**Short title:** NTRC is a master regulator of plastid metabolism

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**One sentence summary:**
NADPH thioredoxin reductase C and 2-Cys peroxiredoxins modulate the redox state of plastidial enzymes in response to light and darkness and affect expression of genes encoding cytosolic chaperones.

**Footnotes:****

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ABSTRACT
In chloroplasts, thiol-dependent redox regulation is linked to light since the disulfide reductase activity of thioredoxins (Trxs) relies on photo-reduced ferredoxin (Fdx). Furthermore, chloroplasts harbor an NADPH-dependent Trx reductase (NTR) with a joint Trx domain, termed NTRC. The activity of these two redox systems is integrated
by the redox balance of 2-Cys peroxiredoxin (Prx), which is controlled by NTRC. However, NTRC was proposed to participate in redox regulation of additional targets, prompting inquiry into whether the function of NTRC depends on its capacity to maintain the redox balance of 2-Cys Prxs or by direct redox interaction with chloroplast enzymes. To answer this, we studied the functional relationship of NTRC and 2-Cys Prxs by a comparative analysis of the triple Arabidopsis (*Arabidopsis thaliana*) mutant, *ntre-2cpab*, which lacks NTRC and 2-Cys Prxs, and the double mutant *2cpab*, which lacks 2-Cys Prxs. These mutants exhibit almost indistinguishable phenotypes: in growth rate, photosynthesis performance, and redox regulation of chloroplast enzymes in response to light and darkness. These results suggest that the most relevant function of NTRC is in controlling the redox balance of 2-Cys Prxs. A comparative transcriptomics analysis confirmed the phenotypic similarity of the two mutants and suggested that the NTRC-2-Cys Prxs system participates in cytosolic protein quality control. We propose that NTRC and 2-Cys Prxs constitute a redox relay, exclusive to photosynthetic organisms, that fine-tunes the redox state of chloroplast enzymes in response to light and affects transduction pathways towards the cytosol.

**INTRODUCTION**

Redox regulation, based on thiol-disulfide exchange, constitutes a universal regulatory mechanism in which the disulfide reductase activity of thioredoxins (Trxs) plays a key role (Balsera and Buchanan, 2019). Trx activity requires reducing power, which in heterotrophic organisms is provided by NADPH with the participation of an NADPH-dependent Trx reductase (NTR) (Jacquot et al., 2009). In plant chloroplasts,
redox regulation plays a key role in the rapid response of photosynthetic metabolism to changes in light intensity (Cejudo et al., 2019; Yoshida et al., 2019; Zaffagnini et al., 2019). However, these organelles present remarkable differences with heterotrophic organisms regarding redox regulation. First, chloroplasts harbor a complex set of up to 20 isoforms of Trxs and Trx-like proteins in clear contrast with the low number of genes encoding either NTRs or Trxs in heterotrophs (Geigenberger et al., 2017; Nikkanen and Rintamaki, 2019). Second, and more importantly, chloroplast Trxs do not rely on NADPH as a source of reducing power, but on photosynthetically reduced ferredoxin (Fdx) with the participation of a Fdx-dependent Trx reductase (FTR) (Schürmann and Buchanan, 2008), which links chloroplast redox regulation to light.

The notion that redox regulation in chloroplasts does not rely on NADPH was challenged by the discovery of an NTR with a joint Trx domain at its C-terminus, termed NTRC (Serrato et al., 2004). This enzyme, which shows high affinity for NADPH (Bernal-Bayard et al., 2012), is localized in any type of plant plastids (Kirchsteiger et al., 2012), though it is a relatively abundant protein in the chloroplast stroma (Serrato et al., 2004). Based on the finding that NTRC is a very efficient reductant of 2-Cys peroxiredoxin (2-Cys Prx), a hydrogen peroxide scavenging enzyme, an antioxidant function was proposed for this enzyme (Moon et al., 2006; Pérez-Ruiz et al., 2006; Alkhalfioui et al., 2007). In line with this proposal, several studies have identified the participation of NTRC in plant response to biotic (Ishiga et al., 2012; Ishiga et al., 2016) and abiotic stresses in plants (Serrato et al., 2004; Chae et al., 2013), green algae (Machida et al., 2012) and cyanobacteria (Sánchez-Riego et al., 2016; Mihara et al., 2017). However, further analyses also showed the participation of NTRC in different redox-regulated processes in plant chloroplasts, which include the biosynthesis of starch (Michalska et al., 2009; Lepistö et al., 2013) and tetrapyrroles
(Stenbaek et al., 2008; Richter et al., 2013; Pérez-Ruiz et al., 2014), and the redox regulation of ATP synthase (Carrillo et al., 2016). In addition, mutant Arabidopsis (Arabidopsis thaliana) plants devoid of NTRC exhibit inefficient use of light energy (Carrillo et al., 2016; Naranjo et al., 2016a).

The participation of NTRC in such a variety of processes raised the question of the mechanism of action of this enzyme. NTRC contains both NTR and Trx domains (Serrato et al., 2004); hence, it could exert both activities simultaneously. It is well established that NTRC interacts with 2-Cys Prxs through its Trx domain (Pérez-Ruiz and Cejudo, 2009; Bernal-Bayard et al., 2014), therefore, concerning the interaction with 2-Cys Prxs, NTRC could be considered as a Trx bearing its own NTR. Moreover, in vitro assays showed that NTRC is not an efficient reductant of plastidial Trxs (Böhrer et al., 2012), suggesting that the enzyme has no NTR activity. However, the overexpression of mutant variants of NTRC (with inactive NTR or Trx domains) partially rescued the phenotype of the Arabidopsis ntrc mutant, suggesting that NTRC interacts with chloroplast Trxs, with f-type being proposed as the most likely partner of the enzyme (Toivola et al., 2013). The interaction of NTRC with Trx f1 was further confirmed by BiFC assays and immunoprecipitation (Nikkanen et al., 2016); this group additionally unveiled the interaction of NTRC with redox regulated enzymes of the Calvin-Benson cycle, such as fructose bisphosphatase (FBPase) and phosphoribulokinase (PRK), and the γ subunit of ATPase (ATPc). In line with these findings, the use of Trx-affinity chromatography confirmed the identification of FBPase as a target of NTRC (Yoshida and Hisabori, 2016); however, no reduction of FBPase by NTRC was detected by in vitro assays (Ojeda et al., 2017). Finally, a double chromatography approach identified additional chloroplast proteins in NTRC-
containing complexes (González et al., 2019), thus extending the function of this enzyme to multiple chloroplast processes.

In a previous report (Pérez-Ruiz et al., 2017), we showed that the phenotype of an Arabidopsis mutant lacking NTRC is highly dependent on the total levels of 2-Cys Prxs since the growth inhibition phenotype of the ntrc mutant was rescued by decreased contents of these enzymes. Based on these results we proposed that the redox balance of 2-Cys Prxs, which is maintained by NTRC, controls the activity of the FTR-Trxs pathway. This proposal suggests that NTRC acts via the control of the redox state of 2-Cys Prxs and provides an explanation for the wide variety of processes affected by the absence of this single redox enzyme. However, as stated above, NTRC interacts with different targets, suggesting that it may regulate redox sensitive enzymes in a 2-Cys Prx-independent manner. Thus, the question arising is, what is the actual mode of action of NTRC in chloroplast redox regulation. To address this question, we have generated mutant plants devoid of 2-Cys Prxs A and B (the 2cpab mutant) and lacking NTRC (the ntrc-2cpab mutant). The high similarity of these two mutants, revealed by physiological, biochemical, and transcriptomic analysis, suggests that NTRC exerts a central function on chloroplast redox regulation by an indirect mechanism, which is the maintenance of the redox balance of the 2-Cys Prxs. In addition, the finding of cytosolic chaperones among the most up-regulated genes in 2cpab and ntrc-2cpab mutants suggests a relevant function of chloroplast redox homeostasis in cytosolic protein quality control.

