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The NADPH-Dependent Thioredoxin Reductase C–2-Cys Peroxiredoxin Redox System Modulates the Activity of Thioredoxin *x* in Arabidopsis Chloroplasts

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The chloroplast redox network is composed of a complex set of thioredoxins (Trxs), reduced by ferredoxin (Fdx) via a Fdx-dependent Trx reductase (FTR), and an NADPH-dependent Trx reductase with a joint Trx domain, NTRC, which efficiently reduces 2-Cys peroxiredoxins (2-Cys Prxs). Recently, it was proposed that the redox balance of 2-Cys Prxs maintains the redox state of *f*-type Trxs, thus allowing the proper redox regulation of Calvin–Benson cycle enzymes such as fructose 1,6-bisphosphatase (FBPase). Here, we have addressed whether the action of 2-Cys Prxs is also exerted on Trx *x*. To that end, an *Arabidopsis thaliana* quadruple mutant, *ntrc-trxx-Δ2cp*, which is knocked out for NTRC and Trx *x*, and contains severely decreased levels of 2-Cys Prxs, was generated. In contrast to *ntrc-trxx*, which showed a severe growth inhibition phenotype and poor photosynthetic performance, the *ntrc-trxx-Δ2cp* mutant showed a significant recovery of growth rate and photosynthetic efficiency, indicating that the content of 2-Cys Prxs is critical for the performance of plants lacking both NTRC and Trx *x*. Light-dependent reduction of FBPase was severely impaired in mutant plants lacking NTRC or NTRC plus Trx *x*, despite the fact that neither NTRC nor Trx *x* is an effective reductant of this enzyme. However, FBPase reduction was recovered in the *ntrc-trxx-Δ2cp* mutant. Our results show that the redox balance of 2-Cys Prxs, which is mostly dependent on NTRC, modulates the activity of Trx *x* in a similar way as *f*-type Trxs, thus suggesting that the activity of these Trxs is highly interconnected.

Keywords: Arabidopsis • Chloroplast • Peroxiredoxin • Redox regulation • Thioredoxin.

Abbreviations: ETR, electron transport rate; FBPase, fructose 1,6-bisphosphatase; Fdx, ferredoxin; FTR, ferredoxin-dependent thioredoxin reductase; NPQ, non-photochemical quenching; NTRC, NADPH-dependent thioredoxin reductase C; Prx, peroxiredoxin; Trx, thioredoxin.

Introduction

Thiol-dependent redox regulation of enzyme activity plays an essential role in the ability of chloroplast metabolism to respond rapidly to unpredictable changes in light intensity. This regulatory mechanism is largely based on the protein disulfide reductase activity of thioredoxins (Trxs). In clear contrast to heterotrophic organisms, which contain a small number of Trxs, the number of these enzymes in chloroplasts is much larger (Meyer et al. 2012, Geigenberger et al. 2017). The first chloroplast Trxs identified were assigned the types *f* and *m*, based on their ability to reduce fructose 1,6-bisphosphatase (FBPase) and NADP-malate dehydrogenase (NADP-MDH), respectively (Meyer et al. 2012, Buchanan 2016). Although *f*-type Trxs have been considered to have a prevalent role in light-dependent redox regulation of enzymes of the Calvin–Benson cycle, recent evidence indicated that *m*-type Trxs also play an important role in the redox regulation of this important metabolic pathway (Okegawa and Motohashi 2015). Indeed, the light-dependent redox regulation of several chloroplast enzymes including FBPase and Trxs *f*2 and *m*2 has recently been reported (Yoshida et al. 2014). The availability of the genome sequence from Arabidopsis and rice led to the identification of three additional types of Trxs termed *x*, *y* and *z*. Initial in vitro analyses revealed better efficiency of Trxs *x* and *y* for 2-Cys peroxiredoxin (2-Cys Prx) reduction, as compared with Trxs *m* and *f*, and thus an antioxidant function was assigned for these Trxs (Collin et al. 2003, Collin et al. 2004), a notion further supported by the finding that type *y* Trxs are efficient reductants of methionine sulfoxide reductase (Vieira Dos Santos et al. 2007) and Prx Q (Yoshida et al. 2015). However, more recent analyses showed similar efficiencies of Trx *f*1, Trx *m*2 and Trx *x* for 2-Cys Prx reduction (Yoshida and Hisabori 2016). Finally, although it was shown that Trx *z* is a component of the plastid-encoded RNA polymerase (PEP) complex playing an essential role in chloroplast development (Arsova et al. 2010), this Trx is also able to reduce Prx Q in vitro (Yoshida et al. 2015). The source of reducing power for chloroplast Trxs is ferredoxin (Fdx) reduced by the photosynthetic electron transport chain with the participation of a plastid-specific Fdx-dependent Trx reductase (FTR), forming the FTR–Trxs redox system, which thus links chloroplast redox regulation to light (Schürmann and Buchanan 2008).

In addition to this redox system, chloroplasts harbor an NADPH-dependent Trx reductase with a joint Trx domain, termed NTRC (Serrato et al. 2004). Initially, it was proposed

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that NTRC exerts an antioxidant function since it is an efficient reductant of 2-Cys Prxs, enzymes with thiol-dependent peroxidase activity (Moon et al. 2006, Pérez-Ruiz et al. 2006, Alkhalfioui et al. 2007, Pérez-Ruiz and Cejudo 2009). However, further analyses of an *Arabidopsis thaliana* mutant knockout for NTRC shows the participation of this enzyme in the redox regulation of chloroplast metabolic pathways previously known to be Trx dependent, which include starch (Michalska et al. 2009, Lepistö et al. 2013) and tetrapyrrole (Stenbaek et al. 2008, Richter et al. 2013, Pérez-Ruiz et al. 2014) biosynthesis. Furthermore, the deficiency of NTRC exerts a severe impairment of the efficiency of light energy utilization (Carrillo et al. 2016, Naranjo et al. 2016a), which might, at least in part, explain the growth inhibition phenotype of the *ntrc* mutant.

Altogether, these findings indicate that chloroplast redox regulation is more complex than previously anticipated, being controlled by two redox systems, the classical FTR–Trxs system, which relies on reduced Fdx, hence being light dependent, and NTRC, which relies on NADPH and might be operative also during the night using NADPH generated from sugars via the oxidative pentose phosphate pathway (Spínola et al. 2008, Cejudo et al. 2012). Thus, a relevant issue in chloroplast redox biology is to establish the relationship between the FTR–Trxs and NTRC redox systems. While Arabidopsis mutants knocked out for *f*-type Trxs show a phenotype very similar to the wild type (Yoshida et al. 2015, Naranjo et al. 2016b), mutants simultaneously devoid of NTRC and Trxs *f* show severe growth inhibition and very decreased photosynthetic performance (Thormählen et al. 2015, Ojeda et al. 2017), indicating that NTRC and Trxs *f* act together, a notion further supported by the finding that mutants simultaneously devoid of NTRC and FTR are inviable when grown under autotrophic conditions (Yoshida and Hisabori 2016). An additional issue to be taken into account is that NTRC is an efficient reductant of 2-Cys Prxs, suggesting a possible relationship between antioxidant mechanisms and redox regulation. In a recent report (Pérez-Ruiz et al. 2017), we have shown that decreased levels of 2-Cys Prxs exert a suppressor effect on the phenotypes of the *ntrc* and *ntrc-trxf1f2* mutants, leading to the proposal that NTRC participates in the redox regulation of chloroplast enzymes that depend on *f*-type Trxs through the control of the redox balance of 2-Cys Prxs.

