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2-Cys Peroxiredoxins Participate in the Oxidation of Chloroplast Enzymes in the Dark

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ABSTRACT

Most redox-regulated chloroplast enzymes are reduced during the day and oxidized during the night. While the reduction mechanism of light-dependent enzymes is well known, the mechanism mediating their oxidation in the dark remains unknown. The thiol-dependent peroxidases, 2-Cys peroxiredoxins (Prxs), play a key role in light-dependent reduction of chloroplast enzymes. Prxs transfer reducing equivalents of thiols to hydrogen peroxide, suggesting the participation of these peroxidases in enzyme oxidation in the dark. Here, we have addressed this issue by analyzing the redox state of well-known redox-regulated chloroplast enzymes in response to darkness in *Arabidopsis thaliana* mutants deficient in chloroplast-localized Prxs (2-Cys Prxs A and B, Prx IIE, and Prx Q). Mutant plants lacking 2-Cys Prxs A and B, and plants overexpressing NADPH-dependent thioredoxin (Trx) reductase C showed delayed oxidation of chloroplast enzymes in the dark. In contrast, the deficiencies of Prx IIE or Prx Q exerted no effect. *In vitro* assays allowed the reconstitution of the pathway of reducing equivalents from reduced fructose 1,6-bisphosphatase to hydrogen peroxide mediated by Trxs and 2-Cys Prxs. Taken together, these results suggest that 2-Cys Prxs participate in the short-term oxidation of chloroplast enzymes in the dark.

Key words: chloroplast, enzyme oxidation, peroxiredoxin, thioredoxin, hydrogen peroxide, darkness

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INTRODUCTION

Redox regulation, based on disulfide–dithiol exchange, constitutes an essential regulatory mechanism that allows the rapid adaptation of chloroplast metabolism to changes in light intensity (Buchanan, 2016). CO_2 fixation via the Calvin–Benson cycle (CBC), which is active during the day and inactive during the night, has become a model system to study light-dependent redox regulation of enzyme activity in plants (Michelet et al., 2013). Early studies, in the 1960s, established that photoreduced ferredoxin (Fdx) activated the CBC enzyme fructose 1,6-bisphosphatase (FBPase) in the light (Buchanan et al., 1967). Further analyses revealed that this activation required a "protein factor" (Buchanan et al., 1971), which turned out to be composed of two components, thioredoxin (Trx) and an Fdx-dependent Trx reductase (FTR) (Wolosiuk and Buchanan, 1977), leading to the notion that the Fdx-FTR-Trx redox system fueled reducing equivalents generated from photochemical reactions for redox regulation of enzyme activity. Initial biochemical approaches (recently reviewed by Buchanan, 2016) identified two isoforms of Trxs in chloroplasts, termed *f* and *m*, based on their ability to preferentially activate FBPase and NADP-malate dehydrogenase (NADP-MDH), respectively. The availability of genome sequence data from a large variety of plant species has allowed establishment of the high complexity of the chloroplast redox network with the identification of 10 canonical Trxs (*f*1, *f*2, *m*1–*m*4, *y*1, *y*2, *x*, and *z*) plus additional Trx-like proteins (Meyer et al., 2012; Balsera et al., 2014; Geigenberger et al., 2017). Moreover, proteomics analyses have identified a large number of targets of Trxs (Montrichard et al., 2009), thus extending the relevance of chloroplast redox regulation far beyond the CBC.

After the discovery of the Fdx-FTR-Trxs redox system, extensive biochemical analyses (reviewed by Schürmann and Buchanan, 2008) have established the molecular basis of the light-dependent redox regulation of enzyme activity in the chloroplast. In particular, it is well established that redox-sensitive CBC enzymes become reduced upon illumination, reducing power being provided by the Fdx-FTR-Trxs system, which thus links CO_2 fixation to light. Further studies revealed that Trxs *f* and *m* play the most relevant role in the redox regulation of this

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pathway (Okegawa and Motohashi, 2015; Yoshida et al., 2015). In contrast, *x*- and *y*-type Trxs (Collin et al., 2003, 2004), and cold, drought, and salt regulated protein of 32 kDa (CDSP32) (Broin et al., 2002) were proposed to have antioxidant function based on their activity as reductants of the hydrogen peroxide scavenging enzyme 2-Cys peroxiredoxin (2-Cys Prx).

More recently, an NADPH-dependent Trx reductase (NTR) with a joint Trx at the C terminus, termed NTRC, was identified (Serrato et al., 2004). NTRC is exclusive of oxygenic photosynthetic organisms (Pascual et al., 2010; Nájera et al., 2017) and shows plastid localization in plants (Serrato et al., 2004; Moon et al., 2006; Kirchsteiger et al., 2012). NTRC displays high affinity for NADPH (Bernal-Bayard et al., 2012), thereby allowing the use of this nucleotide for chloroplast redox homeostasis (Spínola et al., 2008; Cejudo et al., 2012). Because 2-Cys Prx is efficiently reduced by NTRC, it was proposed that this enzyme has an antioxidant function (Moon et al., 2006; Pérez-Ruiz et al., 2006; Alkhalfioui et al., 2007; Pérez-Ruiz and Cejudo, 2009). However, further analyses have shown that NTRC affects redox regulation of a large variety of chloroplast metabolic pathways including starch (Michalska et al., 2009; Lepistö et al., 2013) and chlorophyll (Richter et al., 2013; Pérez-Ruiz et al., 2014) biosynthesis. In addition, *Arabidopsis thaliana* NTRC knock-out mutant plants show low efficiency in light energy utilization (Carrillo et al., 2016; Naranjo et al., 2016b), indicating that NTRC plays a pivotal function in redox regulation of multiple processes in chloroplasts.

Besides the extensive knowledge of molecular basis of the light-dependent reduction of CBC enzymes, it has long been known that these enzymes become rapidly oxidized shortly after darkness. This is the case for FBPase (Leegood and Walker, 1980) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Sparla et al., 2002). Several lines of evidence support the involvement of hydrogen peroxide in the dark-dependent oxidation of chloroplast enzymes (Kaiser, 1979; Tanaka et al., 1982); however, the mechanism of their oxidation remains poorly understood. In a recent report, we have proposed that the redox balance of 2-Cys Prx exerts a critical role in maintaining the redox state of Trx-regulated enzymes, which is essential for photosynthetic performance (Pérez-Ruiz et al., 2017). Hence, 2-Cys Prx, which is a thiol-dependent peroxidase, establishes a link between chloroplast redox homeostasis and hydrogen peroxide detoxification. These findings prompted us to consider the hypothesis that 2-Cys Prxs might also participate in the oxidation of chloroplast enzymes in the dark. *Arabidopsis* chloroplasts contain two almost identical 2-Cys Prxs, A and B, which are among the most abundant proteins of this organelle (Konig et al., 2002). In addition, chloroplasts contain so-called atypical Prxs of types IIE and Q (Horling et al., 2002). In contrast to 2-Cys Prxs, which are homodimers, Prx IIE and Prx Q are monomeric, thus containing two cysteine residues in their catalytic active site, peroxidatic and resolving, in the same polypeptide (Dietz, 2011). In this work, we have analyzed the participation of chloroplast Prxs in enzyme oxidation in the dark by a combination of genetic and biochemical approaches. Our results show the significant participation of 2-Cys Prxs, but not of Prx IIE and Prx Q, in dark-dependent enzyme oxidation.

