, levels of H₂O₂ were found to be 3.0, 10.7 and 14.0 µmol g⁻¹ FW under dark, moderate and high light conditions (6 h), respectively (Fig. 5). While the wild type and the tapx mutant maintained H₂O₂ levels below or at 5 µmol g⁻¹ FW upon high light treatment, 2cpa 2cpb and 2cpa 2cpb tapx mutants were unable to confine H₂O₂ levels below the 5 µmol g⁻¹ FW wild type limit already under moderate light conditions (Fig. 5). H₂O₂ levels further increased in both mutants when exposed to high light.

Hence, with respect to high light-induced accumulation of H₂O₂ (Fig. 5), lack of tAPX appeared to be fully compensated by 2-Cys PRX. In contrast, lack of 2-Cys PRX could only partially be compensated by tAPX, since mutants lacking both 2-Cys PRX and tAPX displayed higher H₂O₂ levels than mutants deficient in 2-Cys PRX under moderate and high light conditions.

2-Cys PRX and tAPX restrict redox-regulated gene expression in response to high light stress

It has been proposed that 2-Cys PRX might be involved in ROS-mediated retrograde signaling. We therefore compared the response of ROS-inducible genes to high light treatment in wild type, tapx, 2cpa 2cpb and 2cpa 2cpb tapx plants. Among ROS-induced genes, many heat shock genes, including HSFA2 and HSP101, are early stress-responsive genes that are transiently up-regulated (displaying maximal expression 1h after onset of high light) not only by heat stress but also by H₂O₂ (Davletova et al., 2005; Maruta et al., 2010). In wild type, tapx and 2cpa 2cpb plants we could not determine a strong induction of HsfA2 and Hsp101 under high light conditions while a transient and dramatic up-regulation of these genes was measured in the 2cpa 2cpb tapx triple mutant plants (Fig. 6). A strong and significant induction of the redox- and H₂O₂-responsive genes OXI1, ZAT12 and At1g49150 (unknown protein) as well as the singlet oxygen-responsive
genes *OXI1* and *BAP1* (Davletova et al., 2005; Queval et al., 2007; Triantaphylides et al., 2008) by high light was also observed in the *2cpa 2cpb tapx* mutant. In contrast, wild type, *tapx* and *2cpa 2cpb* plants did not or only slightly up-regulate these redox- and ROS-responsive genes under high light conditions (Fig. 6). These data suggest that lack of either 2-Cys PRX or tAPX alone is not sufficient to trigger these genes in *A. thaliana* in response to high light.

### 2-Cys-PRX A and B are essential for HL-induced anthocyanin accumulation

We observed that wild type and *tapx* plants accumulated purple pigments, indicative for anthocyanin biosynthesis, after two days of high light treatment. In contrast, there was little increase of pigmentation in *2cpa 2cpb* and *2cpa 2cpb tapx* mutant plants (Supplemental Fig. S4A). Anthocyanin quantification revealed that anthocyanin levels increased 8.8-fold in the wild type and only 2.6-fold in *2cpa 2cpb* mutant plants after 3 days of high light exposure (Fig. 7A). To discern differences in the regulation of anthocyanin biosynthesis between wild type and *2cpa 2cpb* mutant plants, gene expression and metabolic profiles were assessed in the dark and after moderate or high light treatment. Expression of the high light-induced regulatory genes *PAP1* and *PAP2* and the structural *CHS* and *F3H* genes increased continuously when wild type plants were exposed for 6 h to high light (Fig. 7B). In contrast, expression of all four genes only transiently increased in the *2cpa 2cpb* double mutant, reaching maximal mRNA abundance after 3 h of high light exposure. In addition, levels of aromatic amino acids including phenylalanine, a precursor of anthocyanins, increased in the wild type but not in *2cpa 2cpb* mutant plants in response to high light (Supplemental Fig. S4B). These data suggest that a sustained activation of anthocyanin biosynthesis genes and accumulation of amino acid precursors does not occur in the *2cpa 2cpb* double mutant.

High light-induced anthocyanin accumulation can be repressed by elevated intracellular H₂O₂ levels (Vandenabeele et al., 2004; Vanderauwera et al., 2005). Since H₂O₂ levels are higher in the *2cpa 2cpb* and *2cpa 2cpb tapx* mutant plants compared to wild type and *tapx* mutant plants, H₂O₂-mediated down-regulation of the entire anthocyanin biosynthesis pathway may suppress anthocyanin accumulation in 2-Cys PRX-deficient plants. Potentially, other mechanisms may contribute to anthocyanin suppression in *2cpa 2cpb* mutant plants. It has been shown that
ascorbate deficiency or perturbations of the cellular redox status (Giacomelli et al., 2006; Page et al., 2012) are associated with reduced anthocyanin accumulation. To this end, analysis of the levels of soluble antioxidants revealed that absolute levels of reduced glutathione and ascorbate were slightly lower in 2cpa 2cpb mutant compared to wild type plants under moderate light conditions but did not significantly differ between the genotypes during high light treatment. Moreover, the ratio of the oxidized and reduced form of both metabolites was similar for wild type and 2cpa 2cpb double mutant plants (Supplemental Fig. S5). Jasmonates have also been implicated in mediating stress-induced anthocyanin accumulation (Shan et al., 2009). Therefore the effect of high light-stress on the levels of these oxylipins was determined in the wild type and the 2cpa 2cpb mutant plants. However, high light-stress induced a similar accumulation of jasmonic acid (JA) and JA-Ile in both wild type and 2-Cys PRX deficient plants (Supplemental Fig. S5).
DISCUSSION

2-Cys PRX enzymes are essential and more important than tAPX for the function of the water-water cycle and photoprotection under high light conditions

Several functions have been proposed for 2-Cys PRX. Since 2-Cys PRX have been shown to reduce a broad range of peroxides including H$_2$O$_2$ and synthetic alkyl hydroperoxides such as butyl or cumene hydroperoxides, they may be involved in detoxification of plastid H$_2$O$_2$ and membrane lipid peroxides (Konig et al., 2002; Rouhier and Jacquot, 2002). It has also been suggested that 2-Cys PRX function in an alternative ascorbate-independent WWC (Konig et al., 2002; Dietz et al., 2006) in addition to the classical WWC, the Mehler-ascorbate peroxidase (MAP) cycle (Fig. 8), as 2-Cys PRX can also use electrons delivered from the photosynthetic electron transport chain to reduce H$_2$O$_2$.