Despite the fact that in vitro assays with purified proteins showed the activity of Trx *x* as a reductant of 2-Cys Prxs (Collin et al. 2003), an Arabidopsis mutant knocked out for Trx *x* shows a wild-type phenotype and no alteration of the redox state of 2-Cys Prxs (Pulido et al. 2010). However, an Arabidopsis mutant simultaneously devoid of Trx *x* and NTRC, the *ntrc-trxx* double mutant, shows a severe growth inhibition phenotype and strongly impaired photosynthetic performance, which is critical during early stages of plant development (Ojeda et al. 2017), thus resembling the phenotype of the *ntrc-trxf1f2* triple mutant. These results suggest that the regulatory action of NTRC is exerted not only on *f*-type Trxs, but also on Trx *x*. However, the mechanism that allows this ubiquitous function of NTRC remains poorly understood. Here, we have addressed this issue by the generation and characterization of the Arabidopsis mutant *ntrc-trxx-Δ2cp*, which is knocked out for NTRC and Trx *x* and contains decreased levels of 2-Cys Prxs. Our results show that the NTRC–2-Cys Prxs system modulates the redox state of different Trx types, and support the central function of 2-Cys Prxs in chloroplast redox regulation.

Results

Decreased contents of 2-Cys Prxs suppress the phenotype of mutant plants lacking NTRC and Trx *x*

In a previous report (Pérez-Ruiz et al. 2017), we have shown that the phenotype of Arabidopsis mutant plants devoid of NTRC or simultaneously devoid of NTRC and *f*-type Trxs is highly dependent on the contents of 2-Cys Prxs. With the purpose of testing whether the redox balance of 2-Cys Prxs might act as a central hub in chloroplasts able to maintain the reducing capacity of different Trxs, we have extended these analyses to plants simultaneously deficient in NTRC and Trx *x*, which also show a severe growth inhibition phenotype (Ojeda et al. 2017). To that end, the Arabidopsis *ntrc-trxx-Δ2cp* quadruple mutant was generated (**Fig. 1A**). This quadruple mutant is knocked out for NTRC, Trx *x* and 2-Cys Prx B but contains a low amount of 2-Cys Prx A (~6% of the content of 2-Cys Prxs in the wild type), as determined by Western blot (**Fig. 1B, C**). Unfortunately, no anti-Trx *x* antibodies are available and, thus, the lack of Trx *x* was tested by quantitative reverse transcription–PCR (RT–qPCR) analysis, which confirmed the absence of transcripts of the gene in the *trxx* mutant backgrounds (**Fig. 1D**). Compared with the *Δ2cp* mutant, the levels of *2CPA* transcripts (**Fig. 1D**) and protein (**Fig. 1B, C**) are increased in the *ntrc-trxx-Δ2cp* mutant, indicating that the absence of NTRC and Trx *x* exerts a positive effect on the expression of this gene in a *Δ2cp* genetic background. Interestingly, the performance of the *ntrc-trxx-Δ2cp* mutant is much better than that of the *ntrc-trxx* mutant, since this mutant recovered a growth phenotype similar to that of the *Δ2cp* mutant (**Fig. 1A**), as shown by the rosette fresh weight (**Fig. 2A**) and leaf Chl content (**Fig. 2B**). The suppressor effect is also exerted at early stages of plant development as seedlings of the *ntrc-trxx-Δ2cp* mutant recovered the wild-type rate of root growth in synthetic media lacking an exogenous carbon source (**Fig. 2C**), and the timing of appearance of true leaves, which was clearly delayed in the *ntrc-trxx* mutant (**Fig. 2D**). Therefore, these results show that decreased contents of 2-Cys Prxs suppress the severe growth inhibition phenotype of plants simultaneously lacking NTRC and Trx *x*.

The analysis of the photochemical parameters indicates that the recovery of the growth phenotype of the *ntrc-trxx-Δ2cp* mutant is in agreement with the recovery of the photosynthetic performance in this line. The *ntrc-trxx* double mutant shows even lower efficiency of light energy utilization than the *ntrc* mutant, as revealed by the severely decreased photosynthetic electron transport rate [ETR(II)] (**Fig. 3A**), reduced quantum yield of PSII photochemistry, Y(II) (**Fig. 3B, C**) and increased non-photochemical quenching [Y(NPQ)] (**Fig. 3B, D**). While

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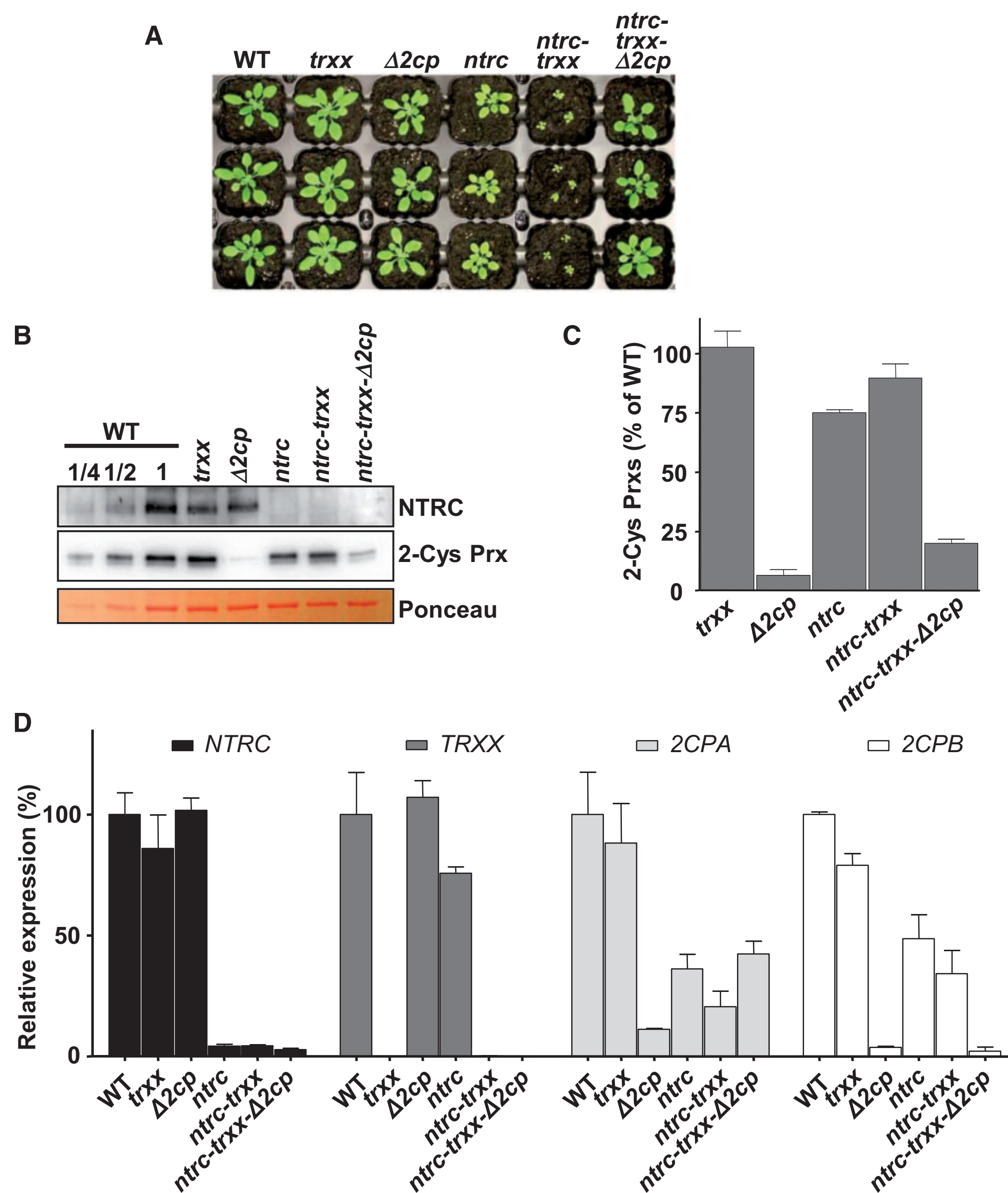