RESULTS

Dark-Induced Oxidation of FBPase, GAPDH, and γ Subunit of ATPase Is Delayed in Mutant Plants Devoid of 2-Cys Prxs

The recent finding of the critical role of 2-Cys Prxs in chloroplast redox regulation (Pérez-Ruiz et al., 2017) implies that disulfide–dithiol exchange of stromal enzymes is linked to the reduction of hydrogen peroxide, which may act as a sink for reducing equivalents. Thus, these results raised the possibility that the oxidation of redox-regulated enzymes occurring shortly after darkness might involve the action of 2-Cys Prxs. To address this possibility, we generated a double knockout mutant devoid of the two 2-Cys Prxs, A and B, present in *Arabidopsis* chloroplasts by manual crossing of the single *2cpa* (Pérez-Ruiz et al., 2017) and *2cpb* (Kirchsteiger et al., 2009) mutants (Supplemental Table 1; Supplemental Figure 1). Similar to the double 2-Cys Prx mutant previously described (Awad et al., 2015), the double mutant generated in this study, termed *2cpab*, contains no detectable 2-Cys Prxs (Supplemental Figure 2A–2C) and shows reduced growth (Supplemental Figure 2D) and lower chlorophyll content (Supplemental Figure 2E) compared with wild-type plants grown either under long- or short-day photoperiod.

To test the involvement of 2-Cys Prxs in the dark-induced oxidation of chloroplast enzymes, we focused on FBPase and the GAPB subunit of GAPDH, two well-known redox-regulated CBC enzymes (Leegood and Walker, 1980; Sparla et al., 2002). Activity of redox-regulated enzymes in chloroplasts depends on their redox state, but also on additional factors, which include pH and the concentration of Mg^{2+} and regulatory metabolites (Schürmann and Buchanan, 2008). Thus, to specifically analyze enzyme oxidation in the dark, the *in vivo* redox state of these enzymes was determined by labeling of thiolic groups with the alkylating agent methylmaleimide polyethylene glycol (MM-PEG_{24}), as previously reported (Naranjo et al., 2016a). It is known that the level of reduction of redox-regulated CBC enzymes depends on light intensity (Yoshida et al., 2014; Naranjo et al., 2016a). Therefore, to assure a high level of reduction of these enzymes in the light, plants grown under a long-day photoperiod, at a light intensity of 125 μE m^{-2} s^{-1}, were taken at the end of a regular 8 h night cycle and incubated during 30 min at a light intensity of 500 μE m^{-2} s^{-1}. This treatment was sufficient to achieve almost complete reduction of FBPase (Figure 1A and 1B) and GAPDH (Figure 1C and 1D) in both wild-type and the *2cpab* mutant. To monitor the dark-induced enzyme oxidation, we transferred light-exposed plants to darkness for 5, 10, and 15 min. As expected, in wild-type plants the two enzymes were rapidly and completely oxidized in response to darkness, so that no reduced enzymes were detected at 5 min of treatment (Figure 1A–1D). In contrast, the oxidation of both enzymes was significantly delayed in the *2cpab* mutant, FBPase being more prone to oxidation than GAPDH (Figure 1A–1D). Nevertheless, full oxidation of both enzymes in the *2cpab* mutant was observed after 15 min of darkness (Figure 1A–1D). Finally, we extended this analysis to the γ subunit of ATPase (CF_1-γ), a redox-regulated protein not belonging to the CBC, which is known to be rapidly reduced in the light (Yoshida et al., 2015). In wild-type plants, CF_1-γ was also oxidized in the dark though at slower rate than FBPase and GAPDH, as the enzyme was

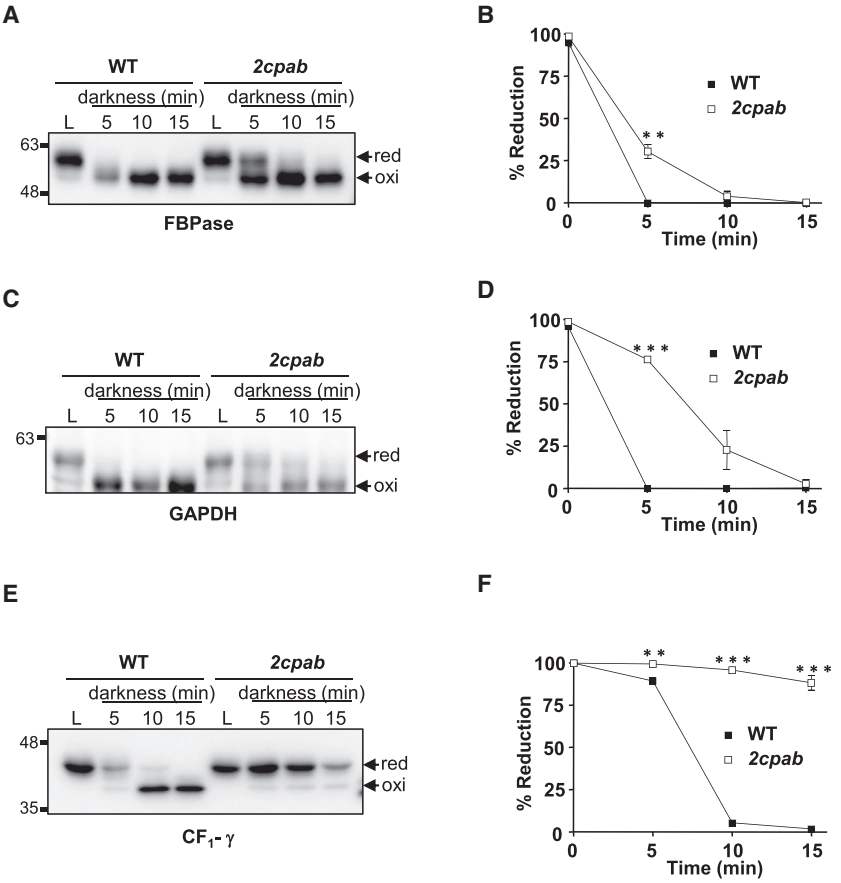

Figure 1. Dark-Dependent Oxidation of FBPase, GAPDH, and CF_1-γ in Wild-Type and the *2cpab* Mutant.

Wild-type and *2cpab* mutant plants were grown under long-day conditions for 4 weeks at a light intensity of 125 μE m^{-2} s^{-1}. At the end of the night period, plants were incubated at a light intensity of 500 μE m^{-2} s^{-1} during 30 min (L), then light was switched off and samples were taken at 5, 10, and 15 min as indicated.

(A, C, and E) The *in vivo* redox state of FBPase **(A)**, GAPDH **(C)**, and CF_1-γ **(E)** were determined with the alkylating agent MM-PEG_{24} as described in Methods.

(B, D, and F) The corresponding band intensities were quantified (GelAnalyzer), and the percentage of reduction is the ratio between the reduced form and the sum of reduced and oxidized forms for each protein. Each value is the mean of three (GAPDH), four (CF_1-γ), or five (FBPase) independent experiments ± SE. Statistical significance (**$p < 0.05$, ***$p < 0.01$) was determined with the Student's *t*-test. red, reduced; oxi, oxidized. Molecular mass markers (kDa) are indicated on the left.

almost fully reduced at 5 min of darkness, and became completely oxidized after 15 min of treatment. On the contrary, CF_1-γ showed almost no oxidation in the *2cpab* mutant during the time tested (Figure 1E and 1F). The fact that the *2cpab* mutant shows delayed oxidation of the enzymes analyzed indicates the participation of 2-Cys Prxs in the short-term oxidation of chloroplast enzymes triggered by darkness. Moreover, these enzymes show different rates and levels of oxidation, indicating their different sensitivity to the redox conditions of the chloroplast.

