NTRC-dependent redox balance of 2-Cys peroxiredoxins is needed for optimal function of the photosynthetic apparatus

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Edited by Bob B. Buchanan, University of California, Berkeley, CA, and approved September 26, 2017 (received for review April 12, 2017)

Thiol-dependent redox regulation allows the rapid adaptation of chloroplast function to unpredictable changes in light intensity. Traditionally, it has been considered that chloroplast redox regulation relies on photosynthetically reduced ferredoxin (Fd), thioredoxins (Trxs), and an Fd-dependent Trx reductase (FTR), the Fd-FTR-Trxs system, which links redox regulation to light. More recently, a plastid-localized NADPH-dependent Trx reductase (NTR) with a joint Trx domain, termed NTRC, was identified. NTRC efficiently reduces 2-Cys peroxiredoxins (Prxs), thus having antioxidant function, but also participates in redox regulation of metabolic pathways previously established to be regulated by Trxs. Thus, the NTRC, 2-Cys Prxs, and Fd-FTR-Trxs redox systems may act concertedly, but the nature of the relationship between them is unknown. Here we show that decreased levels of 2-Cys Prxs suppress the phenotype of the *Arabidopsis thaliana ntrc* KO mutant. The excess of oxidized 2-Cys Prxs in NTRC-deficient plants drains reducing power from chloroplast Trxs, which results in low efficiency of light energy utilization and impaired redox regulation of Calvin–Benson cycle enzymes. Moreover, the dramatic phenotype of the *ntrc-trxf1f2* triple mutant, lacking NTRC and *f*-type Trxs, was also suppressed by decreased 2-Cys Prxs contents, as the *ntrc-trxf1f2-Δ2cp* mutant partially recovered the efficiency of light energy utilization and exhibited WT rate of CO_2 fixation and growth phenotype. The suppressor phenotype was not caused by compensatory effects of additional chloroplast antioxidant systems. It is proposed that the Fd-FTR-Trx and NTRC redox systems are linked by the redox balance of 2-Cys Prxs, which is crucial for chloroplast function.

chloroplast | peroxiredoxin | NTRC | redox signaling | thioredoxin

Photosynthesis is a source of metabolic intermediates that inevitably generates reactive oxygen species (ROS), which may cause oxidative damage but have also an important signaling function (1). The dual role of chloroplasts as source of metabolic precursors and second messengers is highly dependent on light. Thiol-based redox regulation plays an important role in the rapid adaptation of chloroplast metabolism to dark/light transitions. A classic example of a chloroplast redox regulated process is the CO_2 fixation by the Calvin–Benson cycle in which key regulatory enzymes are reduced and fully active during the day and oxidized, and hence inactive, during the night (2–4).

While in heterotrophic organisms redox regulation relies on NADPH via a low number of thioredoxins (Trxs) and an NADPH-dependent Trx reductase (NTR), chloroplasts harbor a specific system composed by photosynthetically reduced ferredoxin (Fd), an Fd-dependent Trx reductase (FTR), and a complex set of Trxs (5). In addition, chloroplasts contain an NTR with a joint Trx domain, termed NTRC (6, 7), which participates in the redox regulation of metabolic pathways previously reported to be regulated by Trxs, such as the biosynthesis of starch (8, 9) and tetrapyrroles (10, 11). These results suggest that both redox systems act concertedly, a notion further supported by the severe growth-retarded phenotype of an *Arabidopsis* double mutant combining the deficiencies of NTRC and *f*- or *x*-type Trxs (12, 13). NTRC is an efficient reductant of 2-Cys peroxiredoxins (Prxs) and was therefore proposed to have antioxidant function (14). However, 2-Cys Prxs act as peroxide scavengers but also as peroxide sensors (15).

Therefore, NTRC is simultaneously involved in redox regulation and antioxidant defense, suggesting that both processes are interconnected; however, the mechanism that allows this central function of NTRC remains poorly understood. Here we have addressed this issue by analyzing the genetic interaction of NTRC and 2-Cys Prxs. Strikingly, the *ntrc-Δ2cp* triple mutant, which lacks NTRC and contains severely reduced levels of 2-Cys Prxs, nearly recovered the WT phenotype, indicating that the deficiency of 2-Cys Prxs suppresses the *ntrc* phenotype. This suppressor effect was characterized, and, based on these results, we propose that the Fd-FTR-Trxs and NTRC redox systems are integrated by the redox balance of the 2-Cys Prxs, which is thus essential for chloroplast redox regulation.

Results

Decreased Levels of 2-Cys Prxs Suppress the Phenotype Caused by the Lack of NTRC in *Arabidopsis*. With the aim of establishing the genetic interaction of NTRC and 2-Cys Prxs, *Arabidopsis* mutants combining the deficiencies of both enzymes were generated. Because *Arabidopsis* contains two almost identical 2-Cys Prxs, termed A and B, the double mutant *Δ2cp*, which lacks 2-Cys Prx B but still contains a small amount of 2-Cys Prx A (16), was manually crossed with the *ntrc* mutant (6). Strikingly, the triple

Significance

Chloroplasts harbor a complex redox network formed by two systems, the FTR- thioredoxins (Trxs), which relies on photoreduced ferredoxin (Fd), and the NADPH-dependent Trx reductase C NTRC. Thus, an important issue in chloroplast biology is to establish the relationship between these redox pathways. Here we propose that the Fd-FTR-Trxs and NTRC redox systems are integrated via the redox balance of 2-Cys peroxiredoxins (Prxs), which therefore has a key role in chloroplast function. NTRC controls the redox balance of 2-Cys Prxs, which maintains the reducing capacity of the pool of chloroplast Trxs and, consequently, proper regulation of photosynthetic carbon assimilation enzymes. Therefore, redox regulation of chloroplast enzymes and hydrogen peroxide reduction are linked by the action of the NTRC-2-Cys Prxs system.