Fig. 1 The phenotype of the Arabidopsis *ntrc-trxx* mutant is partially suppressed by a decreased content of 2-Cys Prxs. (A) Plants of the wild type and mutant lines, as indicated, grown under long-day conditions for 4 weeks. (B) Western blot analysis of the levels of NTRC and 2-Cys Prxs. Protein extracts were obtained from leaves of plants grown as stated in (A) and aliquots (3 μg of protein) from wild-type (WT) (1) and mutant lines, as well as 1/2 and 1/4 dilutions from the WT extract, as indicated, were subjected to SDS–PAGE under reducing conditions, transferred to nitrocellulose filters and probed with anti-NTRC or anti-2-Cys Prx antibodies. (C) Band intensities of 2-Cys Prxs and Ponceau were quantified (ScionImage) and the ratios between them, referred to the WT sample of 3 μg (arbitrarily assigned a value of 100), are indicated as the average ± SD of four determinations (two technical replicates of two independent biological replicates). (D) The levels of transcripts of the genes encoding NTRC, Trx *x*, 2-Cys Prx A and 2-Cys Prx B were determined by RT–qPCR using the oligonucleotides indicated in Supplementary Table S3 and normalized against two reference genes (as indicated in the Materials and Methods). Relative expression was referred to the level of transcripts of each of the genes in wild-type plants, arbitrarily considered as 100%. Determinations were performed three times and mean values ± SEM are represented.

the *ntrc-trxx-Δ2cp* mutant showed higher Y(II) and lower Y(NPQ) than the *ntrc* mutant, the photosynthetic ETR at growth light intensities (125 μE m^{-2} s^{-1}) was similar to that observed in the *ntrc* mutant (**Fig. 3A**). Interestingly, the photosynthetic performance was improved in the *Δ2cp* mutant and diminished in the *trxx* mutant when compared with wild-type plants as shown by the ETR (**Fig. 3A**), Y(II) (**Fig. 3B, C**) and Y(NPQ) (**Fig. 3B, D**). Finally, the ratio of variable fluorescence to maximal fluorescence (F_v/F_m) was clearly reduced in the *ntrc* and, to a higher extent, in *ntrc-trxx* mutants, but not in the *ntrc-trxx-Δ2cp* suppressed line (**Table 1**), further indicating the suppressor effect of decreased contents of 2-Cys Prxs on the phenotype of plants simultaneously devoid of NTRC and Trx *x*. Overall, these results indicate that the dose of 2-Cys Prxs is critical for the phenotype of NTRC-deficient plants, which is not strictly dependent on the photosynthetic performance.

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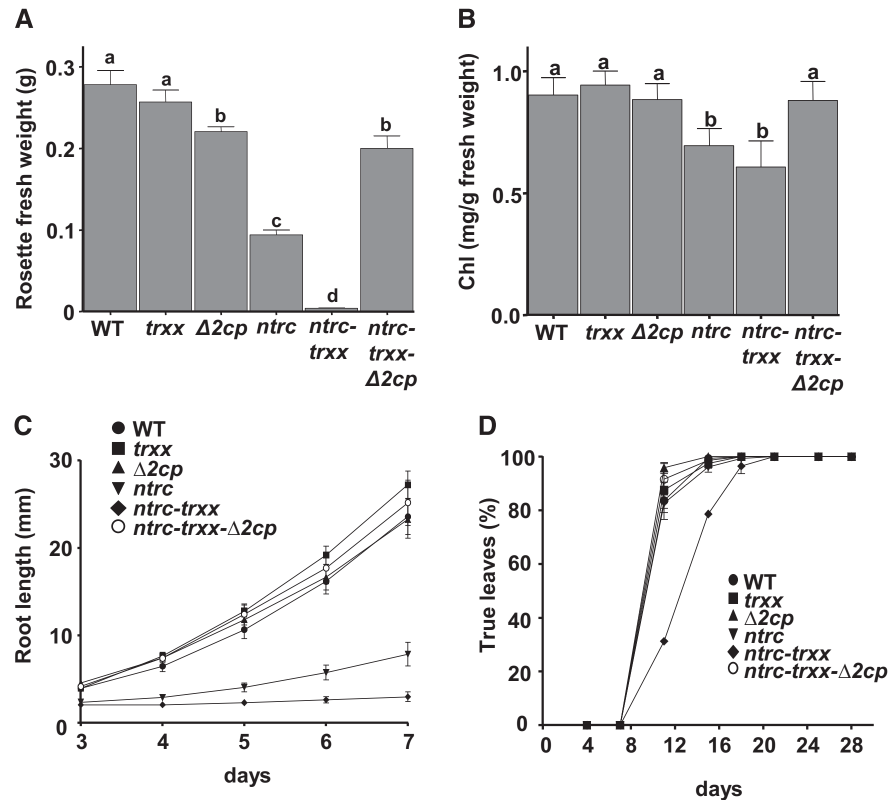

Fig. 2 The suppressor effect of decreased contents of 2-Cys Prxs is exerted during early stages of plant development. (A) The weight of the rosette from 11 plants per line grown under long-day conditions for 4 weeks, is represented as average values ± SEM, with letters indicating significant differences (Student's *t*-test at a 95% confidence interval). (B) Chl contents determined from the same plants are represented as average values ± SEM. Letters indicate significant differences with the Tukey test at a 99% confidence interval. (C) Seedlings of the wild type and mutant lines, as indicated, were grown for 7 d on MS synthetic medium in the absence of sucrose under continuous light (125 μE m^{-2} s^{-1}). Root growth was monitored at the indicated time and mean values ± SEM from three replicates are represented. (D) Seeds of the wild type and mutant lines were allowed to germinate on soil. For each line, the percentage of seedlings with emerging true leaves on the indicated day were determined and represented as means values ± SEM from three replicates.

For further testing the relevance of the content of 2-Cys Prxs for the function of the chloroplast redox systems, the Arabidopsis *ntrc-trxx-Δ2cp* mutant was transformed with a construct driving the expression of a cDNA encoding 2-Cys Prx A by the *Cauliflower mosaic virus* (CaMV) 35S promoter. Two independent transgenic lines with increased contents of 2-Cys Prx A in the *ntrc-trxx-Δ2cp* mutant background (**Fig. 4A–C**) partially restored the growth inhibition phenotype displayed by the *ntrc-trxx* line, as shown by the reduced rosette size (**Fig. 4A**), decreased Y(II) and increased Y(NPQ) (**Fig. 4D**) in these transgenic lines. Therefore, these results further confirm that the contents of 2-Cys Prxs determine the phenotypic traits of plants devoid of NTRC and Trx *x*, in line with the previously reported effect on the phenotype of plants lacking NTRC and *f*-type Trxs (Pérez-Ruiz et al. 2017) and allow the conclusion that the regulatory action of 2-Cys Prxs is exerted on both types of Trx.