RESULTS

The lack of 2-Cys Prxs or NTRC plus 2-Cys Prxs causes very similar phenotypic effects
To analyze the functional relationship of NTRC and 2-Cys Prxs, we have generated a mutant line of Arabidopsis with knockout of both enzymes. We took advantage of the previously reported 2cpab double mutant (Ojeda et al., 2018a), a knockout for the two 2-Cys Prxs, A and B, present in Arabidopsis, which was manually crossed with the ntrc mutant (Serrato et al., 2004) and the triple mutant ntrc-2cpab was selected among the progeny. Western blot analysis confirmed the absence of NTRC and 2-Cys Prxs in the ntrc-2cpab triple mutant (Fig. 1A, B). The ntrc-2cpab plants nearly mimic the phenotype of the 2cpab mutant in terms of leaf biomass when grown under either under short-day (Fig. 1A, C) or long-day conditions (Supplemental Fig. S1A, B). Because the growth phenotype of the ntrc mutant is highly dependent on light availability, the lines under study were challenged with further reduction of the day length by growing plants in a 4 h light/20 h dark regime and light intensity of 125, 400 or 800 µE m\(^{-2}\) s\(^{-1}\) (Supplemental Fig. S2A). Except for the ntrc mutant, which showed a subtle increase of biomass at higher light intensities, the rosette fresh weight of wild-type, 2cpab and ntrc-2cpab plants increased with increasing light intensities. Notably, leaf biomass in the ntrc-2cpab and 2cpab mutants was similar and lower than in the wild type (Supplemental Fig. S2A, B). In line with the above-described growth phenotypes, the high similarity of the 2cpab and ntrc-2cpab mutants was also observed in terms of chlorophyll levels under any of the tested conditions (Fig. 1D and Supplemental Figs. S1C and S2C). Unlike the ntrc mutant, the ntrc-2cpab mutant accumulates chlorophyll to similar or slightly increased levels compared to 2cpab plants (Fig. 1D and Supplemental Figs. S1C and S2C).

Seedlings of a mutant lacking 2-Cys Prxs were reported to bleach during early developmental stages when grown on sucrose-supplemented medium (Awad et al., 2015), though no quantification of this phenotype was performed. To address the role of
NTRC and 2-Cys Prxs during early seedling development, we sought to examine cotyledon phenotype in seedlings with altered levels of these enzymes. Unlike wild-type and ntrc lines, in which nearly all seedlings presented green cotyledons, seedlings from both 2cpab and ntrc-2cpab mutants showed a variety of cotyledon phenotypes from properly developed green to completely albino (Fig. 2A). Furthermore, we also observed that seedlings of these mutants displayed defects in pigmentation ranging from pale green, yellowish or variegated cotyledons, as well as abnormally shaped, asymmetric cotyledons, categorized here in the same class (pale/variegated/asymmetric) for simplicity. Interestingly, the percentage of green (ca. 20%), albino (ca. 50%) and pale/variegated/asymmetric (ca. 30%) phenotypes in seedlings of the 2cpab mutant was almost indistinguishable of that shown by the ntrc-2cpab mutant (Fig. 2B). These results suggest that the absence of 2-Cys Prxs, rather than NTRC, is responsible for these seedling phenotypes and extends the similarity between the 2cpab and ntrc-2cpab mutant lines to early stages of plant development.

For further comparing the phenotypes of the 2cpab and ntrc-2cpab mutants, we analyzed their photosynthetic performance. First, the maximum potential quantum efficiency of photosystem II (PSII), as determined by the Fv/Fm ratio, was more affected in the ntrc than in the 2cpab mutant, which showed Fv/Fm values indistinguishable of those in the ntrc-2cpab mutant (Fig. 3A). A characteristic feature of the ntrc mutant is the inefficient use of light energy as determined by the high non-photochemical quenching Y(NPQ) at low light intensity (Fig. 3B), which is in line with the poor photosynthetic electron transport rate (ETR) at PSII (Fig. 3C). Notably, the 2cpab mutant shows even lower levels of NPQ than the wild type (Fig. 3B) and higher ETR (II) (Fig. 3C). Again, these photosynthetic parameters were almost indistinguishable in the 2cpab and ntrc-2cpab mutants (Fig. 3B, C). The efficient light utilization, i.e., lower
values of NPQ and higher ETR (II), in the 2cpab and ntrc-2cpab mutants suggests a beneficial effect of the lack of 2-Cys Prxs on photosynthesis efficiency; however, these mutants showed lower rates of CO₂ assimilation (A₀) than the wild type at increasing light intensities (Fig. 3D). In this regard, it is worth mentioning that mutant plants lacking 2-Cys Prxs show lower stomatal conductance and enhanced stomatal closure (Montillet et al., 2021), indicating the multilevel effect of 2-Cys Prxs on photosynthetic performance. Taken together, these results indicate that the addition of the ntrc mutation to plants lacking 2-Cys Prxs results in minor, if any, effect on plant growth and development.

NTRC and 2-Cys Prxs have opposing effects on the redox state of chloroplast enzymes

The functional relationship between NTRC and 2-Cys Prxs was also determined by analyzing the in vivo redox state of well-established redox-regulated chloroplast enzymes. Thiol labelling assays by the alkylating agents methyl maleimide polyethylene glycol (MM-PEG₂₄) or iodoacetamide (IAA) showed full oxidation of the Calvin-Benson cycle enzymes FBPase, PRK, and Rubisco activase (RCA) in dark-adapted plants (Fig. 4A, C, E). In agreement with previous results (Thormählen et al., 2015; Ojeda et al., 2017; Pérez-Ruiz et al., 2017), light-dependent reduction of FBPase (Fig. 4A, B), and PRK (Fig. 4C, D) was severely impaired in the ntrc mutant, which also displayed decreased reduction of RCA when compared to the wild type (Fig. 4E, F). On the contrary, the 2cpab and ntrc-2cpab mutants showed wild type levels of reduction of PRK (Fig. 4C, D) and even higher reduction of FBPase (Fig. 4A, B) and RCA (Fig. 4E, F). Finally, we evaluated the impact of combined NTRC and 2-Cys Prxs mutations on the redox state of plastid Trxs. To that end, we selected f-type Trxs, which were
previously shown to be more reduced in plants overexpressing NTRC (Nikkanen et al., 2016) or those with decreased levels of 2-Cys Prxs (Pérez-Ruiz et al., 2017; Ojeda et al., 2018b). Light-dependent reduction of Trxs f was impaired in the ntrc mutant as compared to the wild type, and slightly, but significantly, increased in both 2cpab and ntrc-2cpab mutants (Supplemental Fig. S3A, B). Overall, these analyses indicate a positive effect of the absence of 2-Cys Prxs on the light-dependent reduction of these enzymes.