Author contributions: J.M.P.-R. and F.J.C. designed research; J.M.P.-R., B.N., V.O., and M.G. performed research; J.M.P.-R., B.N., V.O., M.G., and F.J.C. analyzed data; and F.J.C. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1706003114/-/DCSupplemental.

mutant, here termed *ntrc-Δ2cp*, despite being affected in redox regulation and antioxidant defense, partially recovered WT phenotype (Fig. 1*A*), as determined by rosette fresh weight (Fig. 1*B*), indicating that the growth phenotype of the *ntrc* mutant was suppressed by decreased contents of 2-Cys Prxs. It should be noted that the content of 2-Cys Prxs, compared with the WT, is higher in the *ntrc-Δ2cp* (21%) than in the *Δ2cp* mutant (4%; Fig. 1*C*), suggesting a compensatory effect triggered by the deficiency of NTRC. To further analyze this suppressor effect, different growth and environmental conditions were tested. The suppressor effect was also observed when plants were grown under long-day photoperiod at different light intensities (Fig. S1*A*), as shown by rosette fresh weight (Fig. S1*B*) and chlorophyll content (Fig. S1*C*). It is known that the *ntrc* mutant is extremely sensitive to treatments of prolonged darkness (14), which exert a deep effect on PSII photochemical efficiency. Interestingly, the response to prolonged darkness of the *ntrc-Δ2cp* mutant was similar to those of the WT and the *Δ2cp* mutant (Fig. S2*A*), as shown by the ratio of variable fluorescence (*Fv*) to maximal fluorescence (*Fm*; Fig. S2*B*). Finally, the *ntrc-Δ2cp* mutant also showed a better response to oxidative stress than the *ntrc* mutant, as determined by the rate of root growth in the presence of methyl viologen (Fig. S2*C*).

We then tested the effect of the two 2-Cys Prxs present in *Arabidopsis* chloroplasts. To that end, the single mutants *2cpa*, still containing residual amounts of 2-Cys Prx A; *2cpb*, devoid of 2-Cys Prx B (17); and a mutant KO for 2-Cys Prx A, here termed *2cpaGK*, were manually crossed with *ntrc*, and double mutants *ntrc-2cpa*, *ntrc-2cpaGK*, and *ntrc-2cpb* were generated (Fig. 1*A*). As expected, the *2cpaGK* and *2cpb* single mutants showed phenotypes indistinguishable from the WT (Fig. 1 *A* and *B*). Interestingly, whereas mutants *ntrc-2cpaGK* and *ntrc-2cpb*, containing lower levels of 2-Cys Prxs (26% and 33%, respectively; Fig. 1*C*), showed growth phenotypes comparable to the *ntrc-Δ2cp* mutant (Fig. 1 *A* and *B*), the *ntrc-2cpa* mutant, with higher content of 2-Cys Prxs (70%; Fig. 1*C*), showed a subtle reversion of the *ntrc* phenotype (Fig. 1 *A* and *B*). Thus, the phenotype caused by the lack of NTRC depends on the dose of 2-Cys Prxs, with isoforms A and B having indistinguishable effect. A different approach was then undertaken to further test the effect of the dose of 2-Cys Prxs. Overexpression of 2-Cys Prx A or B in the *ntrc* mutant background showed aggravated growth retardation phenotype (Fig. S3 *A* and *B*), but had no visible effect in the WT background (Fig. S3 *C* and *D*). In contrast, an increased expression of 2-Cys Prxs A or B in the *ntrc-Δ2cp* background led to phenotypes intermediate between those of *ntrc* and *ntrc-Δ2cp* (Fig. S3 *E* and *F*). Overall, these results confirm that the phenotype of plants devoid of NTRC is highly dependent on the level of 2-Cys Prxs.

The *ntrc* mutant shows low efficiency of light energy utilization as revealed by the extensive nonphotochemical quenching (NPQ) at low light intensity and the decreased photosynthetic electron transport rate (ETR) (18). Thus, to further characterize the suppression of the *ntrc* phenotype by decreased levels of 2-Cys Prxs, these photosynthetic parameters were also analyzed. The *ntrc-Δ2cp* line recovered almost WT levels of NPQ (Fig. 2*A*) and photosynthetic ETR (Fig. 2*B*), which is in line with the recovery of the rate of CO_2 fixation (Fig. S4).

2-Cys Prxs Cause Imbalance of Chloroplast Redox Regulation in NTRC Deficient Plants. The severe growth inhibition phenotype caused by higher levels of 2-Cys Prxs in the *ntrc* background suggests that the harmful effect is caused by the accumulation of oxidized 2-Cys Prxs. Thus, we established the hypothesis that the increased level of oxidized 2-Cys Prxs in NTRC-deficient plants might act as a sink for electrons from reduced chloroplast electron carriers. Clear candidates for transferring electrons to the 2-Cys Prxs are Trxs because, albeit with low efficiency, different Trxs are able to reduce 2-Cys Prxs (19–24). If this were the case, such redirection of photosynthetic reducing power from the pool of Trxs would affect the light-dependent reduction of Trx-regulated enzymes. To test this possibility, the light-dependent changes of the redox state of Trxs *f*, chosen as representatives of the pool of Trxs, and fructose-bisphosphate phosphatase (FBPase) and phosphoribulokinase (PRK), well-known redox-regulated enzymes of the Calvin–Benson cycle, were analyzed by thiol labeling with methylmaleimide polyethylene glycol$_{24}$ [MM(PEG)$_{24}$]. Dark-adapted WT plants showed the previously reported double band (25), corresponding to fully oxidized Trxs *f*, and an additional nonspecific band (Fig. 3*A*) also detected in extracts from the *trxf1f2* double KO mutant (Fig. S5*A*). In response to light, Trxs *f* were partially reduced, the level of reduction being similar to the WT in the *ntrc-Δ2cp* mutant, higher in the *Δ2cp* mutant, and lower in the *ntrc* mutant (Fig. 3 *A* and *B*). The changes in the redox state of FBPase and PRK resembled those of Trxs *f* (Fig. 3 *A* and *B*), except that PRK became fully reduced upon illumination in all lines except *ntrc*. Therefore, decreased levels of