The classical ascorbate-dependent WWC involves plastid stromal and thylakoid APX, from which the thylakoid-bound tAPX at the site of H$_2$O$_2$ generation appears to be most important (Maruta et al., 2010; Miyake, 2010). However, recent studies using Arabidopsis sapx tapx double mutants suggested that plastid APX are not key enzymes in photoprotection since the double knock-out mutants exhibited no visible symptoms of stress after long term (1-14 d) high light (up to 2,000 µmol photons m$^{-2}$ s$^{-1}$) exposure (Giacomelli et al., 2007; Kangasjarvi et al., 2008; Maruta et al., 2010). At least under standard growth conditions, double mutant 2-Cys PRX deficient plants with <5% of 2-Cys PRX content did also not display visible photooxidative damages (Pulido et al., 2010).

To clarify the role of tAPX and 2-Cys PRX in the WWC, we analyzed wild type plants and mutants deficient in tAPX (tapx), 2-Cys PRX (2cpa 2cpb) or tAPX and 2-Cys PRX (2cpa 2cpb tapx) not only under normal but also under high light conditions. Under these conditions, the WWC is important for the dissipation of excess photon energy and to maintain the electron flow for building up a pH gradient that facilitates other mechanisms of non-photochemical energy quenching. It also serves as electron sink preventing electron carriers from being over-reduced which would facilitate O$_2^-$ formation and damage development (Miyake, 2010).
In agreement with the literature (Giacomelli et al., 2007; Kangasjarvi et al., 2008; Maruta et al., 2010), tapx mutant plants displayed no or little apparent phenotypic differences to wild type plants under moderate and high light conditions. As previously noted in antisense suppressed 2-Cys PRX lines (Baier et al., 2000) we observed growth retardation of 2cpa 2cpb and 2cpa 2cpb tapx mutant plants under standard growth conditions (Fig. 1A). Under these conditions, mutant plants almost deficient in 2-Cys PRX were shown to accumulate slightly more H₂O₂ and 4.7-fold more carbonylated proteins than wild type plants (Pulido et al., 2010). However, increased levels of H₂O₂ and carbonylated proteins in the mutant were not associated with PSII photoinhibition (as indicated by the Fv/Fm value) and photobleaching under moderate light (Pulido et al., 2010). We obtained similar results when studying mutants completely deficient in 2-Cys PRX. However, we did not observe an accumulation of carbonylated proteins under our standard growth conditions.

When compared under high light conditions, 2cpa 2cpb mutant plants showed an impairment in PSII photochemical efficiency (Fig. 1B), an increased formation of superoxide anion radicals (Fig. 2) and H₂O₂ (Fig. 5). As a consequence, 2cpa 2cpb mutants suffered from massive oxidative stress as indicated by elevated autoluminescence and protein carbonylation (Fig. 3) and are ultimately photobleached under high light conditions (Fig. 1). As described in the literature (Giacomelli et al., 2007; Kangasjarvi et al., 2008), tapx mutant plants behaved like the wild type and did not show any of these symptoms under high light conditions. These experiments indicate that 2-Cys PRX are essential and more important than tAPX in preventing excessive O₂⁻ and H₂O₂ formation (Fig. 2 and 5) as well as photo-oxidative damage (Fig. 1 and 3) under high light conditions. Notably, transcription of several APX genes including tAPX as well as the enzyme activities of Cu/ZnSOD and APX have been shown to be down-regulated in 2-Cys PRX deficient mutants already under normal light conditions (Pulido et al., 2010). Notably, knockdown Arabidopsis plants with suppressed expression of Cu/Zn-SOD display growth retardation and reduced chlorophyll content and photosynthetic activity (Rizhsky et al., 2003). Hence, the constitutive reduced capacity to detoxify O₂⁻ and H₂O₂ by these enzymes in the mutants may contribute to ROS toxicity and photosensitivity.
In contrast to tAPX, 2-Cys PRX have been proposed to be involved in the reduction of reactive lipid peroxides thereby providing an additional layer of antioxidative defense and photosystem protection. However, we did not observe massive increase of lipid peroxides under the applied high light conditions in both wild type and 2cpa 2cpb mutant plants (Fig. 4B). Moreover, the ratio of major thylakoid lipid peroxides to their reduced counterparts was not found to be affected in the 2cpa 2cpb mutant (Fig. 4C) suggesting that H$_2$O$_2$ rather than lipid peroxide reduction is the major biochemical function of 2-Cys Prx enzymes in vivo.