The function of 2-Cys Prxs in the short-term oxidation of chloroplast enzymes in the dark has been recently reported (Ojeda et al., 2018a; Vaseghi et al., 2018; Yoshida et al., 2018). In line with this finding, NTRC, which modulates the redox balance of 2-Cys Prxs, also affects the rate of oxidation of chloroplast enzymes in the dark (Ojeda et al., 2018a). To further explore the involvement of the NTRC-2-Cys Prxs system in thiol oxidation, the redox state of FBPase, ATPc, and RCA were analyzed in light-to-dark transitions. The 2cpab and ntrc-2cpab mutants showed indistinguishable and delayed rates of FBPase oxidation in the dark as compared with the wild type, whereas no significant effect was observed in the ntrc mutant (Fig. 5A, B). ATPc and RCA, which are less sensitive to oxidation than FBPase, remained fully and partially reduced, respectively, in the 2cpab and ntrc-2cpab mutants during the time tested (Fig. 5C-F). Interestingly, ATPc oxidation was accelerated in the ntrc mutant, which was observed only when 2-Cys Prxs are present (Fig. 5C, D). The positive effect of the lack of NTRC on enzyme oxidation in the dark was further supported by the rate of oxidation of RCA, which was accelerated in the ntrc mutant, as compared with the wild type (Fig. 5E, F). Overall, these results demonstrate the opposing effects of NTRC and 2-Cys Prxs on chloroplast enzyme oxidation.
Global gene expression profiles of the 2cpab and the ntrc-2cpab mutants

Once we established the effects of NTRC and 2-Cys Prxs on plant performance and the redox state of chloroplast enzymes, we analyzed how chloroplast redox control influences the nuclear transcriptome. To that end, we performed a comparative analysis of the 2cpab and ntrc-2cpab genome wide transcriptomes. RNA sequencing (RNA-Seq) analysis was carried out on three independent biological samples collected from young leaves of both mutant lines and the wild type. Sequencing quality and absence of contamination were assessed by the high percentage of mapped reads. Scatterplots comparing the expression levels of individual genes between biological replicates showed correlations around 99% (Supplemental Fig. S4A-C). The comparison of the global transcriptomes of the 2cpab or ntrc-2cpab mutants with the wild type, using a fold change in gene expression of ± log2(2) and p-value < 0.05, identified a remarkably low number of differentially expressed genes (DEG), 45 and 99 in the 2cpab and ntrc-2cpab mutants, respectively (Fig. 6A). The lists of upregulated and downregulated genes in the 2cpab and ntrc-2cpab mutants, as compared to the wild type, are available as supplemental information (Supplemental Appendix S1). As expected, genes encoding 2-Cys Prxs A and B were among the most downregulated genes in the 2cpab mutant, and these genes, as well as the NTRC gene were downregulated in the ntrc-2cpab mutant, which confirms the consistency of the RNA-Seq analysis data. Of interest, the lack of chloroplast 2-Cys Prxs exerts a remarkable impact in processes taking place outside the organelle, as shown by the subcellular distribution of proteins encoded by DEGs. In the 2cpab mutant, none of the 45 DEG, excluding 2-CYS PRXA and 2-CYS PRXB, encodes chloroplast localized proteins (Supplemental Fig. S5A). Similarly, genes coding for chloroplast proteins, excluding NTRC and 2-CYS PRX A and B, were also absent in the group of DEGs in ntrc-2cpab (Supplemental Fig. S5B).
A principal component analysis (PCA) revealed that the 2cpab and ntrc-2cpab replicates clustered together and separately from the wild type (Supplemental Fig. S6), indicating similar transcriptional profiles between the mutants. Indeed, a substantial percentage (ca. 73%) of DEG in 2cpab were also differentially expressed in the ntrc-2cpab mutant. Thus, of the 28 genes upregulated in the 2cpab mutant, 20 were also induced in the ntrc-2cpab mutant (Fig. 6B). Moreover, an additional group of 32 genes specifically upregulated in the ntrc-2cpab mutant was detected (Fig. 6B). To determine whether these genes constituted truly specific transcriptomic differences between the two mutants, the distribution of their expression fold changes with respect to the wild type was analyzed (Fig. 6C). This analysis revealed that most of the upregulated genes in the ntrc-2cpab mutant were also upregulated, although to a lower level, in the 2cpab mutant, since they exhibit fold changes greater than one. Similarly, most of the downregulated genes in the 2cpab mutant were also downregulated in the ntrc-2cpab mutant (Fig. 6D), and most of the 34 genes specifically downregulated in this mutant were also repressed, though to a lower level, in the 2cpab mutant (Fig. 6E), which was confirmed by bar plots showing the expression levels of genes specifically upregulated (Supplemental Fig. S7) or downregulated (Supplemental Fig. S8) in the mutants. This expression pattern was further verified by RT-qPCR analysis of selected genes (Supplemental Fig. S9).

Transcriptomic analysis of the 2cpab and the ntrc-2cpab mutants suggests a role of chloroplast redox homeostasis in cytosolic protein quality control

Gene Ontology (GO) term enrichment was performed to obtain a better understanding of the biological processes affected in the 2cpab and ntrc-2cpab mutants (Supplemental Appendix S2). Among the upregulated genes, response to endoplasmic
reticulum (ER) stress was the most enriched GO term in both mutants (Supplemental Fig. S10A, B). In addition, the GO terms heat-, hydrogen peroxide-, high light- and oxidative stress-response were specifically enriched in the 2cpab mutant (Supplemental Fig. S10A) whereas GO terms related to biotic stress, such as defense response to pathogens, systemic acquired resistance and salicylic acid biosynthesis, were overrepresented in the ntrc-2cpab mutant. Regarding the downregulated genes, no GO term enrichment was found in the 2cpab mutant, whereas several GO terms related to biosynthesis and metabolism of secondary metabolites containing sulfur (glucosinolates) were significantly enriched in the ntrc-2cpab mutant (Supplemental Fig. S11).

Given the similarity of 2cpab and ntrc-2cpab transcriptomes, we sought to investigate the impact of these mutations in more detail by focusing on genes commonly upregulated and downregulated in both mutants (Supplemental Table S1). Notably, the four most highly induced genes in the 2cpab mutant, which were also upregulated in the ntrc-2cpab mutant, encode heat-shock proteins (HSP), namely HSP70-4, HSP17.6II, HSP23.5 and HSP90-1. In line with this finding, the gene encoding FK506-binding protein 65 (FKBP65), an HSP-interacting co-chaperone (Aviezer-Hagai et al., 2007), was also upregulated, suggesting impairment of protein quality control in these mutants. Both mutants also displayed increased expression of individual members of the phi class of glutathione transferases (GSTF), GSTF3, GSTF7 and GSTF6, suggesting enhanced oxidative stress in these plants. In addition, several genes commonly downregulated in both mutants were identified, including those encoding the ABA-induced transcription repressor 1 (AITR1), xyloglucan endotransglycosylase/hydrolase 31 (XTH31) and β-amylase 5 (BAM5), which are involved in ABA signaling, cell wall remodeling and starch degradation, respectively. Notably, the MTO 1 RESPONDING
DOWN 1 (MRD1) gene, with unknown function, showed the highest fold change among the repressed genes. The expression patterns revealed by the transcriptome analysis were confirmed by RT-qPCR analysis on selected upregulated (HSP17.6, HSP23.5 and GST7) and downregulated genes (MRD1, BAM5 and XTH31) (Supplemental Fig. S12 A, B).