Overexpression of *NTRC* Mimics the Effect of 2-Cys Prx Deficiency

To further analyze the function of 2-Cys Prxs in the short-term oxidation of chloroplast enzymes in the dark, we sought to imbalance the transfer of reducing equivalents to 2-Cys Prxs by overexpressing NTRC, the most efficient reductant of these enzymes *in vivo* (Pulido et al., 2010). To this end, the *Arabidopsis NTRC* cDNA was expressed in wild-type plants under the control of *Cauliflower mosaic virus* (CaMV) 35S promoter, and two independent transgenic lines showing high levels of NTRC were selected for further analysis (Figure 2A and 2B). Interestingly, transgenic lines overexpressing NTRC display a growth inhibition phenotype resembling that of the *2cpab* mutant (Figure 2A), as shown by the fresh weight of long-day-grown plants (Figure 2C), although the level of 2-Cys Prxs in these plants was similar to those in the wild-type (Figure 2B). Strikingly, it was not possible to determine the growth rates of these transgenic plants under short-day photoperiod because these growth conditions caused a heterogeneous phenotype of the transgenic lines. Beside plants with high levels of NTRC, we observed also plants exhibiting green and yellowish leaves containing wild-type or non-detectable levels, respectively, of NTRC (Supplemental Figure 3A and 3B), indicating that under short-day conditions the transgenic lines show pronounced *NTRC* gene silencing. To test the impact of NTRC levels on the dark-induced oxidation of chloroplast enzymes, we performed the same analysis as described for the *2cpab* mutant. In plants accumulating high levels of NTRC, the dark-induced oxidation of FBPase (Figure 3A and 3B) and GAPB subunit of GAPDH (Figure 3C and 3D) was significantly delayed as compared with those of the wild-type plants. In transgenic lines, CF_1-γ remained predominantly reduced after 15 min of darkness, whereas it became completely oxidized at this time of treatment in wild-type plants (Figure 3E and 3F). Overall, the rates and levels of dark-induced enzyme oxidation determined for NTRC-overexpressing plants resemble those observed in the *2cpab* mutant, suggesting that high dosage of NTRC phenocopies the *2cpab* defects in dark-induced oxidation of chloroplast enzymes.

Prx Q and Prx IIE Are Not Involved in Dark-Induced Enzyme Oxidation

The delayed rate of enzyme oxidation in the *2cpab* mutant uncovers the participation of these thiol-dependent peroxidases in chloroplast enzyme oxidation in the dark. However, with the exception of CF_1-γ, which remained almost completely reduced during the time tested (Figure 1E and 1F), both FBPase and GAPDH were fully oxidized at 15 min of darkness in the *2cpab* mutant (Figure 1A–1D), indicating that additional mechanism(s) for dark-induced enzyme oxidation may be operative. Clear candidates to perform this function are additional thiol-dependent peroxidases localized in chloroplasts, such as the atypical Prxs of the types Q and IIE (Dietz, 2011). To test the participation of

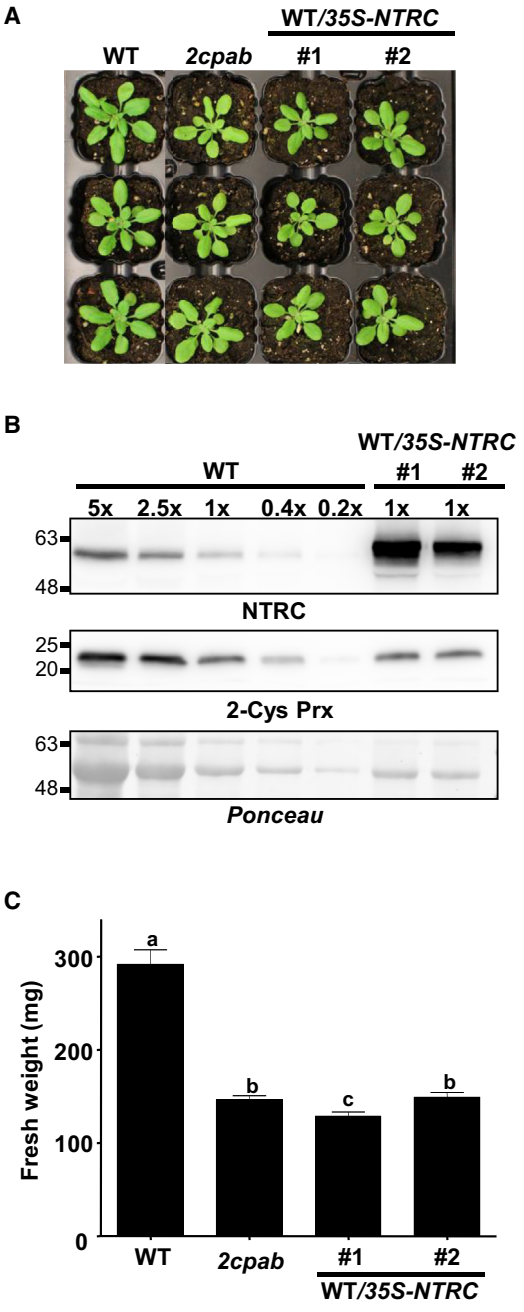

Figure 2. *Arabidopsis* Plants Overexpressing NTRC Display a Growth Phenotype Similar to that of the *2cpab* Mutant.

(A) Wild-type (WT), *2cpab* mutant, and two independent transgenic lines (#1, #2) overexpressing NTRC in the wild-type background (WT/*35S-NTRC*) were grown under long-day conditions for 4 weeks.

(B) Western blot analysis of the content of NTRC and 2-Cys Prxs. Protein extracts were obtained from leaves of plants grown as stated in **(A)** and aliquots of 15 μg (1×), from all lines, and increased (5× and 2.5×) or decreased (0.4× and 0.2×) levels of WT proteins, as indicated, were subjected to SDS–PAGE under reducing conditions, transferred to nitrocellulose filters, and probed with anti-NTRC or anti-2-Cys Prxs antibodies. Even loading was monitored by Ponceau staining of the Rubisco large subunit. Molecular mass markers (kDa) are indicated on the left.

(C) The weight of the rosette leaves from 15 (WT) or 20 (*2cpab*, WT/*35S-NTRC* #1 and #2) plants of each line is represented as average values ± SE; letters indicate significant differences with the Student's *t*-test at 95% confidence interval (CI).

these Prxs in enzyme oxidation, previously described *Arabidopsis* mutant lines knockdowning either Prx IIE (Romero-Puertas et al., 2007) or Prx Q (Lamkemeyer et al., 2006) (Supplemental Table 1) were manually crossed with the *2cpab* double mutant to obtain *2cpab-prxIIE* and *2cpab-prxQ* triple mutants (Figure 4A). Western blot analysis showed that both *2cpab-prxIIE* and *2cpab-prxQ* triple mutants were knocked out for 2-Cys Prxs and knocked down for Prx IIE and Prx Q, respectively (Figure 4B). In agreement with previous results (Lamkemeyer et al., 2006; Romero-Puertas et al., 2007), single mutants *prxIIE* and *prxQ* displayed growth phenotypes very similar to that of the wild-type, as shown by the fresh weight of the rosette leaves (Figure 4C) and chlorophyll content (Figure 4D). The growth phenotypes of both triple mutants were indistinguishable from that of the *2cpab* mutant (Figure 4A, 4C, and 4D), indicating that in the *2cpab* background, decreased levels of either Prx IIE (*2cpab-prxIIE* mutant) or Prx Q (*2cpab-prxQ* mutant) has negligible phenotypic effects in the conditions tested. In line with their growth phenotypes, the *prxIIE* and *prxQ* mutants showed efficiency of light energy utilization very similar to that of the wild type, as shown by the photosynthetic electron transport rate (Figure 5A), yield of photosystem II (Y(II)) (Figure 5B), and non-photochemical quenching (Y(NPQ)) (Figure 5C). Interestingly, plants lacking 2-Cys Prxs, namely the *2cpab*, *2cpab-prxIIE*, and *2cpab-prxQ* mutants, showed improved photosynthetic performance compared with wild-type plants (Figure 5A–5C), indicating that 2-Cys-Prxs affect the regulation of photochemical reactions. Overall, these results indicate that, under the conditions tested, decreased contents of Prx IIE or Prx Q have no phenotypic effects in the *2cpab* mutant. To test the involvement of Prxs types IIE and Q on enzyme oxidation in the dark, we analyzed the redox state of FBPase and CF_1-γ, as representatives of CBC and non-CBC enzymes, respectively. The rates of dark-oxidation of these enzymes in the *2cpab-prxIIE* and *2cpab-prxQ* mutants, almost indistinguishable from those observed in the *2cpab* mutant, were significantly delayed compared with those of the wild-type and the mutants *prxIIE* and *prxQ* (Figure 6A–6D). While these mutants still contain low contents of Prx IIE and Prx Q, respectively, these results suggest a minor contribution, if any, of these Prxs to the dark-dependent oxidation of the chloroplast enzymes analyzed.