Besides detoxification of H$_2$O$_2$, another function of the WWC peroxidases is to serve as electron transmitters that constitute an electron flow in addition to the electron flow provided through the Mehler reaction (Fig. 8): When the linear electron transfer chain becomes over-reduced such as under high light conditions, the electron pressure can partially be relieved through transfer of two electrons from PSI to O$_2$ (e.g. the Mehler reaction) leading to the formation of O$_2$^-•. In addition, the electron pressure at PSI can also be decreased by transfer of two electrons to H$_2$O$_2$ by using 2-Cys PRX and tAPX as electron transmitters (Fig. 8). We hypothesize that deficiency in 2-Cys PRX increases the electron pressure at PSI leading to an accelerated Mehler reaction and excessive superoxide accumulation (Fig. 2). As a consequence, more H$_2$O$_2$ is produced through Cu/Zn-SOD-catalyzed or non-enzymatic dismutation of O$_2$^-• and accumulates because tAPX and other H$_2$O$_2$ detoxifying enzymes cannot fully compensate the deficiency in 2-Cys PRX. In the 2cpa 2cpb mutant, we determined an increase of H$_2$O$_2$ levels from 2.5 µmol g$^{-1}$ FW (in the dark) to 8.8 µmol g$^{-1}$ FW after 6h under high light conditions (Fig. 5). Since chloroplast enzymes are extremely sensitive to H$_2$O$_2$, plastid H$_2$O$_2$ levels are low and have been estimated to be around 0.8 µM in non-stressed leaves (Asada, 1999). The high levels of H$_2$O$_2$, that we and others (Pulido et al., 2010) determined already under normal light conditions in whole leaf extracts (in the mM range), is much too high to represent chloroplast H$_2$O$_2$ levels. Hence, basal and high light-induced H$_2$O$_2$ accumulates to a major part outside chloroplasts and may originate only to a minor part – due to the reduced H$_2$O$_2$ detoxification capacity in the mutants – from chloroplasts. In photosynthetic cells, photorespiratory production of H$_2$O$_2$ in peroxisomes has been shown to be the major source of H$_2$O$_2$ (Foyer et al., 2009) and to increase under photooxidative stress conditions (Kangasjarvi et al., 2012). The site and mechanism of excessive H$_2$O$_2$ generation in 2-
Cys PRX deficient mutants is not known but appears to be triggered by disturbance of redox homoeostasis or ROS accumulation in chloroplasts. In addition, constitutive down-regulation of sAPX and tAPX as well as at least 5 non-plastidic APX including cytosolic APX1, APX3, APX4, APX5 and APX6 (Pulido et al., 2010) may limit the capacity to reduce high light induced H₂O₂ in several compartments.

Notably, photorespiratory overproduction of H₂O₂ in mutants deficient in peroxisomal catalases has been shown to inhibit light-induced accumulation of anthocyanins (Vandenabeele et al., 2004; Vanderauwera et al., 2005). In 2-Cys PRX deficient plants, we observed an inhibition of high light-induced anthocyanin accumulation which is compatible with the hypothesis that H₂O₂ overproduction in 2-Cys PRX deficient mutants could be responsible for the down-regulation of anthocyanin biosynthesis.

**2-Cys PRX and tAPX synergistically restrict photooxidative stress signaling under high light conditions**

A peculiar characteristic of 2-Cys PRX and tAPX is that the enzymes are sensitive to over-oxidation and inactivation by H₂O₂ (Kitajima, 2008). A H₂O₂ floodgate hypothesis has been proposed according to which hydrogen peroxide acts as a signal in eukaryotic organisms when excessively formed H₂O₂ can no longer be detoxified by sensitive 2-Cys PRX due to over-oxidation of the peroxidative cysteine at the active site of 2-Cys PRX (Wood et al., 2003; Puerto-Galan et al., 2013). Therefore, we tested the floodgate hypothesis in the 2-Cys PRX deficient mutant. Although we observed higher H₂O₂ levels and photo-oxidative damage in 2-Cys PRX deficient mutants, we did not observe an over-induction of typical H₂O₂-responsive marker genes (Fig. 6) in the *2cpa 2cpb* mutant compared to wild type plants under high light conditions. Hence, the postulated floodgate preventing H₂O₂-signalling does not collapse in the absence of 2-Cys PRX in the mutants.

With respect to tAPX, this enzyme becomes rapidly inactivated by H₂O₂ when ascorbate levels fall below a threshold level (Shigeoka et al., 2002). However, we did not measure a significant difference in ascorbate levels between the *2cpa 2cpb* mutant and the wild type (Supplemental Fig. S5). To investigate the functional redundancy of 2-Cys PRX and tAPX we analyzed *2cpa*
2cpb tapx triple mutant plants. The triple mutant was more photosensitive than the 2cpa 2cpb double mutant (Fig. 1) and all indicators of photo-oxidative damage including impairment in photosynthetic efficiency, photobleaching, O$_2^-$ and H$_2$O$_2$ accumulation were found to be exacerbated (Fig. 1, 2, 3 and 5). Hence, tAPX is at least partially functional in the 2cpa 2cpb double mutant and attenuates the effects of 2-Cys PRX deficiency. The compensatory function of tAPX may in particular be important for maintaining the WWC when 2-Cys Prx become over-oxidized and inactivated.

When tAPX become inactivated in addition to 2-Cys PRX, plants may resemble 2cpa 2cpb tapx triple mutants that display strong up-regulation of photo-oxidative stress- and H$_2$O$_2$-responsive marker genes in response to high light. According to the H$_2$O$_2$-floodgate hypothesis, 2-Cys PRX and tAPX appear to restrict activation of H$_2$O$_2$–responsive genes potentially through preventing over-accumulation of H$_2$O$_2$ in response to high light (Fig. 7). However, despite pronounced differences in the activation of H$_2$O$_2$-responsive genes, global H$_2$O$_2$ levels were not found to be that much different between 2cpa 2cpb and 2cpa 2cpb tapx mutant plants after 1 and 3 h under high light conditions. Unfortunately, plastid levels of H$_2$O$_2$ could not be measured directly and may differ more strongly between the mutants than global H$_2$O$_2$ levels suggest. Therefore, a potential plastid H$_2$O$_2$ signal may be hidden by the bulk of H$_2$O$_2$ produced in extra-chloroplastic compartments (Fig. 8). Alternatively, the altered plastid redox state or over-reduction of photosynthetic electron transport components may generate an H$_2$O$_2$-independent retrograde plastid to nucleus signal in 2cpa 2cpb tapx mutant plants that are severely compromised in the WWC alternative electron flow. Recently it has been shown that over-reduction of the plastoquinone pool by the photosynthetic electron transport inhibitor DBMIB (2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone) dramatically induces photooxidative stress- and H$_2$O$_2$-responsive genes including HSFA2, HSP101, OXI1 and ZAT12 without increasing levels of H$_2$O$_2$ (Jung et al., 2013).