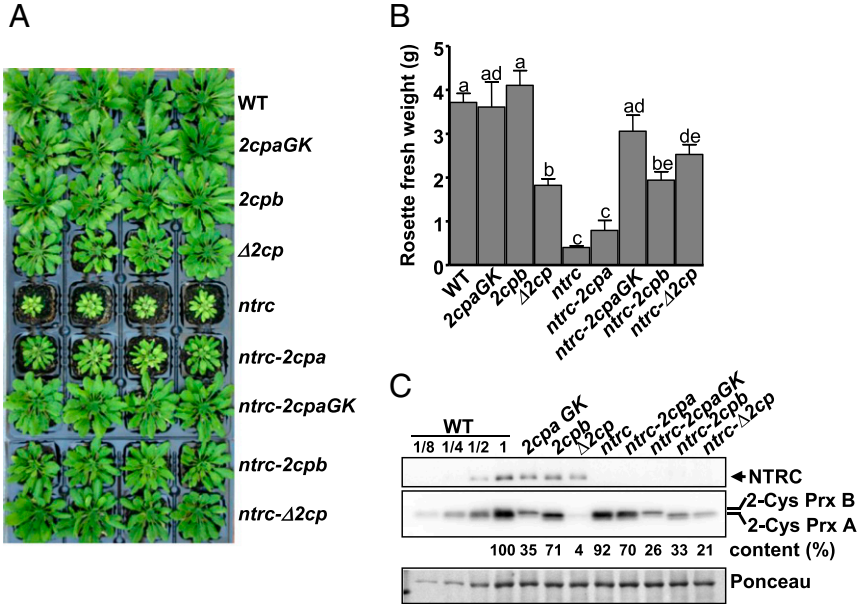

Fig. 1. The phenotype of the *Arabidopsis ntrc* mutant is suppressed by decreased levels of 2-Cys Prxs. (*A*) WT and mutant lines were grown under short-day conditions for 9 wk. The weights of rosette leaves from at least seven plants are presented as average values ± SE. Letters indicate significant differences by Student's *t* test at a 95% CI (*B*). (*C*) 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, as well as 1:2, 1:4, and 1:8 dilutions from WT, 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. Band intensities of 2-Cys Prxs and Ponceau were quantified (ScionImage), and the ratio between them, referred to the WT 1× sample (arbitrarily assigned a value of 100), are indicated.

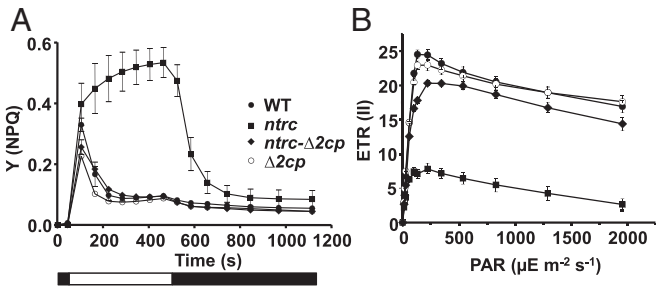

Fig. 2. NPQ and linear photosynthetic ETR in *Arabidopsis* WT and mutant lines. (*A*) Quantum yield of NPQ [Y(NPQ)] was performed using attached leaves from plants grown under short-day conditions for 8 wk. White and black blocks indicate light (75 μE m^{-2}·s^{-1}) and darkness periods, respectively. Each data point is the average of at least four determinations, and SEs are represented by error bars. (*B*) Relative ETRs of PSII, ETR(II), were determined during stepwise increasing photosynthetically active radiation (PAR) in plants grown as in *A*. Each data point is the mean of the ETR(II) from at least five leaves from different plants, and SEs are presented.

2-Cys Prxs suppressed the impairment of redox regulation of Calvin–Benson cycle enzymes in NTRC-deficient plants.

As our hypothesis implies the redirection of reducing power to the 2-Cys Prxs, it would be expected that there would be light-dependent reduction of these enzymes in plants devoid of NTRC. In alkylation assays, the oxidized form of 2-Cys Prxs (oxi) was detected as a band with a mobility similar to nonalkylated samples (mock), whereas the two shifted bands corresponded to the half-reduced (1SH) and fully reduced (2SH) forms, respectively (Fig. 3*C*). Interestingly, 2-Cys Prxs were predominantly reduced (1SH plus 2SH) in WT plants, reaching similar levels in the dark and upon illumination (Fig. 3 *C* and *D*). Although the *ntrc* and *ntrc-Δ2cp* mutants showed a significant decrease of the reduced forms of 2-Cys Prxs in darkness, a significant light-dependent increase of the reduced forms was observed (Fig. 3 *C* and *D*). It should be noted that the amount of 2-Cys Prxs in the *Δ2cp* mutant is too low to be reliably quantified; however, in the *ntrc-Δ2cp* mutant, the content of 2-Cys Prxs is made higher (Fig. 1*C*) by the compensatory effect of the deficiency of NTRC, thus making quantification possible. Oxidant conditions favor the aggregation of 2-Cys Prxs, which shows chaperone activity (26). Thus, an additional possibility is that the aggregation state of 2-Cys Prxs is affected in the *ntrc-Δ2cp* suppressed line. However, native gel electrophoresis showed that most of the 2-Cys Prxs are in dimeric form, and no significant differences in the aggregation state were observed in this preliminary test in any of the lines under analysis in dark/light transitions (Fig. S5*B*).