The redox balance of 2-Cys Prxs underlies the regulatory function of Trxs *x* and *f*

To gain more insight into the functional relationship of NTRC, Trx *x* and *f*-type Trxs in the redox regulation of chloroplast enzymes, we analyzed the in vivo redox state of FBPase, a well-established redox-regulated enzyme of the Calvin–Benson cycle which is known to be target of *f*-type Trxs, but not of Trx *x* (Serrato et al. 2013). In agreement with previous results (Naranjo et al. 2016b), labeling of the reduced form of FBPase with the alkylating agent methyl maleimide polyethylene glycol [(MM(PEG_{24})] showed that this enzyme is fully oxidized in dark-adapted leaves and becomes partially reduced after 30 min of illumination at a growth light intensity of 125 μE m^{-2} s^{-1} (**Fig. 5A, B**). Compared with the wild type, the level of light-dependent reduction of FBPase, which was slightly lower (though not statistically significant) in the *trxx* mutant, was clearly increased in the *Δ2cp* mutant (**Fig. 5A, B**). Mutant plants lacking NTRC or NTRC plus Trx *x* showed a very severe impairment of light-dependent FBPase reduction, which was recovered up to *trxx* levels in the quadruple mutant *ntrc-trxx-Δ2cp* (**Fig. 5A, B**). Likewise, the degree of reduction of *f*-type Trxs in response to light reflected that of FBPase in all lines analyzed. It was slightly decreased and enhanced, respectively, in the *trxx* and *Δ2cp* mutants, severely impaired in the *ntrc* and *ntrc-trxx* mutants, and partially recovered in the *ntrc-trxx-Δ2cp* mutant (**Fig. 5A, B**). Since in vitro assays showed that FBPase is

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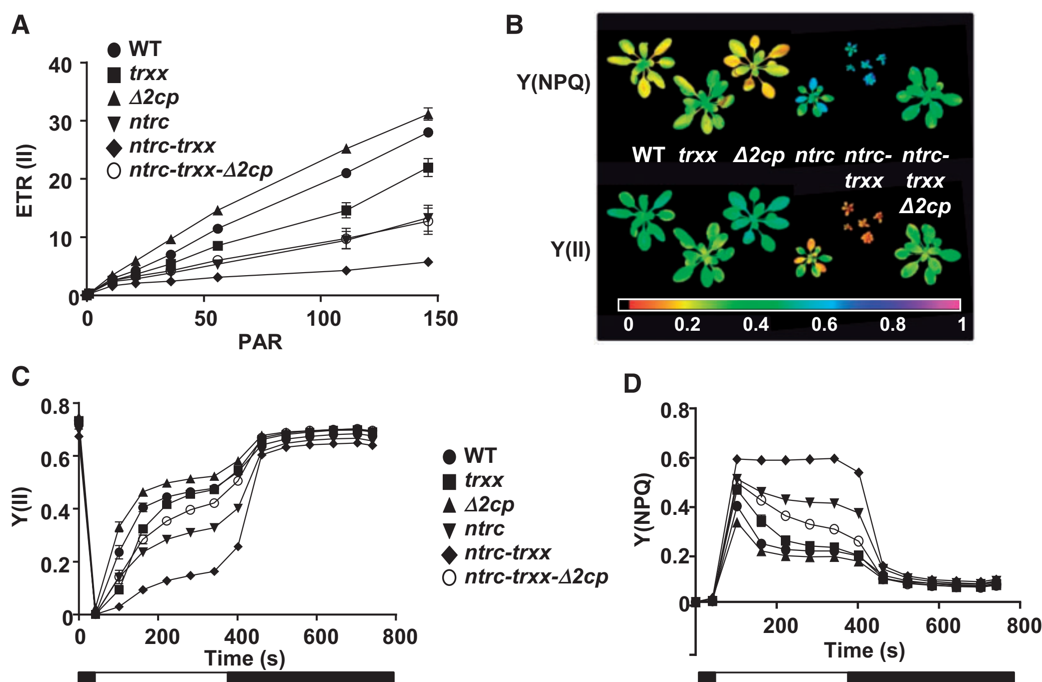

Fig. 3 Photosynthetic performance in Arabidopsis wild type and mutant lines. (A) The linear photosynthetic electron transport rate, ETR(II), was determined at increasing photosynthetically active radiation (PAR). Each value is the average of four determinations, except for *ntrc-trxx* which is the average of five determinations, and the SEM is represented as error bars. (B) False-color images representing quantum yields of non-photochemical quenching, Y(NPQ), and PSII photochemistry, Y(II), of plants of the wild type and mutant lines grown under long-day conditions for 4 weeks. For each parameter, signal intensities (from 0 to 1.0) are indicated according to the color scale bar. (C) Quantum yields of PSII photochemistry, Y(II) and (D) non-photochemical quenching, Y(NPQ), 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, except for mutant *ntrc-trxx* which were performed 15 times, and each data point is the mean ± SEM. White and black blocks indicate periods of illumination with actinic light (81 µE m^{-2} s^{-1}) and darkness, respectively.

Table 1 Effect of the combined deficiencies of NTRC, Trx *x* and 2-Cys Prxs on F_v/F_m

	WT	*trxx*	*Δ2cp*	*ntrc*	*ntrc-trxx*	*ntrc-trxx-Δ2cp*
F_v/F_m	0.76 ± 0.01a	0.76 ± 0.01a	0.76 ± 0.01a	0.70 ± 0.02b	0.67 ± 0.01c	0.76 ± 0.01a

The maximum PSII quantum yield was determined as the ratio of variable fluorescence (F_v) to maximal fluorescence (F_m), F_v/F_m, in dark-adapted leaves of plants grown under long-day conditions. The F_v/F_m values (±SD) are the average of 12 measurements. Letters indicate significant differences with the Student's *t*-test at a confidence interval of 99.9%.

not reduced by NTRC and poorly reduced by Trx *x* (Ojeda et al. 2017), the severe impairment of the light-dependent redox regulation of FBPase, and *f*-type Trxs, in the *ntrc* and *ntrc-trxx* mutants and to a lower extent in the *trxx* mutant, suggests an indirect effect of NTRC on the redox regulation of FBPase via *f*-type Trxs.

Remarkably, decreased contents of 2-Cys Prxs show a positive effect on the level of reduced FBPase and *f*-type Trxs in response to light, as occurs in the *Δ2cp* and the *ntrc-trxx-Δ2cp* mutants when compared with the wild type and *ntrc-trxx* mutant, respectively (**Fig. 5A, B**). These pieces of evidence suggest an important function of 2-Cys Prxs in the light-dependent redox regulation of these chloroplast enzymes. As suggested by our previous proposal (Pérez-Ruiz et al. 2017), a likely possibility is that the content of 2-Cys Prxs determines the extent of the draining of reducing equivalents from the pool of Trxs, hence affecting the redox state of Trxs *f*. To test this possibility, the changes in the in vivo redox state of 2-Cys Prxs were determined in dark–light transitions. Alkylation experiments identified two shifted bands of 2-Cys Prxs (**Fig. 6A**). Arabidopsis 2-Cys Prxs A and B contain exclusively the two cysteine residues, peroxidatic and resolving, that form part of their active site. The catalytic form of the enzyme is arranged as a head-to-tail homodimer, which in its fully oxidized form is linked by two intermolecular disulfide bridges involving the peroxidatic cysteine of one subunit with the resolving cysteine of the other subunit (Puerto-Galán et al. 2013), and no shifted bands are expected in alkylation assays. In the fully reduced form of the enzyme, the two disulfides are reduced; thus, the two cysteine residues in each monomer are in its thiolic form (2SH) and are labeled by the alkylating agent, hence producing a shifted band with the lowest electrophoretic mobility. In addition, the enzyme can be detected in half reduced form, when only one of the disulfides is reduced; thus, one cysteien residue in each