**Conclusion**

2-Cys PRX appear to be essential for optimal function of an alternative electron flow, the water-water cycle, that is important for photoprotection under high light conditions. tAPX seems to be
dispensable as long as the 2-Cys PRX system is functional. Since 2-Cys PRX are sensitive to H₂O₂-mediated over-oxidation, they may become inactivated under severe photooxidative stress conditions. In this situation, tAPX may partially compensate for the loss of 2-Cys PRX in the WWC. Under severe oxidative stress conditions associated with low ascorbate levels, tAPX may also become inactivated by H₂O₂. Loss of both WWC peroxidase systems, the ascorbate-independent 2-Cys PRX and the ascorbate-dependent tAPX, leads to a condition in which high light strongly activates H₂O₂- and photooxidative stress-responsive genes.
MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis thaliana wild type (ecotype Columbia, Col-0) and T-DNA insertion mutants were grown in soil in culture chambers under short day conditions and moderate light intensity (160 µmol m^-2 s^-1, 9 h light/15 h darkness) at 22°C and 20°C during light and dark periods, respectively. A. thaliana T-DNA insertion lines GK-295C05 (2-Cys PRXA gene At3g11630, referred to as 2cpa-2 mutant), SALK_017213C (2-Cys PRX B gene At5g06290, referred to as 2cpb-1 mutant) and WiscDsLox457-460A17 (thylakoidal ascorbate peroxidase, tAPX gene At1g77490, referred to as tapx mutant) were obtained from the Nottingham Arabidopsis Stock Centre (NASC, University of Nottingham, UK). Homozygous 2cpa-2 and 2cpb-1 mutant plants were crossed to generate the 2cpa 2cpb double mutant (Supplemental Fig. S1).

The homozygous 2cpa 2cpb double mutant was crossed with the homozygous tapx mutant, resulting in generation of the 2cpa 2cpb tapx triple mutant. Progeny obtained from these crosses were genotyped for homozygosity in T-DNA insertions using PCR analysis of genomic DNA and primer pairs listed in Supplemental Table 1. Primers, recommended by T-DNA Express: Arabidopsis Gene Mapping Tool, were used for genotyping in combination with LBb1.3, 8409 and p745 for SALK, GK and WiscDsLox lines, respectively. Genomic DNA was extracted using a modified cetyltrimethylammonium bromide-based method (Clarke, 2009). A Nanodrop ND-1000 UV-Vis Spectrophotometer was used for DNA quantification.

Homozygous 2cpa 2cpb double mutant and 2cpa 2cpb tapx triple mutant plants were selected on MS medium containing 1% (w/v) sucrose; MS medium with 3% (w/v) sorbitol or without sucrose served as controls. Seedlings were grown under sterile and short day conditions (160 µmol m^-2 s^-1; 9 h light, 22°C/15 h darkness, 20°C).

Plants (6 weeks old) were grown under moderate light (ML, 160 µmol m^-2 s^-1) or high light stress (HL, 900 µmol m^-2 s^-1). Under HL, leaf surface temperature increased to between 28°C and 32°C.

Heterologous expression and purification of recombinant 2-Cys peroxiredoxin A
Escherichia coli BL21(DE3) cells expressing His-tagged 2-Cys PRX A (Horling et al., 2003) were kindly provided by K.J. Dietz (University of Bielefeld, Germany). His6-tagged 2-Cys PRX A protein was expressed in E. coli, isolated and purified through immobilized metal ion affinity chromatography as described (Horling et al., 2003).

Measurement of chlorophyll fluorescence

Pulse amplitude modulation (PAM) fluorometry was used to measure chlorophyll fluorescence in plants grown under ML or under HL. Chlorophyll fluorescence was measured with a Maxi Imaging PAM chlorophyll fluorometer (Walz GmbH, Effeltrich, Germany) using the saturation pulse method as described (Schreiber, 2004; Bonfig et al., 2006). The optimal quantum yield of PSII (Fv/Fm) was determined using the software ImagingWin V2.41a (Walz GmbH, Germany) as described (van Kooten and Snel, 1990).

Carbon dioxide uptake measurements

The uptake of net carbon dioxides (CO2) by plants was recorded in an open gas exchange system consisting of two parallel water-cooled whole plant cuvettes using an infrared gas analyzer (IRGA, HCM-1000, Walz, www.walz.com). Illumination was provided by three LEDs, providing light at 655 nm at a photon fluence rate of 100 µmol m⁻² s⁻¹, at 455 nm and at 395 nm at 8 µmol m⁻² s⁻¹ (Bauer et al., 2013). To avoid introduction of gas exchange due to soil microorganisms, the potting soil was covered with a black plastic disc and water-tight foil. The gas flow rate through each cuvette was adjusted and controlled by mass flow meters (red-y smart series; www.voegtlin.com). Conditioned air with defined CO₂ concentration (380 ppm) and gas flow rate (1 L min⁻¹) was pumped into the two cuvettes over the leaf rosettes at 24°C and 42% relative humidity. Light conditions were used as described (Bauer et al., 2013). CO₂ uptake was measured in dark adapted plants after switching on the light. To determine the CO₂ uptake, CO₂ levels determined in the light were subtracted from the initial ambient CO₂ value (380 ppm) and related to the leaf-rosette surface area calculated using ImageJ software (http://imagej.nih.gov/ij).