As the *ntrc* mutant shows lower efficiency of light energy utilization (Fig. 2), the poor reduction of Trx-regulated enzymes might be the result of the lower photosynthetic ETR rather than the redirection of reducing power from the pool of Trxs to the 2-Cys Prxs. To distinguish between these possibilities, WT and *ntrc* mutant plants were exposed to different light intensities, and the effects on the photosynthetic ETR and the redox state of FBPase and PRK were analyzed. As expected, the photosynthetic ETR (Fig. S6*A*) and, accordingly, the level of reduction of FBPase and PRK (Fig. S6*B*), were impaired in the *ntrc* mutant at all light intensities tested. However, at 100 μE m^{-2}·s^{-1}, which produced a photosynthetic ETR in the *ntrc* mutant slightly higher than that produced at 27 μE m^{-2}·s^{-1} in the WT (Fig. S6*A*), the *ntrc* mutant showed lower level of reduction of FBPase and PRK than the WT (Fig. S6*B*), indicating that the level of reduction of these enzymes does not solely respond to photosynthetic ETR.

As 2-Cys Prxs have peroxidase activity, an additional possibility is that the suppression of the *ntrc* phenotype in the *ntrc-Δ2cp* mutant is the result of compensatory effects of additional antioxidants. Minor differences were observed in the total content of ascorbic acid (AsA) plus dehydroascorbic acid (DHA) and reduced glutathione (GSH) plus oxidized glutathione (GSSG) in leaves of the lines under analysis (Table S1). Although both antioxidants were slightly more oxidized in the *ntrc* mutant, the *ntrc-Δ2cp* suppressed line partially recovered levels of the reduced form of both antioxidants (Table S1). Therefore, compensatory effects of nonenzymatic antioxidants could be excluded.

Decreased Levels of 2-Cys Prxs Suppress the Phenotype Caused by the Combined Deficiencies of NTRC and *f*-Type Trxs. Recently, it was reported that the *Arabidopsis* triple mutant *ntrc-trxf1f2*, defective in NTRC and *f*-type Trxs, has a very severe growth inhibition phenotype (13). We then tested whether the suppressor effect of decreased levels of 2-Cys Prxs is also exerted in these plants. These experiments were performed with plants grown under long-day conditions because the *ntrc-trxf1f2* mutant showed reduced viability under the short-day conditions used for the previous experiments. In line with its severe growth retardation phenotype (Fig. 4*A* and Fig. S7 *A* and *B*), the use of light energy by the *ntrc-trxf1f2* mutant was very inefficient, showing the highest NPQ (Fig. S7*C*) and the lowest ETR (Fig. 4*C*) of the lines analyzed. Interestingly, the *ntrc-trxf1f2-Δ2cp* quintuple mutant, which is KO for NTRC and Trxs *f* and contains decreased levels of 2-Cys Prxs (32%; Fig. 4*B*), showed partial recovery of NPQ (Fig. S7*C*) and the photosynthetic ETR (Fig. 4*C*), whereas the rate of CO_2 fixation (Fig. S4) and growth phenotype were similar to those of the WT (Fig. 4*A* and Fig. S7 *A* and *B*). In

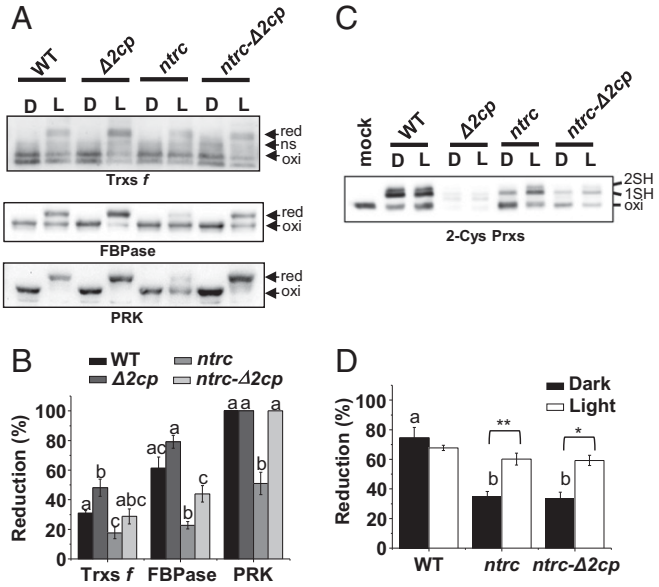

Fig. 3. In vivo redox changes of representative chloroplast enzymes during dark/light transitions in *Arabidopsis* WT and mutant plants. (*A*) In vivo redox state of Trxs *f*, FBPase, and PRK determined from leaves of 8-wk-old short-day grown plants at the end of the dark period (marked as "D") and after 30 min of illumination at 125 μE m^{-2}·s^{-1} ("L"). (*B*) Band intensities were quantified (ScionImage), and the percentage of reduction is the ratio of the reduced form and the sum of reduced and oxidized forms. Each value is the mean of three independent experiments ± SE. Letters indicate significant differences between mutants by Student's *t* test at a 95% CI. (*C*) In vivo redox state of 2-Cys Prxs. (*D*) Reduction level is the ratio between the sums of the half reduced (1SH) and fully reduced (2SH) forms and the sum of reduced and oxidized forms. Each value is the mean of at least three independent experiments ± SE. Letters indicate significant differences between mutants in darkness by Tukey test at a 99% CI (there are no significant differences in light). Statistical significance (*$P < 0.05$ and **$P < 0.01$) determined with the Student's *t* test comparing dark and light values for each line. ns, nonspecific; Oxi, oxidized; red, reduced. 1SH and 2SH indicate reduction of one or the two cysteines, respectively, of 2-Cys Prxs.