Participation of 2-Cys Prxs in Chloroplast Enzyme Oxidation Likely Occurs through an Indirect Mechanism Involving Trxs

The delayed dark-induced oxidation of chloroplast metabolic enzymes in the *2cpab* mutant supports a pathway in which reducing equivalents from enzyme thiols are enzymatically transferred to hydrogen peroxide via 2-Cys Prxs. Two possibilities could be considered for this pathway: the direct oxidation of reduced enzymes by 2-Cys Prxs or an indirect mechanism involving Trxs. To distinguish between these possibilities, we expressed recombinant FBPase, as a classical redox-regulated enzyme, Trxs *f*1 and *f*2, as representatives of Trxs involved in carbon

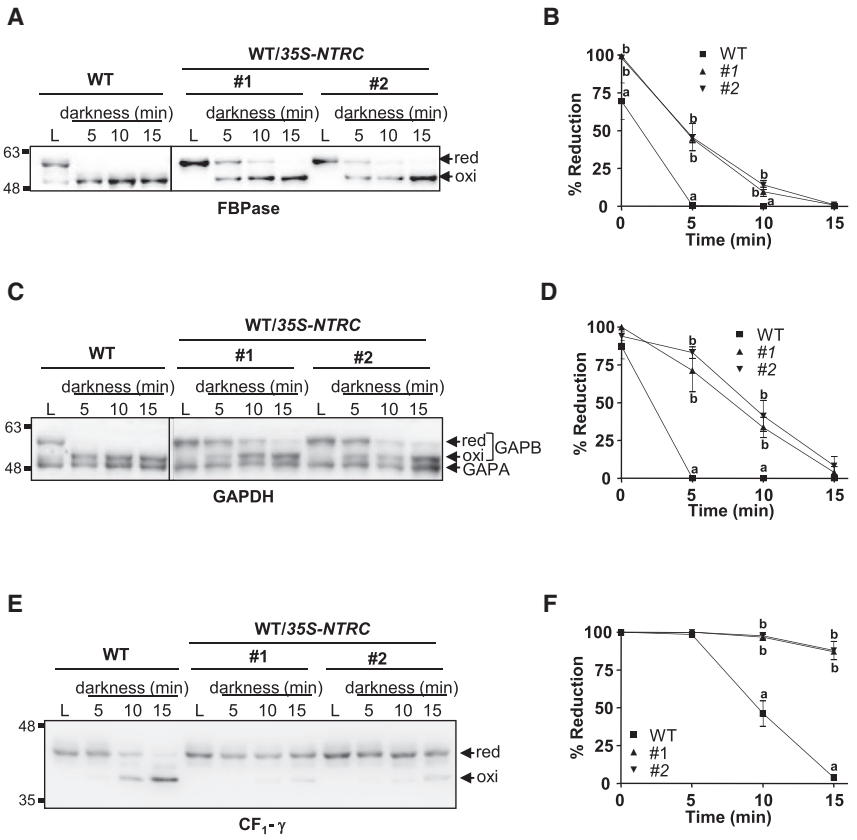

Figure 3. Effect of High Contents of NTRC on Dark-Dependent Oxidation of FBPase, GAPDH, and CF_1-γ.

Wild-type (WT) and transgenic lines overexpressing NTRC were grown under long-day conditions for 4 weeks at a light intensity of 125 μE m^{-2} s^{-1}. At the end of the night period, plants were incubated at a light intensity of 500 μE m^{-2} s^{-1} during 30 min (L), then light was switched off and samples were taken at 5, 10, and 15 min as indicated.

(A, C, and E) The *in vivo* redox state of FBPase **(A)**, GAPDH subunits GAPA and GAPB **(C)**, and CF_1-γ **(E)** were determined with the alkylating agent MM-PEG_{24} as described in Methods.

(B, D, and F) The corresponding band intensities were quantified (GelAnalyzer), and the percentage of reduction is the ratio between the reduced form and the sum of reduced and oxidized forms for each protein. The vertical lines indicate non-adjacent lanes from the same blot. Each value is the mean of three independent experiments ± SE; letters indicate significant differences with the Student's *t*-test at 95% CI. red, reduced; oxi, oxidized. Molecular mass markers (kDa) are indicated on the left.

metabolism, and 2-Cys Prx in *Escherichia coli* with a His tag at the N terminus and purified them by affinity chromatography in nitrilotriacetic acid (NTA) columns (Supplemental Figure 4). First, we performed thiol-labeling assays to test the ability of FBPase to reduce 2-Cys Prxs or Trxs *f in vitro* (Figure 7A). In the absence of a reductant, recombinant FBPase showed increased electrophoretic mobility, probably reflecting the formation of the regulatory and additional, non-regulatory, disulfide bond(s), as previously reported (Serrato et al., 2018). After reduction of FBPase with the non-physiological reducing agent dithiothreitol (DTT), which was then removed by gel filtration, most of the enzyme was detected in the reduced form as determined by *N*-ethylmaleimide (NEM) labeling (Figure 7A). FBPase remained in reduced state when incubated during 15 min in the presence of 2-Cys Prx and hydrogen peroxide (Figure 7A). This result indicates that no direct transfer of electrons occurs between the FBPase and 2-Cys Prxs, and that the hydrogen peroxide, here used at a 100 μM concentration, is not able to directly oxidize FBPase. In contrast, FBPase was almost completely oxidized after the addition of Trx *f*1 or *f*2 (Figure 7A), indicating the transfer of electrons from reduced FBPase to Trxs. Finally, the reconstitution of the FBPase oxidation pathway *in vitro* was followed by hydrogen peroxide reduction (Figure 7B). The rate of hydrogen peroxide consumption, which was low in reaction mixtures containing either 2-Cys Prx, 2-Cys Prx plus FBPase or 2-Cys Prx plus Trxs *f*, increased by the addition of reduced FBPase to either Trx *f*1 or *f*2 and 2-Cys Prx (Figure 7B). It should be noted that after two rounds of gel filtration of reduced FBPase, a residual concentration of DTT (~15 μM) was still detected in the FBPase preparation (Supplemental Figure 5A). Nevertheless, control experiments in which reduced FBPase was replaced by 15 μM DTT showed that this concentration of DTT resulted in basal levels of hydrogen peroxide consumption (Supplemental Figure 5B). Therefore, these results support a pathway in which the reducing equivalents from reduced FBPase are transferred to hydrogen peroxide via the sequential participation of Trxs *f* and 2-Cys Prx.

DISCUSSION

A long-lasting question in redox regulation of chloroplast metabolism is the mechanism that allows the rapid oxidation of enzymes involved in biosynthetic metabolic pathways, such as the CBC, in the dark. Schürmann and Buchanan (2008) proposed two key concepts to be considered in the light/darkness redox regulation of enzyme activity in chloroplasts. First, for maintaining enzyme activity in the light, the Fdx-FTR-Trx redox system needs to be kept in a highly reduced state. Second, this "electron pressure" has to be relieved to allow enzyme deactivation in the dark. In a recent report (Pérez-Ruiz et al., 2017), we proposed a central function of 2-Cys Prxs in chloroplast redox regulation. According to this proposal, the redox balance of the 2-Cys Prxs modulates the "electron pressure" of the Fdx-FTR-Trx system (see scheme in Figure 8). In the light, the flux of electrons from NADPH via NTRC controls the redox balance of 2-Cys Prxs. Different chloroplast Trxs are able to transfer reducing equivalents to 2-Cys Prxs (Broin et al., 2002; Collin et al., 2003; Dangoor et al., 2012; Cheng et al., 2014; Eliyahu et al., 2015; Hochmal et al., 2016). However, NTRC is the most efficient reductant of these enzymes (Pérez-Ruiz et al., 2006; Pulido et al., 2010); thus, reducing equivalents are drained from the Fdx-FTR-Trx system to a lesser extent. Consequently, Trx targets, including FBPase, GAPDH, and CF_1-γ, are