In situ superoxide analysis via nitroblue tetrazolium staining
For visualization of superoxide accumulation in leaves the nitroblue tetrazolium (NBT) staining method (Jabs et al., 1996) was used with modifications. Prior light treatment, dark adapted leaves were detached and vacuum infiltrated with freshly prepared 0.1% (w/v) NBT (Sigma Aldrich, St. Louis, MO, USA) in water. To prevent dehydration, leaves were watered through their petioles and kept in darkness for 2 h to allow absorption of the infiltrated NBT solution. Thereafter, NBT infiltrated leaves were kept in darkness or treated with moderate or high light for 1 h. For evaluation of NBT staining, leaves were boiled in a glycerol:lactatophenole:ethanol (1:1:4, v/v/v) solution to remove chlorophyll for 6 min, washed with water and photographed on white background. Image processing was performed with Corel Draw X5 (for blue color extraction) and ImageJ (National Institute of Mental Health, Bethesda, Maryland, USA) for integration of the blue pixel density per leaf area (Nguyen, 2013).

**Autoluminescence measurements**

Autoluminescence of plants was detected with a CCD camera (Hamamatsu C4742-98, Hamamatsu Photonics, Japan). Plants were first exposed to ML (160 µmol m⁻² s⁻¹) or HL (900 µmol m⁻² s⁻¹) for 1 d or 2 d, then dark-adapted for 40 min for chlorophyll luminescence to decay. The Hokawo 2.1 Imaging Software (Hamamatsu Photonics, Japan) was used for image acquisition; the exposure time was 20 min. Images were converted into pseudo colors with an intensity range from 0 to 300.

**Detection of carbonylated proteins**

Proteins were extracted as described (Lehtimaki et al., 2011) with modifications. Leaves frozen in liquid nitrogen were covered with argon to prevent oxidation, then homogenized in liquid nitrogen and extracted with ice-cold extraction buffer (1:1, w/v) containing 10 mM HEPES-KOH (pH 7.6), 5 mM sucrose, and 5 mM MgCl₂ using a bead mill for homogenization (21 Hz, 1 min). The homogenate was filtered through miracloth (mesh size: 210 µm) to remove cell debris. Protein concentration was determined using Bradford reagent and BSA as standard (Spector, 1978).
Protein samples were separated by SDS PAGE (12.5% acrylamide). After electrophoresis, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Carbonylated proteins were detected using the OxyBlot™ Protein Oxidation Detection Kit (S7150) (Millipore, Billerica, MA, USA) based on derivatization of carbonyl groups in protein side chains to 2,4-dinitrophenylhydrazone (DNP-hydrazone) by reaction with 2,4-dinitrophenylhydrazine (DNPH). Primary antibody specific for the DNP moiety was used for detection.

**Hydrogen peroxide quantification**

H₂O₂ was determined by the homovanillic acid oxidation assay (Creissen et al., 1999) with modifications. Leaves were detached, watered through their petioles to prevent dehydration and kept in the dark or treated with moderate or high light for 1, 3 or 6 hrs. Thereafter, pre-weighed leaves were shock frozen in liquid nitrogen and ground in a bead mill. The frozen tissue powder (50 mg) was extracted with 25 mM HCl (250 µL) at 4°C. After centrifugation (5 min at 5000g at 4°C), the supernatant was mixed with activated charcoal (7 mg) to remove pigments. Centrifugation was repeated and 200 µL of the supernatant was cleared through glass fiber filter. For H₂O₂ determination, 25 µl aliquots of the extract were mixed with 445 µL of 50 mM Hepes (pH 7.5) and incubated with or without catalase (20 U) at 22°C for 1 min. Thereafter, 15 µL of 50 mM homovanillic acid in 50 mM Hepes (pH 7.5) were added and the reaction was started by adding 15 µl of 4 µM horse radish peroxidase. After incubation at 22°C for 30 min, the relative fluorescence (excitation at 315 nm; emission at 425 nm) was measured. In parallel, a dilution series of H₂O₂ was measured to generate a standard curve spanning from 0 - 25 nmol of H₂O₂. Total H₂O₂ concentrations were calculated from the difference in apparent H₂O₂ concentrations between the non-catalase and catalase treated samples.

To determine the substrate specificity of 2-Cys PRX A, enzymatic conversion of H₂O₂ and tert-butyl hydroperoxide (t-BOOH) was measured using the Ferrous Oxidation-Xylenol Orange (FOX) assay (Jiang et al., 1990; Nourooz-Zadeh et al., 1994). The assay was performed at 25°C and started by addition of the peroxide solution. The final reaction mixture (250 µl) contained 5 µM 2-Cys PRX A, 25 µM peroxide (H₂O₂ or t-BOOH) and 100 µM DTT in 25 mM potassium
phosphate buffer (pH 7.2). After different time points (0 - 10 min), 10 µL of the reaction mixture was added to 200 µL FOX working reagent (250 mM sulfuric acid, 1M sorbitol, 2.5 mM ferrous ammonium sulfate, and 1.25 mM xylene orange in H$_2$O) and the absorbance was measured spectrophotometrically at 595 nm. As negative controls, assays were performed without enzyme or with heat-inactivated enzyme (30 min at 95°C).

**Gene expression analysis**

Total RNA was extracted from leaves using the E.Z.N.A. plant RNA kit (Omega Bio-Tek, Norcross, GA). Potential DNA contamination was removed using on-column digestion with DNase I. Following quantification using a ND-1000 UV-Vis Spectrophotometer (NanoDrop, Wilmington, DE), 1 µg of total RNA was used for cDNA synthesis with M-MLV RNase H minus reverse transcriptase (Promega, Madison, WI, USA). A SYBR-Green Capillary Mix (ThermoFisher Scientific) was used for quantitative PCR with a CFX 96 Real-Time System C1000 Thermal Cycler (Bio-Rad) or a Mastercycler ep gradient S (Eppendorf, Wesseling-Berzdorf, Germany) using recommended cycle conditions. The primer pairs used are listed in Supplemental Table 1. Gene expression relative to *Actin 2/8* (Mueller et al., 2008) was measured by using the delta Ct-method (Pfaffl et al., 2004).