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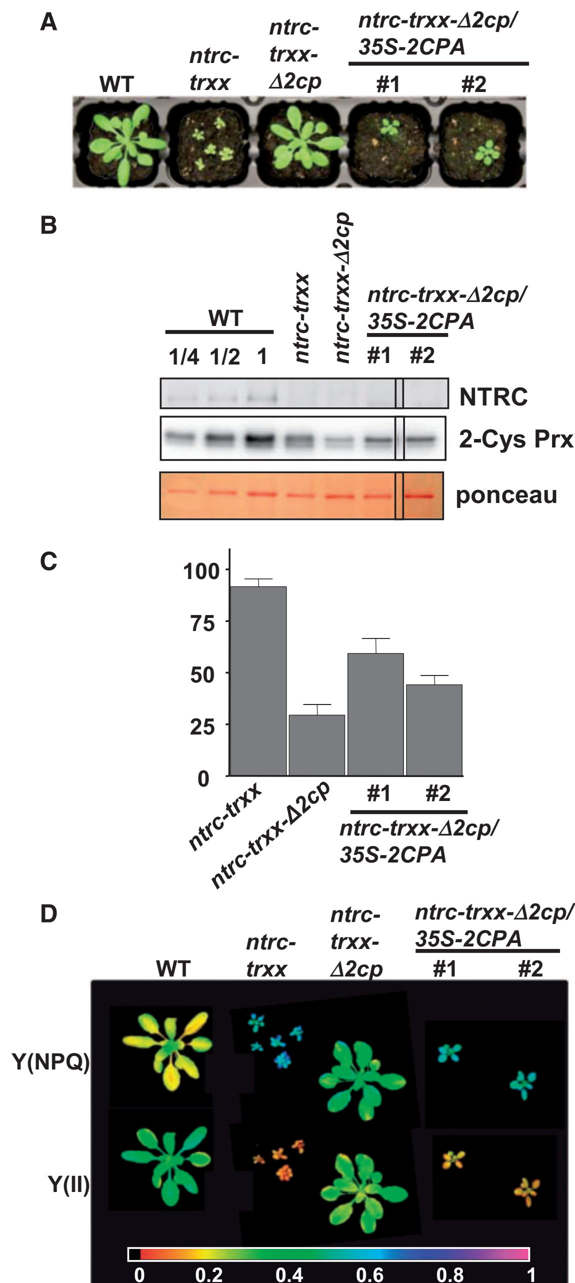

Fig. 4 The effect of the overexpression of 2-Cys Prx A on the *ntrc-trxx-Δ2cp* suppressed line. (A) Wild type, mutant lines and two independent transgenic lines (T_1 generation), #1 and #2, expressing 2-Cys Prx A under the CaMV 35S constitutive promoter in the *ntrc-trxx-Δ2cp* genetic background were grown under a long-day photoperiod for 4 weeks. (B) Western blot analysis of the contents of NTRC and 2-Cys Prxs in the indicated lines. Protein extracts were obtained from leaves, and aliquots (3 µg of protein, corresponding to dilution 1) from wild-type and mutant lines were loaded. In addition, 1/2 and 1/4 dilutions from the wild-type extracts, as indicated, were loaded and samples were subjected to SDS–PAGE under reducing conditions. (C) Band intensities of 2-Cys Prxs and Ponceau were quantified (ScionImage) and the ratios between them, referred to the wild-type sample of 3 µg (arbitrarily assigned a value of 100), are indicated as the average ± SD of two independent experiments. (D) False-color images representing quantum yields of non-photochemical quenching, Y(NPQ), and PSII photochemistry, Y(II). For each parameter, signal intensities (from 0 to 1.0) are indicated according to the color scale bar.

monomer is in its thiolic form (1SH), producing a shifted band of intermediate mobility in alkylation assays. Under dark and light conditions, NTRC-deficient plants exhibited significantly lower levels of the reduced form of 2-Cys Prxs (1SH plus 2SH forms) as compared with the wild type, which showed similar levels to those in *trxx* (**Fig. 6A, B**). The dark–light transition led to increased levels of reduced 2-Cys Prxs in all lines under analysis, the increase being higher in the *ntrc* and *ntrc-trxx-Δ2cp* plants.

Discussion

In a recent report, we have shown that the activity of the FTR–Trxs *f* and NTRC redox systems is integrated by the redox balance of the 2-Cys Prxs, which controls the redox regulatory network of the chloroplast (Pérez-Ruiz et al. 2017). This proposal is based on the finding that the deficiency of 2-Cys Prxs exerts a suppressor effect of the *ntrc* and *ntrc-trxf1f2* phenotypes. Similar to the *ntrc-trxf1f2* mutant, the *ntrc-trxx* mutant also shows a very severe growth inhibition phenotype and impairment of photosynthetic performance (Ojeda et al. 2017), suggesting that the regulatory action of NTRC is not only exerted on *f*-type Trxs, but also on Trx *x*. Since it is considered that Trxs *f* and *x* have different functions in chloroplast redox regulation, the finding that the lack of either Trx type exerts such a severe phenotypic effect in the absence of NTRC suggests that the activities of these Trxs are somehow interconnected by an NTRC-dependent way. According to our model of redox regulation in chloroplasts, the most likely candidates to exert this interconnecting function are 2-Cys Prxs, the redox balance of which is mainly controlled by NTRC (Kirchsteiger et al. 2009). Here, we have addressed this possibility by the generation of the Arabidopsis *ntrc-trxx-Δ2cp* quadruple mutant. This mutant, which is devoid of NTRC and Trx *x* and contains decreased levels of 2-Cys Prxs (**Fig. 1B–D**), shows a much better growth rate than the *ntrc-trxx* or even the *ntrc* mutant (**Figs. 1A, 2A, B**), and a better performance also at early stages of plant development (**Fig. 2C, D**). The suppressor effect of the *ntrc-trxx* phenotype by decreased contents of 2-Cys Prxs in the *ntrc-trxx-Δ2cp* mutant is in agreement with the better efficiency of light energy utilization by this mutant (**Fig. 3A–D**). The ETR of the suppressed line, *ntrc-trxx-Δ2cp*, is similar to that in the *ntrc* mutant (**Fig. 3A**), thus indicating partial recovery of this photosynthetic parameter. Therefore, the partial recovery of the efficiency of light energy utilization of the *ntrc-trxx-Δ2cp* mutant is sufficient to nearly restore the wild-type growth rate, at least under the controlled growth conditions used in this work. Altogether, these results further confirm the central role of 2-Cys Prxs in chloroplast redox regulation and, consequently, in plant performance.

The results reported here showing the effect of the dose of 2-Cys Prxs on the phenotype of the *ntrc-trxx* mutant extend previous results showing a similar effect on the phenotype of the *ntrc* and *ntrc-trxf1f2* mutants (Pérez-Ruiz et al. 2017) and indicate that the redox balance of 2-Cys Prxs modulates the activity of Trxs *f* and *x*. Furthermore, since Trxs *f* and *x* are considered to perform different functions in chloroplast

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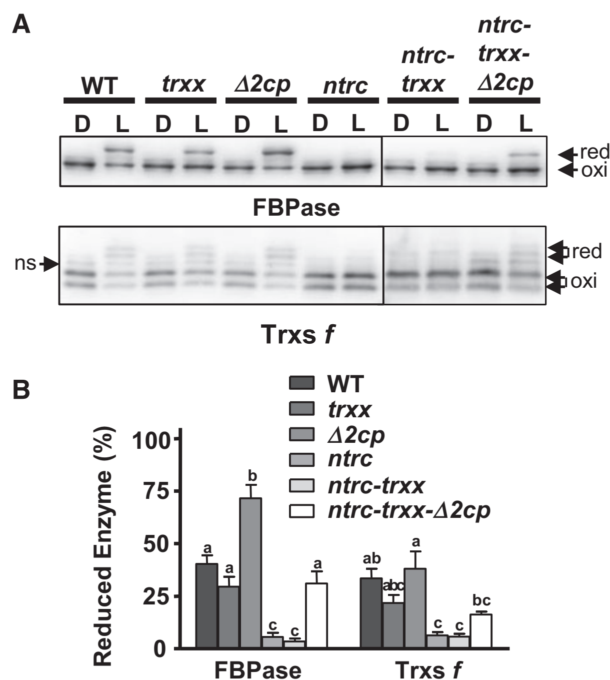

Fig. 5 In vivo redox state of FBPase and *f*-type Trxs in dark–light transitions. (A) The in vivo redox state of FBPase and *f*-type Trxs were determined at the end of the dark period (D), and after 30 min of illumination at 125 μE m^{-2} s^{-1} (L) by labeling of the thiol groups with the alkylating agent $MM(PEG)_{24}$. (B) Band intensities were quantified (ScionImage) and the percentage of reduction, as the ratio between the reduced form and the sum of reduced and oxidized forms, of FBPase and *f*-type Trxs is represented as the mean of three independent experiments ± SEM. Letters indicate significant differences between lines (Tukey test at a 95% confidence interval). red, reduced: oxi, oxidized; ns, non-specific band. The vertical lines mark different filters.