agreement with previous results (12, 25), the light-dependent redox regulation of FBPase was more affected by the deficiency of NTRC than by the deficiency of *f*-type Trxs, as was the redox regulation of PRK (Fig. 4 *D* and *E*). Hardly any light-dependent reduction of FBPase or PRK was observed in the *ntrc-trxf1f2* mutant, whereas the *ntrc-trxf1f2-Δ2cp* quintuple mutant recovered levels of light-dependent reduction of both enzymes, similar to those of the *trxf1f2* mutant (Fig. 4 *D* and *E*). Finally, the determination of FBPase activity from leaf extracts showed higher activity under reducing (i.e., DTT) than under oxidant (i.e., $CuCl_2$) conditions in all lines analyzed (Fig. 4*F*), reflecting the activating effect of reducing conditions on the chloroplastic redox-sensitive isoform of the enzyme. The finding that decreased levels of 2-Cys Prxs suppress the dramatic growth phenotype of plants simultaneously devoid of NTRC and Trxs *f* uncovers the key role of 2-Cys Prxs in chloroplast redox regulation. This notion was further confirmed by the overexpression of 2-Cys Prx A or B in the *ntrc-trxf1f2-Δ2cp* quintuple mutant, which resulted in phenotypes intermediate between *ntrc-trxf1f2* and *ntrc-trxf1f2-Δ2cp* (Fig. S3 *G* and *H*).

NTRC-deficient plants lack the major pathway of 2-Cys Prxs reduction, which might compromise H_2O_2 detoxification and provoke its accumulation. However, as previously reported (14), leaves of the *ntrc* mutant showed similar levels of H_2O_2 as WT (Fig. S8*A*). Even the *ntrc-trxf1f2* mutant, which shows the most severe phenotype, did not show any increase of H_2O_2 (Fig. S8*A*). Such a finding might be caused by a compensatory effect exerted by additional antioxidants within chloroplasts. As the content of nonenzymatic antioxidants was very similar in the *ntrc* mutant and *ntrc-Δ2cp* suppressed line (Table S1), we analyzed the expression of genes encoding plastid-localized enzymatic peroxidases. Although the *Δ2cp* mutant contained the expected lower levels of *2CPA* and almost undetectable levels of *2CPB* transcripts, the content of *2CPA* transcripts was increased in the *ntrc-Δ2cp* and *ntrc-trxf1f2-Δ2cp* mutants (Fig. S8*B*), indicating that the higher level of 2-Cys Prx A in these mutants (Fig. 1*C*) is the result of the up-regulation of the *2CPA* gene triggered by the deficiency of NTRC. Minor differences were observed in the content of transcripts of genes encoding the other plastid-localized Prxs, *PRXQ* and *PRXIIE*, and peroxidases *GPX1*, *sAPX*, and *tAPX*, except in the *ntrc-trxf1f2* triple mutant, which showed reduced amounts (Fig. S8 *B* and *C*). Interestingly, partial (*PRXIIE* and *sAPX*) or complete (*PRXQ*, *GPX1*, and *tAPX*) recovery of the WT levels of these transcripts were observed the *ntrc-trxf1f2-Δ2cp* quintuple mutant (Fig. S8 *B* and *C*). The *GPX7* gene was induced in *ntrc* and *ntrc-trxf1f2* mutants, with induction also suppressed in *ntrc-Δ2cp* and *ntrc-trxf1f2-Δ2cp* mutants (Fig.