**Analysis of galactolipids and hydroperoxy fatty acids**

For galactolipid quantification, leaves from 6-week-old plants (100 mg) were harvested, immediately shock frozen, ground in liquid nitrogen and extracted with 500 µL of 2-propanol containing the radical scavenger butylated hydroxytoluene (BHT, 4.5 mM) and (18:0, 18:0)MGDG (15 µM, internal standard). For quantification of total oxidized endogenous MGDGs (hydroperoxy- and hydroxy-MGDG’s), triphenyl phosphine (TPP, 5 mg) was added to the extraction solvent to reduce hydroperoxy-MGDG’s to the corresponding hydroxy-MGDG’s. For determination of endogenous hydroxy-MGDG’s, TPP was not included in the extraction solvent. Levels of hydroperoxy-MGDGs were calculated by subtracting the levels of hydroxy-MGDGs from the level of total oxidized MGDGs. Samples were sonicated for 5 min, incubated for 15 min on ice, centrifuged and the supernatant was recovered. After re-extraction of the
residue using the same extraction procedure, the supernatants were pooled, taken to dryness under vacuum, reconstituted in 100 µL of 2-propanol and 5 µL were analyzed by UPLC-ESI-qTOF-MS.

UPLC-MS analyses were performed on an ultra-performance liquid chromatograph (Acquity UPLC, Waters, Milford, MA, USA) coupled to a hybrid quadrupole orthogonal time-of-flight mass spectrometer equipped with electrospray ionization source (ESI-qTOF-MS, SYNAPT G2 HDMS, Waters, Miford, MA, USA). Lipids were separated on a BEH C18 analytical column (particle size of 1.7 µm, dimension of 2.1 × 100 mm, Waters, Eschborn, Germany) using a solvent gradient from 30% (v/v) to 100% (v/v) eluent B over 10 min at a temperature of 60°C and a flow rate of 0.3 mL min⁻¹. Eluent A consisted of 10 mM ammonium acetate in water:acetonitrile (60:40, v/v) and eluent B consisted of 10 mM ammonium acetate in 2-propanol:acetonitrile (90:10, v/v). For the detection of the analytes, negative ESI was applied using capillary voltage of 0.8 kV. The flow rate of the desolvation gas (nitrogen) was 800 L/h and the desolvation temperature was kept at 350°C. MassLynx and QuantLynx software (version 4.1, Waters, Manchester, U.K.) were applied for data analysis (processing of chromatograms, peak detection and integration). For determination of response factors, (18:3, 18:3)MGDG was isolated from pumpkin leaves and (18:3, 18:3-OH)MGDG was produced by photo-oxidation and TPP reduction as described (Triantaphylides et al., 2008).

For in vitro analysis of the substrate specificity of recombinant 2-Cys Prx A, 13-hydroperoxy octadecatrienoic acid (13-HPOTE, Cayman Chemicals, Ann Arbor, MI, USA) was quantified by UPLC-ESI-qTOF-MS. The reversed phase separation was performed on a BEH C18 analytical column (1.7 µm, 2.1 × 100 mm, Waters, Eschborn, Germany) using a linear solvent gradient from water:acetonitrile (30:70, v/v) to water:acetonitrile (0:100, v/v) containing 0.1% formic acid over 10 min at 30°C. Quantification was performed via calibration using standard solutions at a concentration of 2.5-25 µM.

**Determination of anthocyanins**

Anthocyanin levels were determined as described (Rabino and Mancinelli, 1986). Briefly, leaves (100 mg) were harvested and shock frozen. After adding 1 mL of methanol containing 1% HCl
and 0.5 mL of water, the sample was ground with a mortar and pestle and incubated over night at 4°C. After centrifugation at 21,000g for 15 min, the supernatant was collected and the absorbance at 657 and 530 nm was measured. Anthocyanin content was calculated from the absorption at 530 nm corrected for the background absorption at 657 nm.

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Figure legends

Figure 1.
High light sensitivity and photosynthetic impairment of 2cpa 2cpb and 2cpa 2cpb tapx mutant plants.
Wild type (WT) and mutant plants were grown under moderate light (ML, 160 µmol m$^{-2}$ s$^{-1}$, 8 h per day) and treated with high light (HL, 900 µmol m$^{-2}$ s$^{-1}$, 8 h per day). (A) 2cpa 2cpb and 2cpa 2cpb tapx plants displayed a smaller habitus with pale green leaves compared to WT and tapx plants. Leaves of WT and tapx plants were tolerant to HL while 2cpa 2cpb and 2cpa 2cpb tapx plants became partially photobleached after 1 d (2cpa 2cpb tapx) or after 2 d (2cpa 2cpb) of HL treatment. (B) Optimal quantum yield (Fv/Fm) of WT (black bars), tapx (dark grey bars), 2cpa 2cpb (white bars) and 2cpa 2cpb tapx (light grey bars) plants grown under ML or HL conditions. Shown are means ± SD, n = 6. (C) Carbon dioxide (CO$_2$) fixation rate in WT (black bars) and 2cpa 2cpb mutant (white bars) plants grown under ML or HL conditions. The rate of CO$_2$ uptake was determined by gas exchange measurements using an infrared gas analyzer. Shown are means ± SD, n = 3. Significant differences between mean values are indicated by asterisks (*, P < 0.05; **, P < 0.01; ***, P < 0.001) using Student’s t test.

Figure 2.
Light-induced superoxide anion radical accumulation in wild type and mutant plants.
O$_2^-$ accumulation in dark adapted leaves of wild type (WT, black bars), tapx (dark grey bars), 2cpa 2cpb (white bars), and 2cpa 2cpb tapx (light grey bars) mutant plants was visualized by NBT staining in the dark or after 1 h of moderate light (ML, 160 µmol m$^{-2}$ s$^{-1}$) or high light treatment (HL, 900 µmol m$^{-2}$ s$^{-1}$). (A) Representative images of stained leaves after chlorophyll extraction. (B) Relative NBT staining (integration of blue color density per leaf area) was determined using the ImageJ image processing software. Shown are means ± SE, n = 10. Significant differences between mean values are indicated by asterisks (*, P < 0.05; **, P < 0.01; ***, P < 0.001) using Student’s t test.
Figure 3.
Sensitivity of wild type and mutant plants to high light stress.