To further explore the impact of the NTRC-2-Cys Prxs redox system on protein quality control, we performed a heat map analysis of individual members of the gene families encoding HSP20s (Fig. S13A), HSP70s (Fig. S13B) and HSP90s (Fig. S13C). Overall, these analyses evidenced that depletion of 2-Cys Prxs, in the presence or absence of NTRC, results in the induction of a specific set of cytosolic chaperones. Of them, we focused on HSP70-4 and HSP90-1 since the levels of these chaperones respond to defective protein import into the chloroplasts (Lee et al., 2009; Wu et al., 2019). Interestingly, the induction of the HSP70-4 and HSP90-1 genes, which was validated by RT-qPCR (Fig. 7A), was reflected in the levels of their encoded proteins. In the 2cpab mutant, increased levels of HSP70 (ca. 1.5-fold) and HSP90-1 (ca. 2-fold) were observed relative to the wild type, whereas slightly higher values were found in ntrc-2cpab (Fig. 7B, C). However, it should be noted that contrary to the specific HSP90-1 antibody, the HSP70 antibody recognizes several HSP70 isoforms, likely resulting in an underestimation of HSP70-4 levels in these plants. Overall, these results suggest that redox modulation exerted by 2-Cys Prxs within chloroplasts impact protein quality control systems outside the organelle.

DISCUSSION

Chloroplast 2-Cys Prxs are efficiently reduced by NTRC (Moon et al., 2006; Pérez-Ruiz et al., 2006; Alkhalfioui et al., 2007; Pulido et al., 2010) and, less
efficiently, by plastid Trxs (Broin et al., 2002; Collin et al., 2003; Dangoor et al., 2012; Cheng et al., 2014; Eliyahu et al., 2015; Hochmal et al., 2016; Jurado-Flores et al., 2020), which allows 2-Cys Prxs to modulate the redox state of Trxs in response to changes in light intensity and during the day-night transition (Pérez-Ruiz et al., 2017; Ojeda et al., 2018b). This model suggests a key role for NTRC as the NADPH-dependent master regulator of chloroplast performance and provides an explanation for the ability of the enzyme to modulate different Trx-dependent processes, yet without directly interacting with these targets. However, several approaches, either in vivo (Nikkanen et al., 2016; González et al., 2019) or in vitro (Yoshida and Hisabori, 2016), have identified putative NTRC targets beside 2-Cys Prxs, suggesting that NTRC might exert its activity through the direct interaction with these enzymes. Thus, it remains as an open question whether NTRC acts exclusively via the regulation of 2-Cys Prxs or by interaction with additional targets. In this study, we addressed this issue with a genetic approach based on a comparative analysis of Arabidopsis lines devoid of 2-Cys Prxs (2cpab mutant) or NTRC plus 2-Cys Prxs (ntrc-2cpab mutant).

Under any of the conditions tested, short-day (Fig. 1A-D), long-day (Suppl. Fig. S1A-C), or ultra-short days (Suppl. Fig. S2A-C), the 2cpab and ntrc-2cpab lines showed very similar growth phenotypes. Therefore, the deficiency of NTRC in Arabidopsis lines that lack 2-Cys Prxs has almost no additional effect, at least in the conditions tested in this work, which supports the notion that NTRC acts exclusively via the regulation of 2-Cys Prxs. A surprising phenotype of the 2cpab mutant is the aberrant development of seedlings, which show a high proportion of albino and pale/variegated/asymmetric cotyledons when grown on MS medium supplemented with sucrose (Fig. 2A, B). The fact that the ntrc-2cpab, but not the ntrc mutant, also exhibits this phenotype, indicates the participation of 2-Cys Prxs, but not of NTRC, in cotyledon
chloroplast biogenesis. On the contrary, at adult stages, the phenotype of the ntrc mutant is more severe than that of the ntrc-2cpab mutant in all of the conditions tested (Fig. 1A-C and Supplemental Figs. S1A-C, S2A-C). Therefore, 2-Cys Prxs exert different effects throughout plant development: NTRC-independent at the early stage and NTRC-dependent at the adult stage. A possibility to explain this behavior is that of the two activities, peroxidase and chaperone (Dietz, 2011), displayed by 2-Cys Prx, one could be more relevant for chloroplast biogenesis and the other in mature chloroplasts, but more work is required to test this possibility.

The availability of mutant lines devoid of NTRC and 2-Cys Prxs has allowed us to address the functional relationship of these enzymes in modulating chloroplast redox homeostasis. While the absence of NTRC provokes lower levels of light-dependent reduction of FBPase, PRK, RCA and Trxs f, the absence of 2-Cys Prxs provokes higher levels of reduction, as compared with the wild type (Fig. 4A-F and Supplemental Fig. S3A-C). However, the rate of oxidation of ATPc and RCA in the dark was accelerated in ntrc mutant but delayed in the 2cpab mutant (Fig. 5C-F). This effect was not as clear for FBPase (Fig. 5A, B), probably due to the rapid oxidation of this enzyme and its poor light-reduction in the ntrc mutant. These results indicate that NTRC favors reduction of chloroplast enzymes, and thus their activation in the light, whereas 2-Cys Prxs favor oxidation, hence their inactivation in the dark. Therefore, in absence of NTRC, a decreased reduction of 2-Cys Prxs is expected to increase oxidation of Trxs, consequently lowering the level of reduction of their targets. In the same line of reasoning, the absence of 2-Cys Prxs is expected to decrease oxidation of Trxs, consequently increasing the level of reduction of their targets, in agreement with previously reported data (Perez-Ruiz el al., 2017). The opposing effects of NTRC and 2-Cys Prxs were also observed on photochemical parameters as shown by the lower
levels of NPQ and higher ETR(II), respectively, in the 2cpab and ntrc-2cpab mutants (Fig. 3B, C). These results reveal that the NTRC-2-Cys Prxs redox system participates in the concerted regulation of stromal enzymes and photochemical reactions, which might be performed by Trxs. In support of this notion, the Trx m4-dependent regulation of photosynthetic cyclic electron transport (Courteille et al., 2013) is exerted via its redox interaction with PROTON GRADIENT REGULATION 5-LIKE PHOTOSYNTHETIC PHENOTYPE (PGRL1) (Okegawa and Motohashi, 2020). The participation of 2-Cys Prxs in enzyme oxidation might be an additional mechanism to dissipate reducing equivalents from reduced stromal enzymes, which might act concertedly with the water-water cycle responsible for photosynthetic control to optimize photosynthetic performance in response to light intensity (Foyer et al., 1990).

As a complementary approach to further explore the functional relationship of NTRC and 2-Cys Prxs, we analyzed the leaf transcriptomes of the 2cpab and ntrc-2cpab mutants. This analysis revealed that depletion of either 2-Cys Prxs or NTRC plus 2-Cys Prxs did not exert a large effect on nuclear gene expression, as shown by the moderate number of DEG identified (Fig. 6A). Given the central role proposed for the NTRC-2-Cys Prxs in chloroplast redox homeostasis, these results may be explained by the fact that this analysis was conducted on plants grown under controlled conditions. Thus, more studies under stressful conditions will be required to fully understand the impact of the NTRC-2-Cys Prxs redox system on plant response to the environment. Remarkably, most of the genes upregulated (Fig. 6B) or downregulated (Fig. 6C) in the 2cpab mutant were coincident in the ntrc-2cpab mutant. Likewise, most of the genes specifically upregulated (Fig. 6C and Supplemental Figs. S7 and S9) or downregulated (Fig. 6D and Supplemental Figs. S8 and S9) in ntrc-2cpab showed a similar trend in the 2cpab mutant, supporting the notion that both mutants have similar gene expression
profiles, as shown by the principal component analysis (Supplemental Fig. S6). Overall, these results demonstrate the high similarity of the 2cpab and ntrc-2cpab mutants at the transcriptome level.