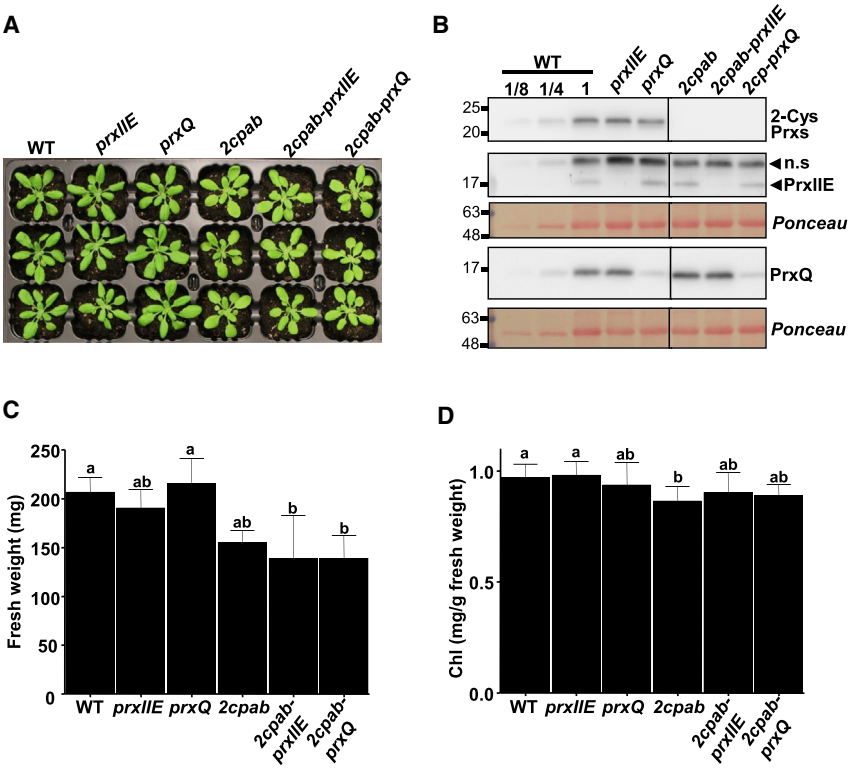

Figure 4. The Phenotype of *Arabidopsis* Mutants Deficient in Chloroplast-Localized Prxs.
(A) Wild-type (WT) and mutant lines, as indicated, were grown under long-day conditions for 4 weeks. **(B–D)** Western blot analysis **(B)** of the content of 2-Cys Prxs, Prx IIE, and Prx Q. Protein extracts were obtained from leaves of plants grown as stated in **(A)** and aliquots of 15 μg (1×), from all lines, and 1/8 and 1/4 dilutions from WT, as indicated, were subjected to SDS–PAGE under reducing conditions, transferred to nitrocellulose filters, and probed with anti-2-Cys Prxs, anti-Prx IIE, or anti-Prx Q antibodies. Even loading was monitored by Ponceau staining of the Rubisco large subunit; molecular mass markers (kDa) are indicated on the left. The vertical lines indicate non-adjacent lanes from the same blot. The weight **(C)** and the content of chlorophylls **(D)** of rosette leaves from at least seven plants of each line are represented as average values ± SE. Letters indicate significant differences with the Tukey test at 95% CI. n.s, non-specific band.

kept in reduced state in the light. 2-Cys Prxs are thiol-dependent peroxidases, hence catalyzing the transfer of electrons from thiolic groups of target enzymes to hydrogen peroxide (Perkins et al., 2015); therefore, 2-Cys Prxs act as intermediates of the redox state of chloroplast stroma enzymes and hydrogen peroxide. Based on these results, we established the hypothesis that the essential function of 2-Cys Prxs in maintaining the ''electron pressure'' of the Fdx-FTR-Trx system in the light might serve to relieve it in the dark. The transfer of reducing equivalents from thiols to hydrogen peroxide, catalyzed by 2-Cys Prxs, would thus provide an explanation for the long-lasting question of how chloroplast enzymes are oxidatively deactivated during the night.

Reports of the deactivation in the dark of well-known redox-regulated CBC enzymes, such as FBPase (Leegood and Walker, 1980), are based on activity assays. However, *Arabidopsis* contains up to three FBPase isoforms that contribute to the detected FBPase activity, but only one of them is redox regulated (Serrato et al., 2009; Rojas-González et al., 2015). Moreover, no indication of the level of reduced and oxidized enzymes can be easily determined with enzyme activity assays. These limitations were overcome in this work by the labeling of thiolic groups with the alkylating agent MM-PEG_{24} followed by the specific detection of the redox-sensitive isoforms of the enzymes by western blotting with specific antibodies. This methodology allowed us to establish that FBPase and GAPDH are highly reduced in light-adapted wild-type and *2cpab* mutant plants and become rapidly oxidized following darkness; however, oxidation was significantly delayed in the *2cpab* mutant (Figure 1A–1D). These results show the participation of 2-Cys Prxs in the short-term enzyme oxidation in the dark, hence lending further support to the central function of 2-Cys Prxs in the control of the chloroplast redox homeostasis in response to light availability, in line with our previous proposal (Pérez-Ruiz et al., 2017).

Given the predominant function of NTRC as the most efficient reductant of 2-Cys Prxs (Pérez-Ruiz et al., 2006; Pulido et al., 2010), it is expected that alterations of the contents of NTRC cause the imbalance of the 2-Cys Prxs, thereby affecting the ''electron pressure'' of the Fd-FTR-Trx system and, thus, of the targets regulated by this system. Indeed, in the *ntrc* mutant, the absence of NTRC provokes the imbalance of the redox state of 2-Cys Prxs, which is more oxidized (Kirchsteiger et al., 2009; Puerto-Galán et al., 2015), hence increasing the drainage of reducing equivalents from the pool of Trxs and, consequently, impairing the redox regulation of their targets (Pérez-Ruiz et al., 2017). In this study, we carried out a different approach to modify the redox balance of 2-Cys Prxs consistent in the overexpression of NTRC. Transgenic lines in which NTRC is in large excess mimic the phenotypes of the *2cpab* mutant in terms of growth (Figure 2A–2C) and delayed short-term oxidation of FBPase and GAPDH in the dark (Figure 3A–3D). Therefore, the short-term dissipation of reducing equivalents from reduced enzymes following darkness is similarly affected by the absence of 2-Cys Prxs and by the additional input of reducing power resulting from the excess of NTRC. These results further support the notion that 2-Cys Prxs participate in the short-term dissipation of reducing equivalents from reduced enzymes following darkness.