(A) Autoluminescence of wild type (WT), tapx, 2cpa 2cpb, and 2cpa 2cpb tapx mutant plants after 8 h of moderate light (ML, 160 µmol m\(^{-2}\) s\(^{-1}\)) or after 1 d and 2 d of high light (HL, 900 µmol m\(^{-2}\) s\(^{-1}\), 8 h HL day) treatment. Images were taken with a CCD camera after 40 min dark adaptation. Representative pictures from 9 measurements were converted to false color images indicating the relative autoluminescence intensity. (B) Effect of light treatment on protein carbonylation in WT (1), tapx (2), 2cpa 2cpb (3) and 2cpa 2cpb tapx (4) plants. Proteins (15 µg of total protein) were derivatized with 2,4-dinitrophenylhydrazine and subjected to a SDS-PAGE. Immunoblotting was performed using an anti-dinitrophenylhydrazone (DNP) antibody. Representative blots from three experiments are shown.

Figure 4.
Lipid peroxide reduction by 2-Cys PRX. (A) The initial rate of peroxide reduction by 2-Cys PRX A was determined in vitro using 100 µM DTT as reductant and the following substrates: H\(_2\)O\(_2\) (25 µM), tert. butyl hydroperoxide (t-BOOH, 25 µM), 13-hydroperoxy octadecatrienoic acid (HOO-18:3, 25 µM) and peroxidized MGDGs (HOO-MGDG, 2.5 µM). Purified recombinant histidine-tagged 2-Cys PRX A (5 µM protein) was used in the assays. (B) Wild type (WT, black bars) and 2cpa 2cpb (white bars) mutant plant leaves were exposed to moderate light (ML, 160 µmol m\(^{-2}\) s\(^{-1}\), 8 h per day) or high light (HL, 900 µmol m\(^{-2}\) s\(^{-1}\), 8 h per day). The ratios of MGDG peroxides (MGDG-OOH) and hydroxides (MGDG-OH) relative to their non-oxidized MGDG precursors were determined for the indicated MGDG species. (C) The ratio of MGDG peroxides relative to total oxidized MGDG is shown for the indicated MGDG species. All values shown are means ± SD (n= 3).

Figure 5.
Light-induced H$_2$O$_2$ levels in different genotypes.

At the end of the night, leaves of wild type (WT), tapx, 2cpa 2cpb and 2cpa 2cpb tapx plants were kept in the dark (black bars) for 3 h or treated with moderate (grey bars, 160 µmol m$^{-2}$ s$^{-1}$) or high (white bars, 900 µmol m$^{-2}$ s$^{-1}$) light for the times indicated. Whole leaf H$_2$O$_2$ levels were determined using the homovanillic acid fluorescence assay. All values shown are means ± SD (n = 10); Significant differences between mean values are indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) using Student’s $t$ test.

Figure 6.

High light-induced expression of redox-regulated genes in wild type and mutant plants. Expression of HSFA2, HSP101, OXI1, ZAT12, At1g49150 and BAP1 in wild type (black bars), tapx (grey bars), 2cpa 2cpb (white bars), and 2cpa 2cpb tapx plants (light grey bars) after moderate light (ML, 160 µmol m$^{-2}$ s$^{-1}$) or high light treatment (HL, 900 µmol m$^{-2}$ s$^{-1}$) for the times indicated. Expression was normalized to the actin gene Act2/8. All values shown are means ± SD (n = 3); significant differences between mean values of wild type and mutant plants are indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) using Student’s $t$ test.

Figure 7.

Light-induced anthocyanin content and anthocyanin-related gene expression in wild type and 2cpa 2cpb plants. (A) Anthocyanin accumulation in wild type (black bars) and 2cpa 2cpb mutant plants (white bars) after the dark period (D, 16 h), moderate light (ML, 8 h, 160 µmol m$^{-2}$ s$^{-1}$) or high light (HL, 8 h per day, 900 µmol m$^{-2}$ s$^{-1}$). (B) Quantitative RT-PCR analysis of regulatory and structural genes contributing to anthocyanin biosynthesis in wild type (black bars) and 2cpa 2cpb mutant plants (white bars) after darkness (D), ML and HL treatment. Expression was normalized to the actin gene ACT2/8. Expression in wild type leaves after dark was arbitrarily set to 1 and all other expression values were expressed relative to it. All values shown are means ±
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**Figure 8.**
Model of the photoprotective function of 2-Cys PRX and tAPX.
The water-water cycle (WWC) and other alternative electron flows (AEF) are proposed to prevent over-reduction of the linear electron transport chain (LET) and to protect the photosystems from oxidative damage. Under high light conditions, 2-Cys PRX (2CP) are essential for maintaining a WWC and are more important than plastid APX involved in the classical WWC for protection of the photosystems. 2-Cys PRX and tAPX synergistically inhibit activation of redox-responsive genes and prevent over-accumulation of $\text{H}_2\text{O}_2$ as well as photooxidative damage under high light conditions.
Supplemental data

The following materials are available in the online version of this article.