2-Cys Prxs reduce hydrogen peroxide using thiol reducing equivalents, hence interconnecting chloroplast antioxidant and redox regulatory mechanisms (Cejudo et al., 2021). In vivo studies showing higher levels of hydrogen peroxide in plants with decreased content of 2-Cys Prxs (Baier et al., 2000; Pulido et al., 2010) support the antioxidant function of these enzymes. In line with this, an Arabidopsis double knock-out mutant lacking both 2-Cys Prxs, A and B, exhibits bleaching in high light, indicating the role of these enzymes in dissipating excess reducing power through the water-water cycle, which protects the photosynthetic apparatus from oxidative stress (Awad et al., 2015). The identification of GO categories of response to heat, hydrogen peroxide, oxidative stress, or light intensity in the 2cpab mutant (Supplemental Fig. S10A) further supports the antioxidant function of 2-Cys Prxs. Moreover, the identification of GO terms related to biotic stress, i.e., defense response to bacterium and fungus or salicylic acid biosynthesis, among the genes overexpressed in the ntrc-2cpab mutant (Supplemental Fig. S10B), point to redox homeostasis as a relevant component of chloroplast function in plant defense, in line with the well-recognized function of chloroplasts in plant pathogen response (Littlejohn et al., 2020).

Remarkably, response to ER stress was the most enriched GO term among the overexpressed genes of the 2cpab and ntrc-2cpab mutants (Supplemental Figure S10A, B), whereas DEGs coding for chloroplast localized proteins were absent from both mutants (Supplemental Figure S5A, B). Thus, the function of 2-Cys Prxs seems to play a relevant role in the signaling function of the organelle, affecting extra-plastidial processes. The predominance of genes encoding cytosolic proteins being among the
most upregulated genes, especially in the 2cpab mutant (Supplemental Figure S5A), uncovers the effect of chloroplast redox homeostasis on the expression of genes encoding proteins not localized in the organelle. In particular, the presence of several cytosolic chaperone proteins among the upregulated genes in both mutants (Supplemental Table S1) indicates that redox impairment within the chloroplast probably affects protein quality control outside the organelle. Heat map analysis showed that, rather than a global effect on chaperone encoding genes, deficiencies of 2-Cys Prxs or NTRC plus 2-Cys Prxs affect the expression profile of specific isoforms of the HSP20, HSP70 and HSP90 families (Supplemental Fig. S13A-C). The most highly upregulated gene in both mutants encodes the chaperone HSP70-4 (Supplemental Appendix S1 and Supplemental Table S1), whereas remaining isoforms of this family, including the chloroplast-localized HSP70-6 and HSP70-7, showed wild type levels of gene expression (Supplemental Fig. S13B). Interestingly, a previous study showed that HSP70-4, but not other HSP70 isoforms, was highly induced in Arabidopsis mutants that accumulate chloroplast preproteins, hence playing a role in the proteolytic degradation of these precursors by the 26S proteasome pathway (Lee et al., 2009). Moreover, 2-Cys Prxs were found to interact with proteins involved in protein folding (Cerveau et al., 2016). Therefore, the activity of the NTRC-2-Cys Prxs system may affect protein import to chloroplasts, supporting the notion that import of preproteins is regulated by the redox status of the organelle (Balsera et al., 2010). In line with this possibility, HSP70-4, but also HSP90-1, induced in both 2cpab and ntrc-2cpab (Supplemental Appendix S1 and Supplemental Table S1), were shown to accumulate in the Arabidopsis double mutant deficient in GENOMES UNCOUPLED 1 (GUN1) and CLIP PROTEASE C1 (CLPC1), chloroplast proteins involved in plastid-to-nucleus retrograde signaling and protein import, respectively (Wu et al., 2019). In this regard, it
is worth mentioning that 2-Cys Prx, but not NTRC, was identified among the interactors of GUN1 in developing seedlings (Wu et al., 2019), raising the possibility that these enzymes might be functionally related, most likely in an NTRC-independent manner; however, the putative role of 2-Cys Prxs in retrograde signaling is yet unknown. As mentioned above, the albino/variegated cotyledons phenotype of 2cpab and ntrc-2cpab seedlings (Fig. 2) indicates defective chloroplast biogenesis in these mutants. Since this phenotype is exclusive to the 2cpab and ntrc-2cpab mutants, but not the ntrc mutant, the function of 2-Cys Prxs in chloroplast biogenesis seems to be NTRC-independent. Therefore, the induction of HSP70-4 and HSP90-1 at both the transcript (Fig. 7A and Supplemental Table S1) and protein levels (7B, C) in the 2cpab and ntrc-2cpab mutants suggests that the NTRC-2-Cys Prxs redox system might influence protein import to fully developed chloroplasts, which may have dramatic effects on chloroplast biogenesis at early stages of development.

In summary, the results presented in this study support the notion that the most relevant function of NTRC is the control of the redox state of 2-Cys Prxs in fully developed chloroplasts. Since NTRC interacts with targets other than 2-Cys Prxs, additional functions of the NTRC via the direct interaction with these targets cannot be ruled out and may be complementary with the role of the enzyme in 2-Cys Prxs regulation. The catalytic cycle of 2-Cys Prx, which implies the use of thiol reducing equivalents (from NTRC and Trxs) to reduce hydrogen peroxide, allows the function of this enzyme to interconnect with antioxidant mechanisms via its contribution to the water-water cycle redox regulation, allowing the rapid control of chloroplast redox regulated processes in response to changes in light intensity and during the day-night transition. Finally, the comparative transcriptomic analysis of the 2cpab and ntrc-2cpab mutants highly suggests the participation of the NTRC-2-Cys Prxs system in protein
quality control outside the organelle. This function may be especially relevant in chloroplast biogenesis at early stages of plant development, as suggested by the aberrant cotyledon phenotypes of the 2cpab and ntrc-2cpab mutants. Interestingly, the ntrc mutant does not show albino cotyledon phenotype, indicating NTRC-independent functions for 2-Cys Prxs, yet unknown, at early stages of development.

MATERIALS AND METHODS

Biological material and growth conditions

Arabidopsis (Arabidopsis thaliana) wild type (ecotype Columbia) and mutant plants (Supplemental Table S2) were routinely grown in soil in growth chambers. Tested photoperiods were long-day (16-h light/8-h darkness), short-day (8-h light/16-h darkness) and ultra-short-day (4-h light/20-h darkness) at 22°C and 20°C during light and dark periods, respectively, and light intensity of 125 µE m⁻² s⁻¹ unless otherwise specified. To generate the ntrc-2cpab triple mutant, the previously described ntrc (Serrato et al., 2004) and 2cpab (Ojeda et al., 2018a) mutants were manually crossed and the triple homozygous line was identified in the progeny by PCR analysis of genomic DNA with oligonucleotides listed in Supplemental Table S3.

Cotyledon phenotype analyses were performed on seedlings grown on Murashige and Skoog medium containing 0.35% (w/v) Gelrite (Duchefa) and 0.5% (w/v) sucrose. In brief, sets of at least 50 seeds of the different lines under analysis were sown on plates and grown under continuous light. The cotyledon phenotypes, grouped as green, albino or pale/variegated/asymmetric, were scored at seven days to determine the percentage of seedlings within each group.