The variability of the phenotypic consequences of plants containing high levels of NTRC should be noted. The transgenic lines reported in this work show growth inhibition phenotype under long-day photoperiod (Figure 2A–2C), and pronounced *NTRC* gene silencing under short-day conditions (Supplemental Figure 3A and 3B). This is in contrast with the enhanced growth of rosette leaves shown by short-day-grown *NTRC* overexpression (Toivola et al., 2013; Nikkanen et al., 2016). This phenotype is highly dependent on the light intensity since it was

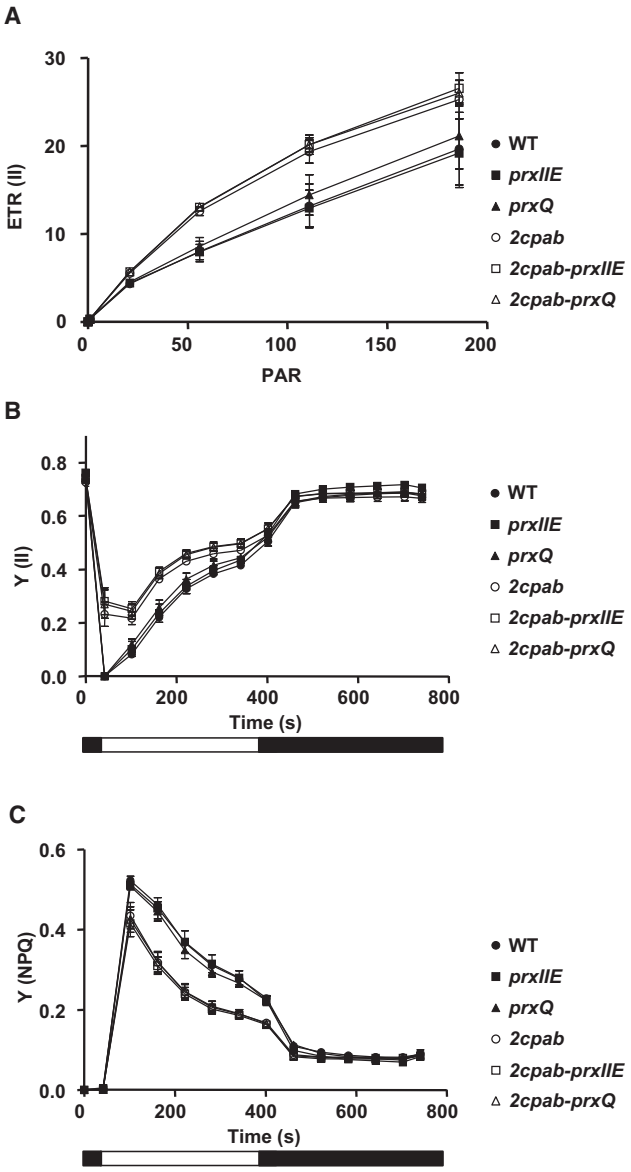

Figure 5. Effect of Deficiencies in Chloroplast Prxs on Photosynthetic Performance.

(A) Linear photosynthetic electron transport rate, ETR (II), was determined at increasing photosynthetic active radiation (PAR). Each value is the average of three determinations and SEs are represented as error bars.

(B and C) Quantum yields of photosystem II, Y(II) **(B)**, and non-photochemical quenching, Y(NPQ) **(C)**, were measured in whole plants grown at 125 μE m^{-2} s^{-1} under long-day conditions and adapted to darkness. Determinations were performed three times and each data point is the mean ± SE. White and black blocks indicate periods of illumination with actinic light (81 μE m^{-2} s^{-1}) and darkness, respectively.

observed when plants were grown at a light intensity of 600 μE m^{-2} s^{-1} (Toivola et al., 2013). In line with these results, the phenotype of plants lacking *NTRC* is highly influenced by growth conditions, such as photoperiod and light intensity (Pérez-Ruiz et al., 2006; Lepistö et al., 2009; Thormählen et al., 2017). Therefore, the phenotypes of plants with different contents of NTRC, ranging from plants devoid of the enzyme to those with high levels, are highly dependent on light intensity, thus confirming the key function of NTRC in the rapid adaptation of chloroplast metabolism to varying light intensity. Furthermore, the similarity of the phenotypes of *NTRC*-overexpressing plants and the *2cpab* mutant suggests that the function of *NTRC* is exerted by the control of the redox balance of 2-Cys Prxs.

The fact that, albeit delayed, chloroplast enzyme oxidation in the dark also occurs in the *2cpab* mutant is indicative that additional mechanisms are operative in the process of dark-dependent oxidation. Here, we have tested the participation of Prx Q and Prx IIE, the other two types of Prxs present in *Arabidopsis* chloroplasts (Liebthal et al., 2018). Single mutants of *Arabidopsis* with severely decreased contents of Prx IIE or Prx Q showed no alteration of the dark-dependent oxidation of chloroplast enzymes (Figure 6A–6D). Moreover, combining these deficiencies with those of 2-Cys Prxs A and B did not modify either the growth phenotype (Figure 4A–4D), photosynthetic performance (Figure 5A–5C), or the redox state of chloroplast enzymes following darkness (Figure 6A–6D), since the phenotypes of the *2cpab-prxIIE* and *2cpab-prxQ* were indistinguishable of the *2cpab* phenotype. Therefore, these results allowed us to discard the possibility that atypical Prxs of the types IIE and Q have a significant function in the mechanism of enzyme deactivation in the dark.

The involvement of 2-Cys Prxs in the dark-induced oxidation of CBC enzymes raises the possibility that these enzymes also participate in the light/darkness redox regulation of additional chloroplast redox-regulated proteins. To explore this issue, we selected the ATPase CF_1-γ subunit, which is rapidly reduced in response to light (Yoshida et al., 2014; Carrillo et al., 2016). The dark-dependent oxidation of the CF_1-γ subunit of ATPase (Figure 1E and 1F) showed remarkable differences with those of FBPase and GAPDH. In wild-type plants, the oxidation of the CF_1-γ subunit in the dark was delayed compared with that of FBPase and GAPDH (Figure 1E and 1F). Interestingly, this protein remained essentially reduced during the time of darkness treatment used in this work in the *2cpab* mutant, indicating that different enzymes of the chloroplast respond in different ways to the redox conditions of the chloroplasts. This behavior of the ATPase CF_1-γ subunit, which is involved in energy production in the chloroplast, may have a physiological meaning since it assures the supply of ATP for biosynthetic metabolic pathways.

Different reports described the inactivation of CBC enzymes by hydrogen peroxide, and based on these results it was proposed that enzyme thiols are directly oxidized by the action of hydrogen peroxide (Kaiser, 1979; Tanaka et al., 1982). If this were the case, it would be expected that in plants devoid of 2-Cys Prxs, which are very abundant chloroplast peroxidases (Konig et al., 2002), hydrogen peroxide would be accumulated at higher levels, thus favoring inactivation of the CBC enzymes. Indeed, previous results have shown that leaves of plants lacking 2-Cys Prxs accumulate increased content of hydrogen peroxide both during the day and at night (Awad et al., 2015). Nevertheless, our results show that enzymes are not directly oxidized by hydrogen peroxide since dark-dependent oxidation of chloroplast enzymes is delayed in the *2cpab* mutant (Figure 1A–1F) and hydrogen

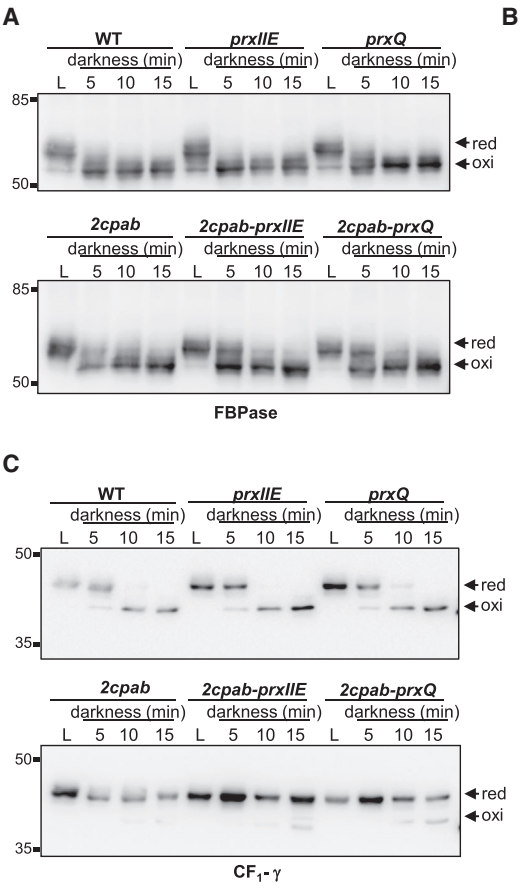

Figure 6. Effect of Deficiencies in Chloroplast Prxs on Dark-Dependent Oxidation of FBPase and CF_1-γ.