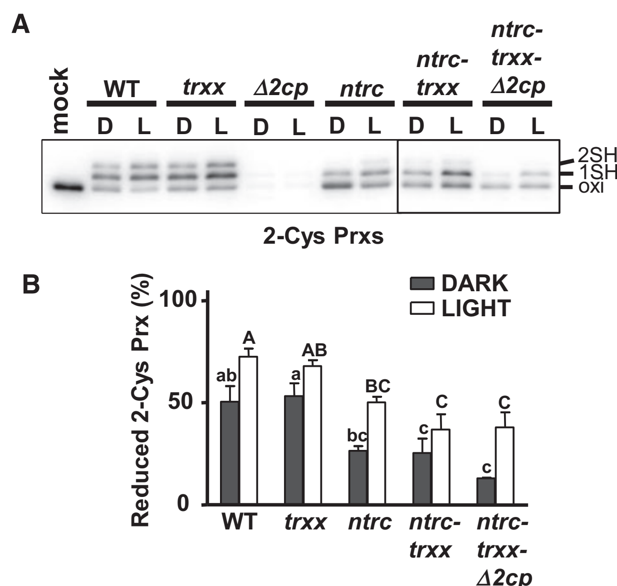

Fig. 6 In vivo redox state of 2-Cys Prxs in dark–light transitions. (A) The in vivo redox state of 2-Cys Prxs was determined at the end of the dark period (D), and after 30 min of illumination at 125 μE m^{-2} s^{-1} (L) by labeling of the thiol groups with the alkylating agent $MM(PEG)_{24}$. (B) Band intensities were quantified (ScionImage) and the level of reduction is the ratio between the sums of the half-reduced (1SH) and fully reduced (2SH), and the sum of reduced and oxidized (oxi) forms is shown as the mean of four (WT, *trxx*, *ntrc* and *ntrc-trxx*) or three (*ntrc-trxx-Δ2cp*) independent experiments ± SEM. Letters above bars indicate significant differences between lines in darkness (lower case letters) and in light (upper case letters) with Tukey test at a 95% confidence interval. Oxi, oxidized; 1SH and 2SH indicate reduction of one or two cysteines, respectively.

redox regulation, these results suggest that the activity of these Trxs is somehow interdependent. In this regard, the analysis of the light-dependent redox state of FBPase in the lines under study revealed that Trxs *f* and Trx *x* exert a concerted action that affects the redox regulation of the enzyme in plants devoid of NTRC. In contrast with mutant plants lacking *f*-type Trxs, which show impaired light-dependent FBPase reduction (Naranjo et al. 2016b), the level of FBPase reduction in plants lacking Trx *x* is nearly similar to that in wild-type plants (**Fig. 5A, B**). These results are in full agreement with in vitro analyses showing that FBPase is efficiently reduced by Trxs *f*1 and *f*2 but not by Trx *x*, which is a poor reductant of the enzyme (Yoshida et al. 2015, Ojeda et al. 2017). However, the level of light-dependent reduction of FBPase was severely impaired in the *ntrc* mutant, as it was in the *ntrc-trxx* mutant (**Fig. 5A, B**), suggesting that NTRC and Trx *x* affect the FBPase redox state indirectly. The light-dependent reduction of Trxs *f* parallels that of FBPase in all lines analyzed (**Fig. 5A, B**), indicating that the deficiencies of NTRC or NTRC plus Trx *x* affects the redox state of *f*-type Trxs, thereby affecting the redox state of FBPase.

The relevant role of NTRC as a reductant of 2-Cys Prxs was confirmed by the lower level of reduction of these enzymes in mutant lines devoid of NTRC, as compared with the wild type and the *trxx* mutant (**Fig. 6A, B**). Though at variable levels, all lines show increased reduction of 2-Cys Prxs upon illumination (**Fig. 6**), a result which is not fully consistent with our previous results (Pérez-Ruiz et al. 2017). This inconsistency is most probably due to the different growth conditions, short-day photoperiod in the previous study (Pérez-Ruiz et al. 2017) and long-day photoperiod in the present study due to the reduced viability of the *ntrc-trxx* mutant under the short-day photoperiod (Ojeda et al. 2017). The fact that the light-dependent reduction of *f*-type Trxs and FBPase was affected in the *ntrc* and *ntrc-trxx* mutants, but not in the *ntrc-trxx-Δ2cp* mutant (**Fig. 5A, B**), which contains decreased contents of 2-Cys Prxs, supports the notion that, in plants devoid of NTRC or NTRC plus Trx *x*, the drainage effect exerted by 2-Cys Prxs affects the light-dependent reduction of *f*-type Trxs and, consequently, of FBPase, in line with the relevant role proposed for 2-Cys in chloroplast redox regulation (Pérez-Ruiz et al. 2017). The suppressor effect observed in the *ntrc-trxx-Δ2cp* mutant indicates that decreased contents of 2-Cys Prxs alleviate the drainage of reducing power, thus restoring the redox state of *f*-type Trxs and FBPase. In support of this notion, transgenic lines recovering the expression of 2-Cys Prx A in the *ntrc-trxx-Δ2cp* mutant recover the mutant phenotype (**Fig. 4A–C**).

The results reported in this study confirm the essential function of 2-Cys Prxs in chloroplast redox regulation, extending the

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action of this hydrogen peroxide-scavenging enzyme to the function of different Trxs such as those of the types *x* and *f*. Moreover, the finding that tetrapyrrole biosynthesis is severely affected in Arabidopsis plants simultaneously deficient in *m*-type Trxs and NTRC (Da et al. 2017) further suggests that the NTRC-dependent redox balance of 2-Cys Prxs may also modulate the function of *m*-type Trxs. Remarkably, the *Δ2cp* mutant, which contains severely decreased levels of 2-Cys Prxs (∼6% of the wild type), showed slightly better efficiency of light energy utilization than the wild type (**Fig. 3A–D**) and higher level of light-dependent reduction of *f*-type Trxs and FBPase (**Fig. 5A, B**). These results suggest that 2-Cys Prxs, which are among the more abundant proteins of the chloroplast (Dietz 2011), exert an oxidizing effect on the pool of Trxs. The reductive activity of each chloroplast Trx is dependent on their redox potential. The *f*-type Trxs are more reducing than Trx *x* (Yoshida et al. 2015), hence some redundancy of these Trxs cannot be excluded. In this regard, several types of chloroplast Trxs are able to reduce 2-Cys Prxs with different efficiencies (Collin et al. 2003, Collin et al. 2004, Yoshida et al. 2015, Yoshida and Hisabori 2016). Moreover, atypical chloroplast Trxs ACHT1 (Dangoor et al. 2012) and ACHT4 (Eliyahu et al. 2015), and the Trx-like CDSP32 (Broin et al. 2003) are also reductants of 2-Cys Prxs. Based on these results, we propose that the NTRC–2-Cys Prxs redox system acts as a central hub playing a critical role in the maintenance of the reducing capacity of the pool of chloroplast Trxs, hence suggesting that the activities of the different types of Trxs of the chloroplast are highly interconnected.