Protein extraction, alkylation assays and western blot analysis

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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) 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 (Pérez-Ruiz et al., 2017) using 10 mM methylmaleimide polyethylene glycol (MM(PEG)$_{24}$) or 60 mM iodoacetamide as indicated. Protein samples were subjected to SDS-PAGE under reducing (NTRC, 2-Cys Prxs, HSP70 and HSP90-1) or non-reducing (FBPase, PRK, RCA, ATPc and Trxs $f$) conditions using acrylamide gel concentration of 9.5% (FBPase, PRK, RCA and ATPc), 12% (NTRC, 2-Cys Prxs, HSP70 and HSP90-1) and 15% (Trxs $f$). 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 and anti-RCA antibodies were kindly provided by Dr. Sahrawy (Estación Experimental del Zaidín, Granada, Spain) and Dr. A. R. Portis (USDA, Urbana, USA), respectively. Antibodies for ATPc, PRK, HSP70 and HSP90-1 were purchased from Agrisera (Sweden).

**Determination of chlorophyll levels, measurements of chlorophyll $a$ fluorescence and determination of carbon assimilation rates**

Chlorophyll levels were measured as previously described (Pérez-Ruiz et al., 2006). Room temperature chlorophyll fluorescence was measured using a pulse-amplitude modulation fluorometer (DUAL-PAM-100; Walz). The maximum quantum yield of PSII was assayed after incubation of plants in the dark for 30 min by calculating the ratio of the variable fluorescence, $F_v$, to maximal fluorescence, $F_m$.\footnotetext{22}
(Fv/Fm). Induction-recovery curves were performed using red (635 nm) actinic light at 75 μE m⁻² s⁻¹ for 8 min. Saturating pulses of red light at 10000 μE m⁻² s⁻¹ intensity and 0.6 s duration were applied every 60 s and recovery in darkness was recorded for up to 10 min. The parameters Y(II) and Y(NPQ), corresponding to the respective quantum yields of PSII photochemistry and non-regulated basal quenching, were calculated according to reported equations (Kramer et al., 2004). Measurements of relative linear electron transport rates (ETR (II)) were based on chlorophyll fluorescence of pre-illuminated plants by applying stepwise increasing actinic light intensities up to 344 μE m⁻² s⁻¹. Net CO₂ assimilation rate (Aₙ) was measured as previously reported (Ojeda et al., 2017), using an open gas exchange system Li-6400 equipped with the chamber head (Li-6400–40) in dark-adapted leaves of plants grown under short-day for eight weeks. Measurements were performed by the Service for Photosynthesis, Instituto de Recursos Naturales y Agrobiología de Sevilla (Spain).

Transcriptomic Analysis by RNA sequencing and RT-qPCR

Eight-week-old short-day grown wild-type, 2cpab and ntrc-2cpab plants were randomly selected and young rosette leaves collected after 2 hours of illumination at 125 μE m⁻² s⁻¹. Our experimental design consisted of three biological replicates for each genotype, each of them containing leaves from three individual plants. RNA extraction was performed from pooled leaf samples using Sure Prep kit (Fisher), following the manufacturer’s instructions. RNA concentration and purity were tested by an Agilent 2100 Bioanalyzer, a microfluidics-based platform that performs quality control of DNA and RNA samples before sequencing. Library construction of cDNA molecules was carried out following the manufacturer’s instructions using the TruSeq Stranded Total RNA with Ribo-Zero kit (Illumina). The generated DNA fragments were sequenced.
with the Illumina HiSeq 4000 platform, yielding approximately 60-80 million 150 bp long paired-end reads for each sample. Quality control was carried out using the software package FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) confirming that all samples were of good quality. No preprocessing of the reads was required to trim low-quality read fragments. The Arabidopsis Col-0 TAIR 10 reference genome was downloaded from the Ensembl Plants Database (https://plants.ensembl.org/). Read mapping to the reference genome and transcript assembly were performed with the software tools HISAT2 and StringTie (Pertea et al., 2016) using default parameters. Differential expression analysis was carried out with the Bioconductor R packages Ballgown (Pertea et al., 2016) and LIMMA (Richtie et al., 2015) using a fold change of ± log2(2) and p-value < 0.05 computed according to a moderated t test. The R package VennDiagram was used to generate Venn diagrams comparing the different sets of differentially expressed genes. Gene Ontology term and KEGG pathways enrichment analysis over the different gene sets was performed with the Bioconductor R package ClusterProfiler (Yu et al., 2012). Subcellular distribution of proteins encoded by DEGs was assigned according to the SUBcellular Arabidopsis consensus (SUBAcon) algorithm (http://suba.live/) (Hooper et al., 2017). Principal component analysis and hierarchical clustering was carried out using the R Packages FactoMineR (Le et al., 2008). Heatmaps representing gene expression fold-changes in the mutants with respect to the wild type were generated using the function heatmap.2 from the R package gplots. Gene members of the HSP20, HSP70, and HSP90 are classified according to Swindel et al. (2007) and listed in Supplementary Table S4. For RT-qPCR analysis, total RNA was extracted using Trizol reagent (Invitrogen) and cDNA synthesis was performed with 1 µg of total RNA using the Maxima first-strand cDNA synthesis kit (Thermo Scientific) according to manufacturer’s instructions. Real-
time quantitative PCR (RT-qPCR) was performed using an IQ5 real-time PCR detection system (Bio-Rad). Oligonucleotides used for RT-qPCR analyses are listed in Supplementary Table S3. Expression levels were normalized using ACTIN2 and UBQ10 as reference genes.

Accession numbers

The RNA-seq data sets generated in this study have been deposited in the Gene Expression Omnibus (GEO) under accession GSE147793.

Supplemental Data

The following supplemental information is available.

Supplemental Figure S1. The phenotype of the Arabidopsis WT and mutants ntrc, 2cpab and ntrc-2cpab grown under long-day photoperiod.

Supplemental Figure S2. Effect of prolonged nights and different light intensities on the growth of the Arabidopsis WT and mutants ntrc, 2cpab and ntrc-2cpab.

Supplemental Figure S3. In vivo redox state of Trxs f in WT and mutants ntrc, 2cpab and ntrc-2cpab in response to light.

Supplemental Figure S4. Correlation between the transcriptomes of the three biological replicates per Arabidopsis line.

Supplemental Figure S5. Subcellular distribution of proteins encoded by differentially expressed genes in the 2cpab and ntrc-2cpab mutants.

Supplemental Figure S6. Principal component analysis of global gene expression.

Supplemental Figure S7. Genes specifically activated in the ntrc-2cpab mutant.

Supplemental Figure S8. Genes specifically repressed in the ntrc-2cpab mutant.
Supplemental Figure S9. RT-qPCR analysis of selected genes specifically upregulated or downregulated in the ntrc-2cpab mutant.

Supplemental Figure S10. Gene ontology (GO) enrichment analysis of upregulated genes in 2cpab and ntrc-2cpab mutants.

Supplemental Figure S11. Gene ontology (GO) enrichment analysis of downregulated genes in the ntrc-2cpab mutant.

Supplemental Figure S12. RT-qPCR analysis of selected genes commonly upregulated or downregulated in the 2cpab and ntrc-2cpab mutants.

Supplemental Figure S13. Heat-map analysis of the differential expression of chaperone encoding genes of the HSP20, HSP70 and HSP90 families in the 2cpab and ntrc-2cpab mutants.