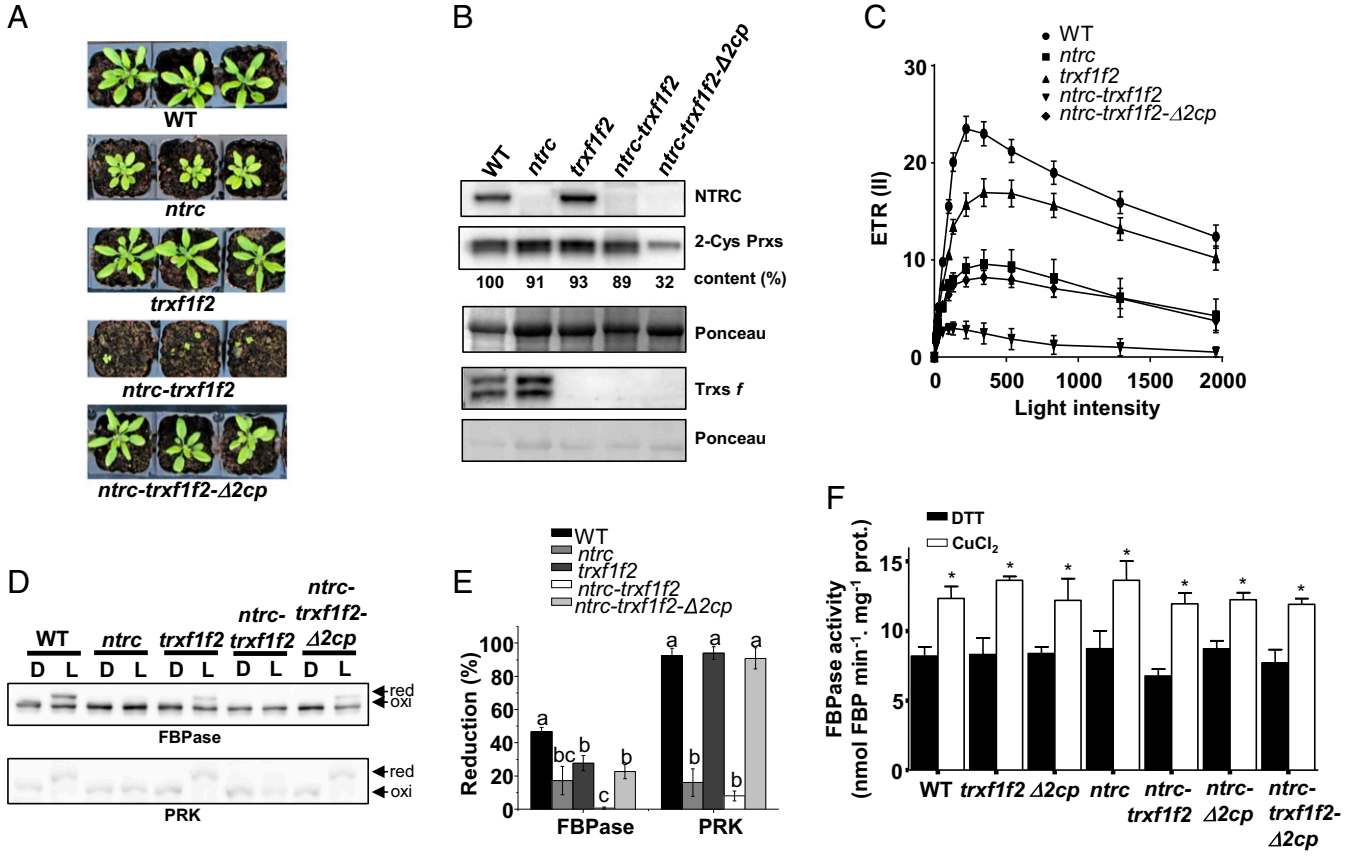

Fig. 4. Decreased levels of 2-Cys Prxs suppress the growth inhibition phenotype of the *ntrc-trxf1f2* mutant. (*A*) WT and mutant lines were grown under long-day conditions for 4 wk. (*B*) Western blot analysis of the content of NTRC, 2-Cys Prxs, and Trxs *f* in WT and mutant lines grown as stated in *A*. Band intensities of 2-Cys Prxs and Ponceau were quantified (ScionImage), and the ratios between them in reference to the WT sample (arbitrarily assigned a value of 100) are indicated. (*C*) Relative ETRs of PSII, ETR(II), were determined during stepwise increasing PAR in plants grown as in *A*. Each data point is the mean of the ETR(II) from at least five leaves from different plants, and SEs are presented. (*D*) In vivo redox state of FBPase and PRK was monitored at the end of the dark period (marked as "D") or after 30 min of illumination at 125 $\mu E\ m^{-2}\cdot s^{-1}$ ("L") using plants grown as in *A*. (*E*) Band intensities were quantified (ScionImage), and the percentage of reduction for each enzyme is the ratio between the reduced form and the sum of reduced and oxidized forms. Each value is the mean of five independent experiments ± SE. Letters indicate significant differences between mutants by Student's *t* test at a 95% CI. oxi, oxidized; red, reduced. (*F*) FBPase activity was assayed in leaf extracts, pretreated with 100 μM $CuCl_2$ (black bars) or 10 mM DTT (white bars), from plants grown as described in *A* and harvested at 12 h of the day period. Results are the mean ± SE from three biological replicates. For each genotype, asterisks indicate significant differences (Student's *t* test at a 95% CI) between oxidant and reducing conditions.

S8C). Altogether, these results suggest that the phenotypic effects caused by the deficiencies of the redox systems studied here are the result of imbalance of the chloroplast redox network rather than oxidative stress or compensatory effects of additional antioxidants.

Discussion

The discovery that chloroplasts harbor an NADPH-dependent redox system (i.e., NTRC) in addition to the light-dependent Fd-FTR-Trxs (6) gave rise to the issue of establishing how these two redox systems interact. Based on the phenotype of *Arabidopsis* double mutants combining the deficiencies of NTRC and *f*- or *x*-type Trxs (12, 13) or NTRC and FTR (27), it was proposed that both redox systems act separately, but with overlapping functions. Here, we propose that these systems are integrated via the redox balance of 2-Cys Prxs as depicted in Fig. 5.