Materials and Methods

Biological material and growth conditions

Arabidopsis thaliana wild-type (ecotype Columbia) and mutant plants (Supplementary Table S1) were routinely grown in soil in growth chambers under long days (16 h light/8 h darkness) at 22 and 20°C during light and dark periods, respectively, and a light intensity of 125 μE m^{-2} s^{-1}. Arabidopsis mutants *ntrc* (Serrato et al. 2004), *Δ2cp*, *trxx* (Pulido et al. 2010) and *ntrc-trxx* (Ojeda et al. 2017) were described previously. To generate the *ntrc-trxx-Δ2cp* quadruple mutant, the *ntrc-trxx* and *Δ2cp* mutants were manually crossed and seeds resulting from this cross were checked for heterozygosity of the T-DNA insertions in the *NTRC*, *TRX X*, *2CPA* and *2CPB* genes. Plants were then self-pollinated and the quadruple homozygous line was identified in the progeny by PCR analysis of genomic DNA with oligonucleotides listed in Supplementary Table S2. For the generation of Arabidopsis transgenic lines, plasmids for overexpressing 2-Cys Prx A driven by the CaMV 35S promoter (Pérez-Ruiz et al. 2017) were transformed into the *Agrobacterium tumefaciens* strain GV301, which were then used for plant transformation by the floral dip method (Clough and Bent 1998).

Root growth assays were performed as previously reported (Ojeda et al. 2017) on Murashige and Skoog medium containing 0.35% (w/v) Gelrite (Duchefa). For seedling development experiments, the formation of true (post-embryonic) leaves was monitored as previously described (Ojeda et al. 2017). In brief, three sets of at least 60 seeds of each line were sown on soil and grown during 28 d under long-day conditions. True leaf formation was scored at 3–4 d intervals and quantified to determine the rate of seedlings reaching the true leaf stage.

RT–qPCR analysis

Total RNA was isolated with Trizol reagent (Invitrogen) and cDNA was synthesized with the Maxima first strand cDNA synthesis kit (Fermentas). Gene expression analyses were performed using an IQ5 system (Bio-Rad) with oligonucleotides listed in Supplementary Table S3 and a standard thermal profile (95°C, 3 min; 40 cycles at 95°C for 10 s, and 60°C for 30 s). After the PCR, a melting curve analysis (55–94°C at 0.5°C/30 s) was performed to confirm the specificity of the amplicon. Relative contents of transcripts were normalized using AT1G13320 and AT5G25760 as reference genes (Czechowski et al. 2005).

Protein extraction, alkylation assays and Western blot analysis

Plant tissues were ground under liquid nitrogen to a fine powder. Extraction buffer [50 mM Tris–HCl pH 8.0, 0.15 M NaCl, 0.5% (v/v) NP-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. 2016b) using $MM(PEG)_{24}$ from Thermo Scientific. Protein samples were subjected to SDS–PAGE under reducing (NTRC, 2-Cys Prxs, *f*-type Trxs) or non-reducing (FBPase) conditions using an acrylamide gel concentration of 9.5% (FBPase alkylation assays), 12% (protein levels of NTRC and 2-Cys Prxs) and 14% (alkylation assays of *f*-type Trxs 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), 2-Cys Prxs (Pérez-Ruiz et al. 2006) and Trxs *f* (Naranjo et al. 2016b) were previously raised in our laboratory. The anti-FBPase antibody was kindly provided by Dr. Sahrawy (Estación Experimental del Zaidín, Granada, Spain).

Determination of chlorophylls and measurements of Chl *a* fluorescence

Chl levels were measured as previously described (Pérez-Ruiz et al. 2006). Room temperature Chl *a* fluorescence and Chl fluorescence imaging of whole rosettes were performed using a pulse-amplitude modulation fluorometer (IMAGING-PAM M-Series instrument, Walz). 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 maximum PSII quantum yield, determined as the ratio of variable fluorescence (F_v) to maximal fluoresence (F_m), F_v/F_m, 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 Chl fluorescence of pre-illuminated plants applying stepwise increasing actinic light intensities up to 146 μE m^{-2} s^{-1}.

Supplementary Data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

References

Alkhalfioui, F., Renard, M. and Montrichard, F. (2007) Unique properties of NADP-thioredoxin reductase C in legumes. *J. Exp. Bot.* 58: 969–978.

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Arsova, B., Hoja, U., Wimmelbacher, M., Greiner, E., Ustun, S., Melzer, M., et al. (2010) Plastidial thioredoxin z interacts with two fructokinase-like proteins in a thiol-dependent manner: evidence for an essential role in chloroplast development in Arabidopsis and *Nicotiana benthamiana*. *Plant Cell* 22: 1498–1515.

Broin, M and Rey, P. (2003) Potato plants lacking the CDSP32 plastidic thioredoxin exhibit overoxidation of the BAS1 2-cysteine peroxiredoxin and increased lipid peroxidation in thylakoids under photooxidative stress. *Plant Physiol.* 132: 1335–1343.

Buchanan, B.B. (2016) The path to thioredoxin and redox regulation in chloroplasts. *Annu. Rev. Plant Biol.* 67: 1–24.

Buchanan, B.B., Holmgren, A., Jacquot, J.P. and Scheibe, R. (2012) Fifty years in the thioredoxin field and a bountiful harvest. *Biochim. Biophys. Acta* 1820: 1822–1829.

Carrillo, L.R., Froehlich, J.E., Cruz, J.A., Savage, L. and Kramer, D.M. (2016) Multi-level regulation of the chloroplast ATP synthase: the chloroplast NADPH thioredoxin reductase C (NTRC) is required for redox modulation specifically under low irradiance. *Plant J.* 87: 654–663.

Cejudo, F.J., Ferrández, J., Cano, B., Puerto-Galán, L. and Guinea, M. (2012) The function of the NADPH thioredoxin reductase C–2-Cys peroxiredoxin system in plastid redox regulation and signalling. *FEBS Lett.* 586: 2974–2980.

Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* 16: 735–743.

Collin, V., Issakidis-Bourguet, E., Marchand, C., Hirasawa, M., Lancelin, J.M., Knaff, D.B., et al. (2003) The Arabidopsis plastidial thioredoxins: new functions and new insights into specificity. *J. Biol. Chem.* 278: 23747–23752.

Collin, V., Lamkemeyer, P., Miginiac, M.M., Hirasawa, M., Knaff, D.B., Dietz, K.J., et al. (2004) Characterization of plastidial thioredoxins from Arabidopsis belonging to the new *y*-type. *Plant Physiol.* 136: 4088–4095.

Czechowski, T., Stitt, M., Altmann, T., Udvardi, M.K. and Scheible, W.R. (2005) Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. *Plant Physiol*. 139: 5–17.

Da, Q., Wang, P., Wang, M., Sun, T., Jin, H., Liu, B., et al. (2017) Thioredoxin and NADPH-dependent thioredoxin reductase C regulation of tetrapyrrole biosynthesis. *Plant Physiol*. 175: 652–666.

Dangoor, I., Peled-Zehavi, H., Wittenberg, G. and Danon, A. (2012) A chloroplast light-regulated oxidative sensor for moderate light intensity in Arabidopsis. *Plant Cell* 24: 1894–1906.

Dietz, K.J. (2011) Peroxiredoxins in plants and cyanobacteria. *Antioxid. Redox Signal.* 15: 1129–1159.

Eliyahu, E., Rog, I., Inbal, D. and Danon, A. (2015) ACHT4-driven oxidation of APS1 attenuates starch synthesis under low light intensity in Arabidopsis plants. *Proc. Natl. Acad. Sci. USA* 112: 12876–12881.

Geigenberger, P., Thormählen, I., Daloso, D.M. and Fernie, A.R. (2017) The unprecedented versatility of the plant thioredoxin system. *Trends Plant Sci.* 22: 249–262.

Kirchsteiger, K., Pulido, P., González, M. and Cejudo, F.J. (2009) NADPH thioredoxin reductase C controls the redox status of chloroplast 2-Cys peroxiredoxins in *Arabidopsis thaliana*. *Mol. Plant.* 2: 298–307.