Supplemental Appendix S1. Differentially expressed genes in the 2cpab and ntrc-2cpab mutant lines.

Supplemental Appendix S2. Gene Ontology (GO) term enrichment in the 2cpab and ntrc-2cpab mutant lines.

Supplemental Table S1. Commonly differentially expressed genes in the 2cpab and ntrc-2cpab mutants.

Supplemental Table S2. Arabidopsis mutants used in this study.

Supplemental Table S3. Oligonucleotides used in this study.

Supplemental Table S4. Members of the HSP20, HSP70 and HSP90 gene families from Arabidopsis thaliana.

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FIGURE LEGENDS

Figure 1. Growth phenotype of Arabidopsis wild type and mutant lines \textit{ntrc}, \textit{2cpab} and \textit{ntrc-2cpab} grown under short-day conditions. (A) Plants of the wild type and mutant lines, as indicated, grown under short-day conditions for 7 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 subjected to SDS-PAGE under reducing conditions, transferred to nitrocellulose filters and probed with anti-NTRC or anti-2-Cys Prx antibodies. Molecular mass markers (kDa) are indicated on the right. The weight of the rosette leaves (C) and chlorophyll content (D) were determined from at least twenty five (C) or ten (D) plants grown as in (A) and represented as average values ± SEM. Asterisks represent significant differences compared with the wild type (**, \( P < 0.01; \) ***, \( P < 0.001, \) Student’s \( t \) test). Hashes indicate significant differences between \textit{2cpab} and \textit{ntrc-2cpab} (#, \( P < 0.05, \) Student’s \( t \) test).

Figure 2. Decreased contents of 2-Cys Prxs affect early stages of plant development. Seedlings of wild type, \textit{ntrc}, \textit{2cpab} and \textit{ntrc-2cpab} mutant lines were grown under continuous light (125 \( \mu \)E m\(^{-2} \) s\(^{-1} \)) on MS synthetic medium supplemented with sucrose. (A) Representative cotyledon phenotypes, grouped as green, albino or pale/variegated/asymmetric, of seven-day-old seedlings. (B) For each genotype, the percentage of seedlings exhibiting the indicated phenotype was determined and represented as mean values ± SEM from three independent replicates (sets of at least 50
seedlings). Asterisks represent significant differences compared with the wild type (**, P < 0.01; ***, P < 0.001, Student’s t test).

Figure 3. Photosynthetic performance of mutant lines devoid of the NTRC/2-Cys Prx system. Photosynthetic parameters were determined in leaves of plants grown under short-day conditions for 7 weeks. (A) 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. The $F_v/F_m$ values (±SEM) are the average of at least twenty-five measurements. Statistical significance compared with the wild type is indicated (***, P < 0.001, Student’s t test). No significant differences were found between 2cpab and ntrc-2cpab. (B) Quantum yields of non-photochemical quenching, Y(NPQ), were measured in five leaves of plants adapted to darkness, except for ntrc which were performed seven times, and each data point is the mean ± SEM. White and black blocks indicate periods of illumination with actinic light (75 µE m$^{-2}$ s$^{-1}$) and darkness, respectively. (C) Linear photosynthetic electron transport rate, ETR (II), was determined at increasing photosynthetic active radiation (PAR). Each value is the average of five determinations, except for ntrc which were performed seven times, and standard errors of the mean (SEM) are represented as error bars. (D) Net CO$_2$ assimilation rate ($A_N$) was measured using an open gas exchange system in dark-adapted leaves. For each line, three leaves were measured and mean ± SD are represented.

Figure 4. In vivo redox state of FBPase, PRK and RCA in the ntrc-2cpab mutant in response to light. Wild type and mutant plants were grown under long-day conditions for 4 weeks at a light intensity of 125 µE m$^{-2}$ s$^{-1}$. The in vivo redox state of FBPase (A) PRK (C) and RCA (E) were determined at the end of the dark period (D), and after 30
min of illumination at 180 µE m$^{-2}$ s$^{-1}$ (L) by labelling of the thiol groups with the
alkylating agent MM(PEG)$_{24}$ (A, C) or iodoacetamide (E). Molecular mass markers
(kDa) are indicated on the left. Band intensities were quantified (Gel Analyzer) and the
percentage of reduction, determined as the ratio between the reduced form and the sum
of reduced and oxidized forms, of FBPase (B), PRK (D) and RCA (E) is represented as
the mean ± SEM of four independent experiments. Statistical significance compared
with the wild type is indicated (***, P < 0.001, Student’s t test). No significant
differences were found between 2cpab and ntrc-2cpab. Red, reduced: Ox, oxidized.

Figure 5. Dark-dependent oxidation of FBPase ATPc and RCA in the wild type
and mutant lines. Wild type and 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 480 µE m$^{-2}$ s$^{-1}$ for 45 min (L): then light was
switched off and samples were taken at the indicated times. The in vivo redox states of
FBPase (A), ATPc (C) and RCA (E) were determined with the alkylating agent
iodoacetamide. Molecular mass markers (kDa) are indicated on the left. The
 corresponding band intensities were quantified (GelAnalyzer) and the percentage of
reduction of FBPase (B), ATPc (D) and RCA (F) is shown as the ratio between the
reduced form and the sum of reduced and oxidized forms for each protein. Values are
the mean ± SEM of three independent experiments, except for 2cpab (FBPase and
ATPC) where values represent mean ± SEM of four independent experiments.
Statistical significance compared with the wild type is indicated (*, P < 0.05; **, P <
0.01; ***, P < 0.001, Student’s t test). No significant differences were found between
2cpab and ntrc-2cpab. Red, reduced: Ox, oxidized.
Figure 6. Transcriptome analysis of wild type and the 2cpab and ntrc-2cpab mutants. (A) Total number of differentially expressed genes (DEG) and distribution of upregulated and downregulated genes in the 2cpab and ntrc-2cpab mutants compared to the wild type (according to a 2-fold change and a P value of 0.05). (B-D) Venn diagrams indicating the number of upregulated (B) and downregulated (D) genes in 2cpab (light blue) and ntrc-2cpab (red). The number of genes that overlap among 2cpab and ntrc-2cpab are indicated in dark blue. The fold changes in expression for specifically upregulated (C) and downregulated (E) genes in the ntrc-2cpab mutant are represented using boxplots. The median (middle horizontal line), upper and lower quartiles (boxes), as well as minimum and maximum values (whiskers) are indicated. Circles represent high and low extreme values outside 1.5 times the interquartile range (box length) above/below the upper/lower quartile.

Figure 7. Transcript and protein levels of HSP70-4 and HSP90-1 in wild type and the mutants 2cpab and ntrc-2cpab. (A) Levels of transcripts of HSP70-4 and HSP90-1 were determined by RT-qPCR using RNA isolated from young leaves of plants grown under short-day conditions for 8 weeks. (B) Western blot analysis of the content of HSP70 and HSP90-1. Protein extracts were obtained from leaves of plants grown as stated in (A) and aliquots of 5 µg (1X), from all lines, and 10 µg (2X) and 15 µg (3X) of WT proteins, as indicated, were subjected to SDS–PAGE under reducing conditions, transferred to nitrocellulose filters, and probed with anti-HSP70 or anti-HSP90-1 antibodies (see methods). Molecular mass markers (kDa) are indicated on the left and even loading was monitored by Ponceau staining of the Rubisco large subunit. (C) Band intensities corresponding to HSP70, HSP90-1 and Rubisco large subunit were
quantified (GelAnalyzer). The contents of HSP70 and HSP90-1, normalized to the levels of Rubisco large subunit, are shown relative to the levels of the WT (1X) sample (arbitrarily assigned a value of 100). Data are given as the mean ± SEM of three independent experiments. Statistical significance compared with the wild type is indicated (*, P < 0.05; **, P < 0.01; ***, P < 0.001, Student’s t test). No significant differences were found between 2cpab and ntrc-2cpab.