According to this model (Fig. 5*A*), NTRC maintains 2-Cys Prxs predominantly reduced in a light-independent manner in WT plants (Fig. 3 *C* and *D*). Chloroplast Trxs reduce 2-Cys Prxs (19–24) with lower efficiency than NTRC (12, 16, 28), so no or minor draining of electrons from the pool of Trxs to 2-Cys Prxs would occur in WT plants, allowing the light-dependent redox regulation of enzymes, such as those of the Calvin–Benson cycle, via the Fd-FTR-Trxs system. In NTRC-deficient plants (Fig. 5*B*), 2-Cys Prxs accumulate in oxidized form, as observed in leaves of the dark-adapted *ntrc* mutant (Fig. 3 *C* and *D*). Upon illumination, oxidized 2-Cys Prxs act as a sink of electrons in the *ntrc* mutant, depleting reducing power from the pool of Trxs, as shown by the light-dependent reduction of 2-Cys Prxs (Fig. 3 *C* and *D*), the impaired reduction of Trxs *f* (Fig. 3 *A* and *B*), and, consequently, of FBPase and PRK (Fig. 3 *A* and *B*). The deficient photosynthetic performance in the *ntrc* mutant (Fig. 2) shows that the NTRC-2-Cys Prxs system is also involved in feedback regulation of photochemical reactions. The mechanism of this feedback regulation is not yet clear; however, stromal Trxs are most likely involved, as suggested by the Trx *f*-dependent regulation of PGRL1, which transfers electrons form reduced Fd to quinones in photosynthetic cyclic electron flow (29). As the content of oxidized 2-Cys Prxs decreases in NTRC-deficient plants (Fig. 5*C*), withdrawal of electrons from the pool of Trxs during the dark/light transition decreases accordingly, keeping sufficiently active the Fd-FTR-Trxs system, here determined by the redox state of Trxs *f*, to permit the light-dependent reduction of redox-regulated enzymes such as FBPase and PRK (Fig. 3 *A* and *B*), as well as the recovery of photochemical parameters (Fig. 2).

The similar aggregation state of 2-Cys Prxs in the *ntrc* mutant and the *ntrc-Δ2cp* suppressed line (Fig. S5*B*) indicates that the effect of 2-Cys Prxs in NTRC-deficient plants is the result of imbalance of redox regulation rather than the possible chaperone activity of the oligomeric form of these enzymes. In addition, 2-Cys Prxs have a nonreductive stimulatory effect on chloroplast FBPase activity (30). However, the *ntrc-Δ2cp* and *ntrc-trxf1f2-Δ2cp* suppressed lines, containing lower levels of 2-Cys Prxs (Figs. 1*C* and 4*B*), showed levels of FBPase activity under oxidant and reducing conditions similar to WT (Fig. 4*F*), suggesting that the nonreductive effect of 2-Cys Prxs on FBPase activity is not relevant to explain the suppressor effect.

Our model is fully supported by the 2-Cys Prxs dose-dependent effect on the *ntrc* phenotype. The content of 2-Cys Prxs in NTRC-deficient plants would determine the extent of reducing power drainage and therefore the redox imbalance of the pool of Trxs. This is the case of the *ntrc* and the *ntrc-2cpa* mutants (Fig. 1*A*), which contain higher levels of 2-Cys Prxs (92% and 70% of WT level, respectively) than the *ntrc-2cpaGK*, *ntrc-2cpb*, and *ntrc-Δ2cp* mutants (26%, 33%, and 21% of WT level, respectively; Fig. 1*C*) and, to a greater extent, of the *ntrc*/2CPA and *ntrc*/2CPB transgenic lines, which contain even higher levels of 2-Cys Prxs (159–461% of *ntrc* level) and show a more severe phenotypic effect (Fig. S3 *A* and *B*). Moreover, overexpression of 2-Cys Prx A or B in the *ntrc-Δ2cp* (Fig. S3 *E* and *F*) and *ntrc-trxf1f2-Δ2cp* (Fig. S3 *G* and *H*) lines

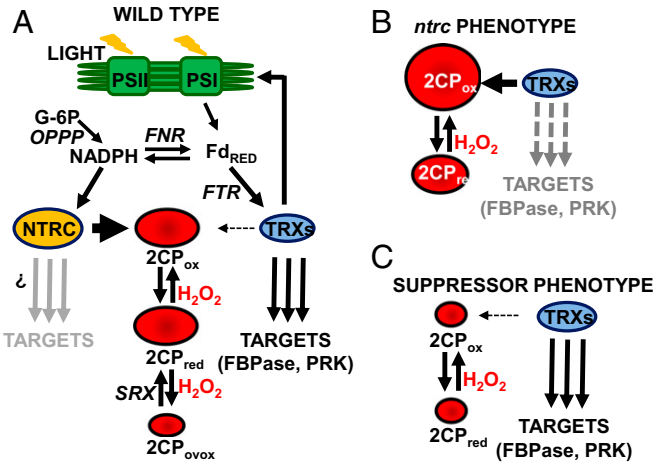

Fig. 5. A proposed model of the control of chloroplast redox homeostasis. (*A*) Light-driven photochemical reactions of photosynthesis generate reduced Fd (Fd_{RED}) and NADPH by the action of Fd-$NADP^+$ reductase (FNR). Additionally, NADPH can be produced by the oxidative pentose phosphate pathway (OPPP). Chloroplast Trxs are responsible for the light-dependent redox regulation of metabolic pathways and the feedback regulation of photochemical reactions. 2-Cys Prxs display three thiol-based redox states: oxidized ($2CP_{ox}$), reduced ($2CP_{red}$), and overoxidized ($2CP_{ovox}$), which is reduced by sulfiredoxin (SRX). (*B*) In NTRC-deficient plants, the accumulation of oxidized 2-Cys Prxs provokes draining of reducing power from the pool of Trxs, and, consequently, redox regulation of their targets is impaired. (*C*) Decreasing the content of 2-Cys Prxs accordingly decreases the draining of reducing power from the pool of Trxs, and the redox regulation of their targets is thereby restored.

partially restored the growth inhibition phenotypes of *ntrc* and *ntrc-trxf1f2*, respectively. The fact that the overexpression of 2-Cys Prx A or B in NTRC-deficient plants provokes similar growth inhibition (Fig. S3 *A*, *B*, and *E*–*H*) indicates that both isoforms have indistinguishable effects.