Kramer, D.M., Johnson, G., Kiirats, O. and Edwards, G.E. (2004) New fluorescence parameters for the determination of Q_A redox state and excitation energy fluxes. *Photosynth. Res.* 79: 209–218.

Lepistö, A., Pakula, E., Toivola, J., Krieger-Liszkay, A., Vignols, F. and Rintamäki, E. (2013) Deletion of chloroplast NADPH-dependent thioredoxin reductase results in inability to regulate starch synthesis and causes stunted growth under short-day photoperiods. *J. Exp. Bot.* 64: 3843–3854.

Meyer, Y., Belin, C., Delorme-Hinoux, V., Reichheld, J.P. and Riondet, C. (2012) Thioredoxin and glutaredoxin systems in plants: molecular mechanisms, crosstalks, and functional significance. *Antioxid. Redox Signal*. 17: 1124–1160.

Michalska, J., Zauber, H., Buchanan, B.B., Cejudo, F.J. and Geigenberger, P. (2009) NTRC links built-in thioredoxin to light and sucrose in regulating starch synthesis in chloroplasts and amyloplasts. *Proc. Natl. Acad. Sci. USA* 106: 9908–9913.

Moon, J.C., Jang, H.H., Chae, H.B., Lee, J.R., Lee, S.Y., Jung, Y.J., et al. (2006) The C-type Arabidopsis thioredoxin reductase ANTR-C acts as an electron donor to 2-Cys peroxiredoxins in chloroplasts. *Biochem. Biophys. Res. Commun*. 348: 478–484.

Naranjo, B., Díaz-Espejo, A., Lindahl, M. and Cejudo, F.J. (2016b) Type-*f* thioredoxins have a role in the short-term activation of carbon metabolism and their loss affects growth under short-day conditions in *Arabidopsis thaliana*. *J. Exp. Bot.* 67: 1951–1964.

Naranjo, B., Mignee, C., Krieger-Liszkay, A., Hornero-Méndez, D., Gallardo-Guerrero, L., Cejudo, F.J., et al. (2016a) The chloroplast NADPH thioredoxin reductase C, NTRC, controls non-photochemical quenching of light energy and photosynthetic electron transport in Arabidopsis. *Plant Cell Environ.* 39: 804–822.

Ojeda, V., Pérez-Ruiz, J.M., González, M., Najera, V.A., Sahrawy, M., Serrato, A.J., et al. (2017) NADPH thioredoxin reductase C and thioredoxins act concertedly in seedling development. *Plant Physiol.* 174: 1436–1448.

Okegawa, Y. and Motohashi, K. (2015) Chloroplastic thioredoxin *m* functions as a major regulator of Calvin cycle enzymes during photosynthesis in vivo. *Plant J.* 84: 900–913.

Pérez-Ruiz, J.M. and Cejudo, F.J. (2009) A proposed reaction mechanism for rice NADPH thioredoxin reductase C, an enzyme with protein disulfide reductase activity. *FEBS Lett.* 583: 1399–1402.

Pérez-Ruiz, J.M., Guinea, M., Puerto-Galán, L. and Cejudo, F.J. (2014) NADPH thioredoxin reductase C is involved in redox regulation of the Mg-chelatase I subunit in *Arabidopsis thaliana* chloroplasts. *Mol. Plant* 7: 1252–1255.

Pérez-Ruiz, J.M., Naranjo, B., Ojeda, V., Guinea, M. and Cejudo, F.J. (2017) NTRC-dependent redox balance of 2-Cys peroxiredoxins is needed for optimal function of the photosynthetic apparatus. *Proc. Natl. Acad. Sci. USA* 114: 12069–12074.

Pérez-Ruiz, J.M., Spínola, M.C., Kirchsteiger, K., Moreno, J., Sahrawy, M. and Cejudo, F.J. (2006) Rice NTRC is a high-efficiency redox system for chloroplast protection against oxidative damage. *Plant Cell* 18: 2356–2368.

Puerto-Galán, L., Pérez-Ruiz, J.M., Ferrández, J., Cano, B., Naranjo, B., Nájera, V.A., et al. (2013) Overoxidation of chloroplast 2-Cys peroxiredoxins: balancing toxic and signaling activities of hydrogen peroxide. *Front. Plant Sci.* 4: 310.

Pulido, P., Spínola, M.C., Kirchsteiger, K., Guinea, M., Pascual, M.B., Sahrawy, M., et al. (2010) Functional analysis of the pathways for 2-Cys peroxiredoxin reduction in *Arabidopsis thaliana* chloroplasts. *J. Exp. Bot.* 61: 4043–4054.

Richter, A.S., Peter, E., Rothbart, M., Schlicke, H., Toivola, J., Rintamaki, E., et al. (2013) Posttranslational influence of NADPH-dependent thioredoxin reductase C on enzymes in tetrapyrrole synthesis. *Plant Physiol.* 162: 63–73.

Schürmann, P. and Buchanan, B.B. (2008) The ferredoxin/thioredoxin system of oxygenic photosynthesis. *Antioxid. Redox Signal.* 10: 1235–1274.

Serrato, A.J., Fernández-Trijueque, J., Barajas-López, J.D., Chueca, A. and Sahrawy, M. (2013) Plastid thioredoxins: a 'one-for-all' redox-signaling system in plants. *Front. Plant Sci.* 4: 463.

Serrato, A.J., Pérez-Ruiz, J.M., Spínola, M.C. and Cejudo, F.J. (2004) A novel NADPH thioredoxin reductase, localized in the chloroplast, which deficiency causes hypersensitivity to abiotic stress in *Arabidopsis thaliana*. *J. Biol. Chem.* 279: 43821–43827.

Spínola, M.C., Pérez-Ruiz, J.M., Pulido, P., Kirchsteiger, K., Guinea, M., González, M., et al. (2008) NTRC new ways of using NADPH in the chloroplast. *Physiol. Plant.* 133: 516–524.

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Stenbaek, A., Hansson, A., Wulff, R.P., Hansson, M., Dietz, K.J. and Jensen, P.E. (2008) NADPH-dependent thioredoxin reductase and 2-Cys peroxiredoxins are needed for the protection of Mg-protoporphyrin monomethyl ester cyclase. *FEBS Lett*. 582: 2773–2778.

Thormählen, I., Meitzel, T., Groysman, J., Ochsner, A.B., von Roepenack-Lahaye, E., Naranjo, B., et al. (2015) Thioredoxin f1 and NADPH-dependent thioredoxin reductase C have overlapping functions in regulating photosynthetic metabolism and plant growth in response to varying light conditions. *Plant Physiol*. 169: 1766–1786.

Vieira Dos Santos, C., Laugier, E., Tarrago, L., Massot, V., IssakidisBourguet, E., Rouhier, N., et al. (2007) Specificity of thioredoxins and glutaredoxins as electron donors to two distinct classes of Arabidopsis plastidial methionine sulfoxide reductases B. *FEBS Lett*. 581: 4371–4376.

Yoshida, K., Hara, S. and Hisabori, T. (2015) Thioredoxin selectivity for thiol-based redox regulation of target proteins in chloroplasts. *J. Biol. Chem*. 290: 14278–14288.

Yoshida, K. and Hisabori, T. (2016) Two distinct redox cascades cooperatively regulate chloroplast functions and sustain plant viability. *Proc. Natl. Acad. Sci. USA* 113: E3967–E3976.

Yoshida, K., Matsuoka, Y., Hara, S., Konno, H. and Hisabori, T. (2014) Distinct redox behaviors of chloroplast thiol enzymes and their relationships with photosynthetic electron transport in *Arabidopsis thaliana*. *Plant Cell Physiol*. 55: 1415–1425.

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