Wild-type (WT) and mutant lines, as indicated, were grown under long-day conditions for 4 weeks at a light intensity of 125 μE m^{-2} s^{-1}. At the end of the night period, plants were incubated at a light intensity of 500 μE m^{-2} s^{-1} during 30 min (L), then light was switched off and samples were taken at 5, 10, and 15 min as indicated.

(A and C) The *in vivo* redox state of FBPase **(A)** and CF_1-γ **(C)** were determined with the alkylating agent MM-PEG_{24} as described in Methods.

(B and D) The corresponding band intensities were quantified (GelAnalyzer), and the percentage of reduction is the ratio between the reduced form and the sum of reduced and oxidized forms for each protein. Each value is the mean of three independent experiments ±SE; letters indicate significant differences with the Student's *t*-test at 95% CI. red, reduced; oxi, oxidized. Molecular mass markers (kDa) are indicated on the left.

peroxide, at a concentration of 100 μM, does not oxidize FBPase *in vitro* (Figure 7A). In contrast, our results are in agreement with the proposal that 2-Cys Prxs relay oxidizing equivalents to redox-regulated enzymes as occurs in human cells (Stocker et al., 2017). The oxidizing action of 2-Cys Prxs in enzyme deactivation may take place by two possible pathways: via the direct interaction with their reduced targets, i.e., FBPase and GAPDH, hence directly transferring reducing equivalents of their thiolic groups to hydrogen peroxide, or with the participation of Trxs as redox intermediates. Among the proteins identified as interacting with 2-Cys Prxs, FBPase was found but not GAPDH (Cerveau et al., 2016). However, *in vitro* assays show neither oxidation of reduced FBPase in the presence of 2-Cys Prxs and hydrogen peroxide (Figure 7A) nor consumption of hydrogen peroxide in the presence of FBPase plus 2-Cys Prxs (Figure 7B). In contrast, the addition of Trxs *f*1 or *f*2 provoked the oxidation of FBPase and the increase in the rate of hydrogen peroxide reduction, indicating the participation of Trxs in the process of enzyme deactivation in the dark. In a previous report it was shown that in plants illuminated with moderate light intensity, 2-Cys Prxs drive the oxidation of chloroplast atypical Trx-like ACHT1 (Dangoor et al., 2012). Moreover, the 2-Cys Prx-driven oxidation of ACHT4 was linked to oxidation of the small subunit of ADP-glucose pyrophosphorylase (Eliyahu et al., 2015), hence contributing to fine-tuning starch biosynthesis in response to variable light intensity. Furthermore, while this article was under review, Yoshida et al. (2018) reported the function of thioredoxin-like2 and 2-Cys Prx in oxidative thiol modulation. Our results are in line with these findings, and suggest that the oxidative function of 2-Cys Prxs in chloroplast might be exerted through the high diversity of the Trxs present in this organelle. This is in contrast with the pathway of thiol oxidation proposed in human cells in which Trx is not needed (Stocker et al., 2017).

In summary, our results demonstrate the significant participation of 2-Cys Prxs, but not of Prx IIE and Prx Q, in the short-term oxidation of chloroplast enzymes in the dark. Moreover, the fact that overexpression of *NTRC* mimics the phenotype and delayed oxidation of FBPase and GAPDH of the *2capb* mutant in the dark further confirms the essential function of 2-Cys Prxs in chloroplast redox homeostasis and indicates that the function of NTRC is the maintenance of redox balance of the 2-Cys Prxs. Our results are compatible with the model proposed in Figure 8. In this proposed model, NADPH, which can be produced from reduced Fdx in the light, by the activity of Fdx-$NADP^+$ reductase (FNR), or from sugars in the dark, by the oxidative pentose phosphate pathway, maintains the redox balance of the NTRC-2-Cys Prxs system. In this way, the "electron pressure" of the FTR-Trxs redox system is maintained and, consequently, the reductive activation of their targets. In the dark, this electron pressure is rapidly relieved. Our results suggest that oxidation occurs by a transfer pathway in which the reducing equivalents of the reduced thiols of the enzymes are transferred via Trxs to 2-Cys Prxs and then to hydrogen peroxide, which is thus the final sink of the electrons (Figure 8). This mechanism is operative in the short term, up to 15 min of darkness, and may have the physiological function of rapid adaptation of the chloroplast electron pressure to rapid and unpredictable changes in light intensity in nature. Our results also show the existence of additional mechanism(s) for enzyme oxidation in the dark. However, although we can rule out a significant role of Prxs IIE and Prx Q in this additional mechanism, its nature remains unknown.

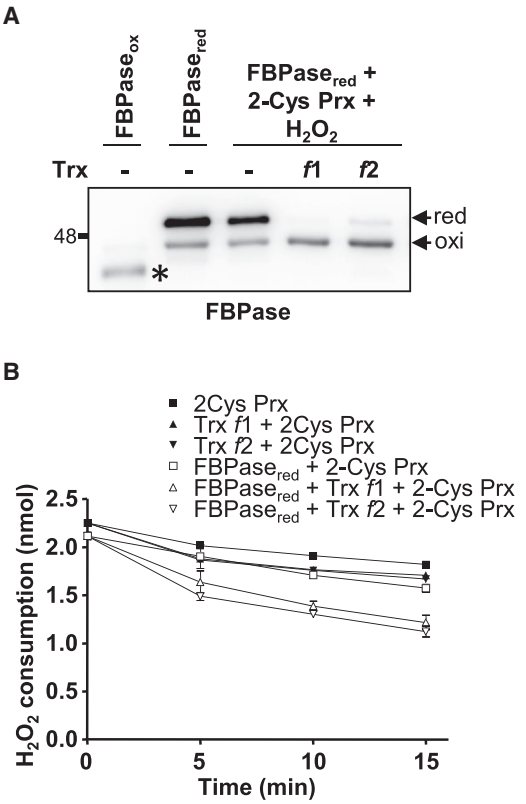

Figure 7. Reconstitution of the Pathway of Oxidation of Reduced FBPase *In Vitro*.

Recombinant FBPase was reduced by treatment with the reducing agent dithiothreitol (DTT) (see Methods). Reaction mixtures contained, as indicated, FBPase (5 μM), in the presence or absence of Trx *f*1 or *f*2 (15 μM), 2-Cys Prx (40 μM), and H_2O_2 (100 μM). After an incubation of 15 min at room temperature, the level of reduced/oxidized FBPase was determined by alkylation with NEM and SDS–PAGE analysis **(A)**. Molecular mass markers (kDa) are indicated on the left. Labels red and oxi refer to the dithiol and disulfide forms of regulatory cysteine residues. Asterisk denotes the fully oxidized form of FBPase, containing regulatory and non-regulatory disulfide bonds. In parallel, the rate of hydrogen peroxide consumption in reaction mixtures containing the indicated proteins was followed by the ferrous ion oxidation assay. Each value is the mean of at least three independent experiments ± SE **(B)**. red, reduced; oxi, oxidized.

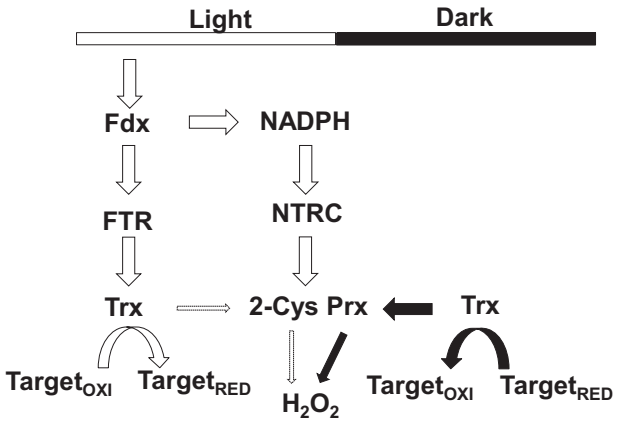

Figure 8. Scheme Depicting the Function of 2-Cys Prxs in Chloroplast Enzyme Redox Regulation in Response to Light and Darkness.