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Figure 1. Growth phenotype of Arabidopsis wild type and mutant lines *ntrc*, *2cpab* and *ntrc-2cpab* grown under short-day conditions. (A) Plants of the wild type and mutant lines, as indicated, grown under short-day conditions for 7 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 subjected to SDS-PAGE under reducing conditions, transferred to nitrocellulose filters and probed with anti-NTRC or anti-2-Cys Prx antibodies. Molecular mass markers (kDa) are indicated on the right. The weight of the rosette leaves (C) and chlorophyll content (D) were determined from at least twenty five (B) or ten (C) plants grown as in (A) and represented as average values ± SEM. Asterisks represent significant differences compared with the wild type (**, P < 0.01; ***, P < 0.001, Student’s t test). Hashes indicate significant differences between *2cpab* and *ntrc-2cpab* (#, P < 0.05, Student’s t test).
Figure 2. Decreased contents of 2-Cys Prxs affect early stages of plant development.

Seedlings of wild type, ntrc, 2cpab and ntrc-2cpab mutant lines were grown under continuous light (125 µE m⁻² s⁻¹) on MS synthetic medium supplemented with sucrose. (A) Representative cotyledon phenotypes, grouped as green, albino or pale/variegated/asymmetric, of seven-day-old seedlings. For each line, the percentage of seedlings with the indicated phenotype were determined and represented as mean values ± SEM from three replicates. Letters indicate significant differences with the Student’s t test at 95% confidence interval.
Figure 3. Photosynthetic performance of mutant lines devoid of the NTRC/2-Cys Prx system. Photosynthetic parameters were determined in leaves of plants grown under short-day conditions for 7 weeks. (A) The maximum PSII quantum yield was determined as variable fluorescence (F_v) to maximal fluorescence (F_m), F_v/F_m, in dark-adapted leaves. The F_v/F_m values (±SEM) are the average of at least twenty-five measurements. Statistical significance compared with the wild type is indicated (***, P < 0.001, Student’s t test). No significant differences were found between 2cpab and ntrc-2cpab. (B) Quantum yields of non-photochemical quenching, Y(NPQ), were measured in five leaves of plants adapted to darkness, except for ntrc which were performed seven times, and each data point is the mean ± SEM. White and black blocks indicate periods of illumination with actinic light (75 µE m^{-2} s^{-1}) and darkness, respectively. (C) Linear photosynthetic electron transport rate, ETR (II), was determined at increasing photosynthetic active radiation (PAR). Each value is the average of five determinations, except for ntrc which were
performed seven times, and standard errors of the mean (SEM) are represented as error bars. (D) Net CO$_2$ assimilation rate ($A_N$) was measured using an open gas exchange system in dark-adapted leaves. For each line, three leaves were measured and mean ± SD are represented.
Figure 4. *In vivo* redox state of FBPase, PRK and RCA in the *ntrc-2cpab* mutant in response to light. Wild type and mutant plants were grown under long-day conditions for 4 weeks at a light intensity of 125 µE m\(^{-2}\) s\(^{-1}\). The *in vivo* redox state of FBPase (A), PRK (C) and RCA (E) were determined at the end of the dark period (D), and after 30 min of illumination at 180 µE m\(^{-2}\) s\(^{-1}\) (L) by labelling of the thiol groups with the alkylation agent MM(PEG)\(_{24}\) (A, C) or iodoacetamide (E). Molecular mass markers (kDa) are indicated on the left. Band intensities were quantified (Gel Analyzer) and the
percentage of reduction, determined as the ratio between the reduced form and the sum of reduced and oxidized forms, of FBPase (B), PRK (D) and RCA (E) is represented as the mean ± SEM of four independent experiments. Statistical significance compared with the wild type is indicated (***, P < 0.001, Student’s t test). No significant differences were found between 2cpab and ntrec-2cpab. Red, reduced: Oxi, oxidized.
Figure 5. Dark-dependent oxidation of FBPase ATPc and RCA in the wild type and mutant lines. Wild type and 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 480 µE m⁻² s⁻¹ during 45 min (L), then light was switched off and samples were taken at the indicated times. The *in vivo* redox state of FBPase (A),
ATPc (C) and RCA (E) were determined with the alkylating agent iodoacetamide. Molecular mass markers (kDa) are indicated on the left. The corresponding band intensities were quantified (GelAnalyzer) and the percentage of reduction of FBPase (B), ATPc (D) and RCA (F) is shown as the ratio between the reduced form and the sum of reduced and oxidized forms for each protein. Values are the mean ± SEM of three independent experiments, except for 2cpab (FBPase and ATPC) which represent mean ± SEM of four independent experiments. Statistical significance compared with the wild type is indicated (*, P < 0.05; **, P < 0.01; ***, P < 0.001, Student’s t test). No significant differences were found between 2cpab and ntre-2cpab. Red, reduced: Oxi, oxidized.
Figure 6. Transcriptome analysis of wild type and the 2cpab and ntrc-2cpab mutants.

(A) Total number of differentially expressed genes (DEG) and distribution of upregulated and downregulated genes in the 2cpab and ntrc-2cpab mutants compared to the wild type according to a 2-fold change and a $P$ value of 0.05. (B-D) Venn diagrams indicating the number of upregulated (B) and downregulated (D) genes in 2cpab (light blue) and ntrc-2cpab (red). The number of genes that overlap among 2cpab and ntrc-2cpab are indicated in dark blue. Distribution of the fold-change in gene expression for genes specifically upregulated (C) and downregulated (E) in the ntrc-2cpab mutant.
Figure 7. Transcript and protein levels of HSP70-4 and HSP90-1 in wild type and the mutants 2cpab and ntrc-2cpab. (A) Levels of transcripts of HSP70-4 and HSP90-1 were determined by RT-qPCR using RNA isolated from young leaves of plants grown under short-day conditions for 8 weeks. (B) Western blot analysis of the content of HSP70 and HSP90-1. Protein extracts were obtained from leaves of plants grown as stated in (A) and aliquots of 5 µg (1X), from all lines, and 10 µg (2X) and 15 µg (3X) of WT proteins,
as indicated, were subjected to SDS–PAGE under reducing conditions, transferred to nitrocellulose filters, and probed with anti-HSP70 or anti-HSP90-1 antibodies (see methods). Molecular mass markers (kDa) are indicated on the left and even loading was monitored by Ponceau staining of the Rubisco large subunit. (C) Band intensities corresponding to HSP70, HSP90-1 and Rubisco large subunit were quantified (GelAnalyzer). The contents of HSP70 and HSP90-1, normalized to the levels of Rubisco large subunit, are shown relative to the levels of the WT (1X) sample (arbitrarily assigned a value of 100). Data are given as the mean ± SEM of three independent experiments. Statistical significance compared with the wild type is indicated (*, P < 0.05; **, P < 0.01; ***, P < 0.001, Student’s t test). No significant differences were found between 2cpab and ntrc-2cpab.
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