The present results strongly suggest that the role of NTRC in chloroplast redox regulation is exerted via its functional relationship with 2-Cys Prxs, and therefore the effect of the deficiency of NTRC on plant phenotype would be indirectly produced by the redox imbalance of the pool of Trxs caused by deregulated 2-Cys Prxs. This would provide an explanation for the fact that the lack of NTRC affects such a large variety of chloroplast processes controlled by Trxs. Indeed, the redox regulation of enzymes such as FBPase, which is not reduced by NTRC (13, 27), is severely affected in mutants lacking NTRC (Figs. 3 *A* and *B* and 4 *D* and *E* and Fig. S6*B*). Nevertheless, it has been reported that NTRC interacts with AGPase (8), the CHLI subunit of Mg-chelatase (11, 27), and different Trxs and Calvin–Benson cycle enzymes (27, 31). Therefore, a direct effect of NTRC in redox regulation of these enzymes cannot be ruled out at the moment.

In agreement with previous reports (25, 32), mutants devoid of *f*-type Trxs show only a slight impairment of light-dependent FBPase or PRK reduction (Fig. 4 *D* and *E*). According to our hypothesis, in mutants lacking *f*-type Trxs, the redox state of FBPase and PRK remain unaltered, as NTRC is present to regulate 2-Cys Prxs (Fig. 5*A*). Thus, NTRC would allow other plastidial Trxs, most likely those of the *m* type (33), to exert a compensatory effect. The severe phenotype of the *ntrc-trxf1f2* triple mutant (Fig. 4*A* and Fig. S7 *A* and *B*) is most probably the result of the increased depletion of reducing power from the pool of remaining Trxs provoked by the simultaneous deficiencies of NTRC and *f*-type Trxs, which are among the most abundant chloroplast Trxs (26). This mutant also exhibits dramatically reduced efficiency of light energy utilization, as shown by the elevated NPQ (Fig. S7*C*) and low ETR (Fig. 4*C*). Remarkably, the suppressed mutant *ntrc-trxf1f2-Δ2cp* recovered WT levels of

PLANT BIOLOGY

CO_2 fixation (Fig. S4) and a significant level of light-dependent redox regulation of FBPase and PRK (Fig. 4 *D* and *E*), in line with its growth phenotype, which resembles that of the WT (Fig. 4*A* and Fig. S7 *A* and *B*). However, NPQ and ETR were only partially recovered in the suppressed *ntrc-trxf1f2-Δ2cp* mutant (Fig. 4*C* and Fig. S7*C*), indicating that this level of efficiency of light energy utilization is sufficient to support WT growth rate.

The key function of 2-Cys Prx as a light-regulated oxidative sensor was already proposed based on the analysis of the atypical Trxs ACHT1 and ACHT4 under low light intensity (21, 23). The suppressor effect exerted by decreased levels of 2-Cys Prxs on the *ntrc* phenotype (Fig. 1 and Figs. S1 and S2) and, most notably, on the more severe *ntrc-trxf1f2* phenotype (Fig. 4 *A*–*E* and Fig. S7), confirms the function of 2-Cys Prxs as oxidative sensor and suggests that this function affects not only the atypical Trxs ACHT1 and ACHT4 but the whole pool of chloroplast Trxs. Moreover, our results show the key function of NTRC as the major modulator of the redox balance of the 2-Cys Prxs in chloroplast redox regulation.

As 2-Cys Prxs act as H_2O_2 scavengers, an additional possibility to be taken into account is that the deficiency of the chloroplast redox systems analyzed here—NTRC, Trxs *f*, and 2-Cys Prxs—affect plant phenotype as a result of the oxidative stress caused by the accumulation of H_2O_2, whereas the suppressor effect might be the result of compensatory antioxidant systems. This was addressed by the analysis of nonenzymatic antioxidants (Table S1), the content of H_2O_2 (Fig. S8*A*), and the expression of genes encoding plastid-localized antioxidant enzymes (Fig. S8 *B* and *C*). The minor differences detected suggest that these parameters have very low effect, if any, on the phenotypes of the mutants analyzed here.

The redox state of chloroplast 2-Cys Prxs is maintained by the reducing activity of NTRC and, to a lesser extent, by the pool of Trxs (Fig. 5*A*). It should be noted, however, that reduced 2-Cys Prxs are recycled by H_2O_2, which is therefore an essential component of chloroplast redox regulation. Given the relevant function of the redox balance of 2-Cys Prxs, a question that arises is whether these enzymes are dispensable for plant life. It was recently shown that the *Arabidopsis 2cpa-2cpb* double-KO mutant showed lower photosynthetic efficiency and high sensitivity to high light (34). However, this mutant is viable, suggesting that additional backup systems operate in chloroplast redox homeostasis in the absence of 2-Cys Prxs. Although a yeast strain lacking all eight thiol peroxidases is also viable (35), clear candidates to act as backup systems are additional thiol peroxidases present in plant chloroplasts.

Materials and Methods

Detailed procedures for biological materials and growth conditions, generation of *Arabidopsis* transgenic plants, alkylation assays, measurements of chlorophyll fluorescence, FBPase activity assays, and CO_2 fixation rates, as well as other methods, are described in *SI Materials and Methods* and Tables S2 and S3.

ACKNOWLEDGMENTS. This work was supported by European Regional Development Fund-cofinanced Grant BIO2013-43556-P from the Spanish Ministry of Innovation and Competitiveness (MINECO) and predoctoral fellowships from MINECO (to B.N. and V.O.). The anti-FBPase antibody was provided by Dr. M. Sahrawy, Estación Experimental del Zaidín, Granada, Spain.

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