**Supplemental Material and Methods**

**Supplemental Table S1:** Primers used for genotyping and quantitative gene expression analysis.

**Supplemental Figure S1.** Isolation of the 2cpa 2cpb double mutant.

**Supplemental Figure S2.** Sucrose sensitivity of wild type, 2cpa, 2cpb and 2cpa 2cpb mutant plants.

**Supplemental Figure S3.** Growth phenotype and lower chlorophyll content of the 2cpa 2cpb mutant.

**Supplemental Figure S4.** Leaf pigmentation and aromatic amino acid levels in wild type and 2cpa 2cpb mutant plants.

**Supplemental Figure S5.** Ascorbate, glutathione and jasmonate levels in wild type and 2cpa 2cpb mutant plants.
Figure 1. High light sensitivity and photosynthetic impairment of 2cpa 2cpb and 2cpa 2cpb tapx mutant plants.
Wild type (WT) and mutant plants were grown under moderate light (ML, 160 μmol m⁻² s⁻¹, 8 h per day) and treated with high light (HL, 900 μmol m⁻² s⁻¹, 8 h per day). (A) $2ca 2cpb$ and $2ca 2cpb tapx$ plants displayed a smaller habitus with pale green leaves compared to WT and tapx plants. Leaves of WT and tapx plants were tolerant to HL while $2ca 2cpb$ and $2ca 2cpb tapx$ plants became partially photobleached after 1 d ($2ca 2cpb tapx$) or after 2 d ($2ca 2cpb$) of HL treatment. (B) Optimal quantum yield ($F_d/F_m$) of WT (black bars), tapx (dark grey bars), $2ca 2cpb$ (white bars) and $2ca 2cpb tapx$ (light grey bars) plants grown under ML or HL conditions. Shown are means ± SD, n = 6. (C) Carbon dioxide (CO₂) fixation rate in WT (black bars) and $2ca 2cpb$ mutant (white bars) plants grown under ML or HL conditions. The rate of CO₂ uptake was determined by gas exchange measurements using an infrared gas analyzer. Shown are means ± SD, n = 3. Significant differences between mean values are indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) using Student’s $t$ test.
Figure 2

Figure 2. Light-induced superoxide anion radical accumulation in wild type and mutant plants. O$_2^-$ accumulation in dark adapted leaves of wild type (WT, black bars), tapx (dark grey bars), 2cpa 2cpb (white bars), and 2cpa 2cpb tapx (light grey bars) mutant plants was visualized by NBT staining in the dark or after 1 h of moderate light (ML, 160 µmol m$^{-2}$ s$^{-1}$) or high light.
treatment (HL, 900 µmol m\(^{-2}\) s\(^{-1}\)). (A) Representative images of stained leaves after chlorophyll extraction. (B) Relative NBT staining (integration of blue color density per leaf area) was determined using the ImageJ image processing software. Shown are means ± SE, n = 10. Significant differences between mean values are indicated by asterisks (*, \(P < 0.05\); **, \(P < 0.01\); ***, \(P < 0.001\)) using Student’s \(t\) test.
Figure 3. Sensitivity of wild type and mutant plants to high light stress.

(A) Autoluminescence of wild type (WT), tapx, 2cpa 2cpb, and 2cpa 2cpb tapx mutant plants after 8 h of moderate light (ML, 160 µmol m$^{-2}$ s$^{-1}$) or after 1 d and 2 d of high light (HL, 900 µmol m$^{-2}$ s$^{-1}$, 8 h HL day) treatment. Images were taken with a CCD camera after 40 min dark adaptation. Representative pictures from 9 measurements were converted to false color images.
indicating the relative autoluminescence intensity. (B) Effect of light treatment on protein carbonylation in WT (1), tapx (2), 2cpa 2cpb (3) and 2cpa 2cpb tapx (4) plants. Proteins (15 µg of total protein) were derivatized with 2,4-dinitrophenylhydrazine and subjected to a SDS-PAGE. Immunoblotting was performed using an anti-dinitrophenylhydrazone (DNP) antibody. Representative blots from three experiments are shown.
Figure 4

A

Peroxide reduction (μmol min⁻¹)

H₂O₂  l-BOOH  18:3-OOH  MGDG-OOH

B

% of precursor

ML  HL 1 d  HL 2 d

18:3-OH  18:3-OH  18:3-OH  18:3-OH

MGDG

C

MGDG-OOH / MGDG-OOH (%)

ML  HL

18:3  18:3ox

MGDG

18:3  18:3ox
Figure 4. Lipid peroxide reduction by 2-Cys PRX. (A) The initial rate of peroxide reduction by 2-Cys PRX A was determined in vitro using 100 µM DTT as reductant and the following substrates: H$_2$O$_2$ (25 µM), tert. butyl hydroperoxide (t-BOOH, 25 µM), 13-hydroperoxy octadecatrienoic acid (HOO-18:3, 25 µM) and peroxidized MGDGs (HOO-MGDG, 2.5 µM). Purified recombinant histidine-tagged 2-Cys PRX A (5 µM protein) was used in the assays. (B) Wild type (WT, black bars) and 2cpa 2cpb (white bars) mutant plant leaves were exposed to moderate light (ML, 160 µmol m$^{-2}$ s$^{-1}$, 8 h per day) or high light (HL, 900 µmol m$^{-2}$ s$^{-1}$, 8 h per day). The ratios of MGDG peroxides (MGDG-OOH) and hydroxides (MGDG-OH) relative to their non-oxidized MGDG precursors were determined for the indicated MGDG species. (C) The ratio of MGDG peroxides relative to total oxidized MGDG is shown for the indicated MGDG species. All values shown are means ± SD (n= 3).
Figure 5. Light-induced H$_2$O$_2$ levels in different genotypes. At the end of the night, leaves of wild type (WT), tapx, 2cpa 2cpb and 2cpa 2cpb tapx plants were kept in the dark (black bars) for 3 h or treated with moderate (grey bars, 160 µmol m$^{-2}$ s$^{-1}$) or high (white bars, 900 µmol m$^{-2}$ s$^{-1}$) light for the times indicated. Whole leaf H$_2$O$_2$ levels were determined using the homovanillic acid fluorescence assay. All values shown are means ± SD (n = 10); Significant differences between mean values are indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) using Student’s $t$ test.
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