In the light, the photosynthetic electron transport chain produces reduced Fdx, which fuels reducing equivalents for the light-dependent reduction of stromal target enzymes via FTR and Trxs. The redox balance of 2-Cys Prxs is maintained by NTRC using NADPH, which is formed from reduced Fdx, by the action of FNR, or from sugars by the oxidative pentose phosphate pathway. Although chloroplast Trxs are able to transfer reducing power to 2-Cys Prxs, the rate is lower than that of NTRC and, thus, the "electron pressure" of the FTR-Trxs is maintained. In the dark, the input of reducing equivalents as reduced Fdx ceases and reduced stromal targets become oxidized by transferring reducing equivalents via Trxs and 2-Cys Prxs to hydrogen peroxide. RED, reduced; OXI, oxidized.

METHODS

Biological Material and Growth Conditions

A. thaliana wild-type (ecotype Columbia) and mutant plants (Supplemental Table 1) were routinely grown in soil in growth chambers under long-day (16 h light/8 h darkness) or short-day (8 h light/16 h darkness) conditions at 22°C and 20°C during light and dark periods, respectively, and light intensity of 125 μE m^{-2} s^{-1}. *Arabidopsis* mutants *prxIIE* (Romero-Puertas et al., 2007) and *prxQ* (Lamkemeyer et al., 2006) were described previously. For generation of the *2cpab* double mutant, the *2cpa* (Pérez-Ruiz et al., 2017) and *2cpb* (Kirchsteiger et al., 2009) single mutants were manually crossed and seeds resulting from this cross were checked for heterozygosity of the T-DNA insertions in the *2CPA* and *2CPB* genes. Plants were then self-pollinated and the double homozygous line was identified in the progeny by PCR analysis of genomic DNA (Supplemental Figure 1) with oligonucleotides listed in Supplemental Table 2. The *2cpab-prxIIE* and *2cpab-prxQ* triple mutants (Supplemental Table 1) were obtained by manual crossing and identified by PCR analysis of genomic DNA (Supplemental Figure 1) with oligonucleotides listed in Supplemental Table 2. *E. coli* and *Agrobacterium tumefaciens* were grown in liquid Miller nutrient at 37°C and 28°C, respectively, with the appropriate antibiotics.

Generation of *Arabidopsis* Transgenic Plants Overexpressing *NTRC*

Total RNA was isolated from wild-type *Arabidopsis* leaves with TRIzol reagent (Invitrogen) and cDNA was synthesized with the Maxima first-strand cDNA synthesis kit (Fermentas). For the generation of transgenic plants, the NTRC cDNA was amplified with iProof High-Fidelity DNA Polymerase (Bio-Rad) using oligonucleotides specified in Supplemental Table 3, which added attB recombination sites at the 5′ and 3′ ends, respectively. PCR product was cloned in the Gateway compatible vector pDONR207 (Invitrogen), sequenced and transferred to the 35S overexpression vector pEARLEYGATE100 using LR clonase (Invitrogen). Plasmids were then transformed into the *A. tumefaciens* strain GV3101, which were used for plant transformation (Clough and Bent, 1998).

Cloning, Expression, and Purification of FBPase and *f*-type Trxs from *Arabidopsis*

For the expression of recombinant proteins, the cDNAs encoding Trx *f*1, Trx *f*2, and the redox-sensitive isoform of FBPase, excluding the predicted transit peptides, were amplified with iProof High-Fidelity DNA Polymerase (Bio-Rad) using oligonucleotides specified in Supplemental Table 3, which added *Bam*HI and *Pst*I sites at the 5′ and 3′ ends, respectively. PCR products were gel-purified, cloned in pGEMt vector (Promega), and sequenced. pGEMt-derived plasmids were digested with *Bam*HI and *Pst*I, subcloned in the pQE30 (Qiagen) expression vector, and introduced into *E. coli* XL1Blue. Overexpressed recombinant proteins, containing a His tag at the N terminus, were purified by NTA affinity chromatography in Hi-Trap affinity columns (GE Healthcare). Recombinant His-tagged

2-Cys Prx from rice was obtained as previously described (Pérez-Ruiz et al., 2006).

Reconstitution of the Oxidation Pathway of FBPase *In Vitro*

Recombinant FBPase was reduced by treatment with 50 mM DTT for 15 min, and excess DTT was removed by two steps of gel filtration using SpinTrap columns (GE Healthcare) following the manufacturer's instructions. After each chromatography step, the concentration of DTT was measured at 412 nm using the DTNB (5,5′-dithiobis(2-nitrobenzoic acid)) assay. The determination of the redox state of FBPase in reaction mixtures was performed as described previously (Ojeda et al., 2017) using 40 mM NEM (Sigma). Aliquots of protein samples were subjected to SDS–PAGE under non-reducing conditions using acrylamide gel concentration of 8.5%. Resolved proteins were transferred to nitrocellulose membranes and probed with the FBPase antibody. The consumption of hydrogen peroxide in reaction mixtures was measured spectrophotometrically at 560 nm using the ferrous ion oxidation assay in the presence of xylenol orange (Wolff, 1994).

Protein Extraction, Alkylation Assays, and Western Blot Analysis

Plant tissues were ground under liquid nitrogen. For SDS–PAGE, extraction buffer (50 mM Tris–HCl [pH 8.0], 0.15 M NaCl, 0.5% [v/v] Nonidet P-40) was immediately added, mixed on a vortex, and centrifuged at 16 100 *g* at 4°C for 20 min. Protein was quantified using the Bradford reagent (Bio-Rad). Alkylation assays were performed as previously described (Naranjo et al., 2016a) using MM-PEG_{24} from Thermo Scientific. Protein samples were subjected to SDS–PAGE under reducing or non-reducing conditions using acrylamide gel concentration of 9.5% (FBPase, GAPDH, and CF_1-γ), 10% (NTRC and 2-Cys Prxs), and 14% (Prx IIE, Prx Q and 2-Cys Prxs). Resolved proteins were transferred to nitrocellulose membranes and probed with the indicated antibody. Specific antibodies for NTRC (Serrato et al., 2004) and 2-Cys Prx (Pérez-Ruiz et al., 2006) were previously raised in our laboratory. The anti-CF_1-γ antibody was purchased from Agrisera (Sweden). The anti-FBPase, anti-GAPDH, and anti-Prx IIE antibodies were respectively provided by Dr. M. Sahrawy (Estación Experimental del Zaidín, Granada, Spain), Prof. R. Scheibe (University of Osnabrück, Germany) and Dr. N. Rouhier (Lorraine University-INRA, Nancy, France).

Determination of Chlorophylls and Measurements of Chlorophyll *a* Fluorescence

Chlorophyll levels were measured as previously described (Pérez-Ruiz et al., 2006). Room-temperature chlorophyll *a* fluorescence and chlorophyll fluorescence imaging of whole rosettes were performed using a pulse-amplitude modulation fluorometer (IMAGING-PAM M-Series instrument; Walz, Effeltrich, Germany). Induction–recovery curves were performed using blue (450 nm) actinic light (81 μE m^{-2} s^{-1}) at the intensities specified for each experiment during 6 min. Saturating pulses of blue light (10 000 μE m^{-2} s^{-1}) and 0.6-s duration were applied every 60 s, and recovery in darkness was recorded for another 6 min. The parameters Y(II) and Y(NPQ), corresponding to the respective quantum yields of PSII photochemistry and non-regulated basal quenching, were calculated by the ImagingWin v2.46i software according to the equations in Kramer et al. (2004). Measurements of relative linear electron transport rates were based on chlorophyll fluorescence of pre-illuminated plants applying stepwise increasing actinic light intensities up to 186 μE m^{-2} s^{-1}.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

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AUTHOR CONTRIBUTIONS

J.M.P.-R. and F.J.C. designed the research; V.O. and J.M.P.-R. performed the experiments; V.O., J.M.P.-R., and F.J.C. analyzed the data; F.J.C. wrote the paper.

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