NADPH Thioredoxin Reductase C and Thioredoxins Act Concertedly in Seedling Development[1][OPEN]

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Thiol-dependent redox regulation of enzyme activity plays a central role in the rapid acclimation of chloroplast metabolism to ever-fluctuating light availability. This regulatory mechanism relies on ferredoxin reduced by the photosynthetic electron transport chain, which fuels reducing power to thioredoxins (Trxs) via a ferredoxin-dependent Trx reductase. In addition, chloroplasts harbor an NADPH-dependent Trx reductase, which has a joint Trx domain at the carboxyl terminus, termed NTRC. Thus, a relevant issue concerning chloroplast function is to establish the relationship between these two redox systems and its impact on plant development. To address this issue, we generated Arabidopsis (*Arabidopsis thaliana*) mutants combining the deficiency of NTRC with those of Trxs *f*, which participate in metabolic redox regulation, and that of Trx *x*, which has antioxidant function. The *ntrc-trxf1f2* and, to a lower extent, *ntrc-trxx* mutants showed severe growth-retarded phenotypes, decreased photosynthesis performance, and almost abolished light-dependent reduction of fructose-1,6-bisphosphatase. Moreover, the combined deficiency of both redox systems provokes aberrant chloroplast ultrastructure. Remarkably, both the *ntrc-trxf1f2* and *ntrc-trxx* mutants showed high mortality at the seedling stage, which was overcome by the addition of an exogenous carbon source. Based on these results, we propose that NTRC plays a pivotal role in chloroplast redox regulation, being necessary for the activity of diverse Trxs with unrelated functions. The interaction between the two thiol redox systems is indispensable to sustain photosynthesis performed by cotyledons chloroplasts, which is essential for early plant development.

Photosynthesis is a central feature of chloroplasts, allowing the use of light and water for the production of organic material, thus being the primary source of biomass and oxygen in the biosphere. Besides their essential function as factories of metabolic precursors, chloroplasts also play a relevant role in harmonizing the growth of the different organs of the plant as well as in its acclimation to changing environmental conditions. Therefore, chloroplast biogenesis and function are deeply integrated with plant growth and development (Inaba and Ito-Inaba, 2010; Jarvis and López-Juez, 2013). A classic example of this integration occurs at the seedling stage, when true leaves are generated (Waters and Langdale, 2009; Pogson et al., 2015). To fulfill these functions, chloroplast metabolism needs to adjust rapidly to unpredictable changes of light availability. In this regard, the thiol-dependent redox regulation of enzyme activity plays a relevant role (Couturier et al., 2013; Balsera et al., 2014), the disulfide reductase activity of thioredoxins (Trxs) being central for this regulatory mechanism (Meyer et al., 2012; Serrato et al., 2013).

Trx-dependent redox regulation is universally found in any kind of organism from bacteria and yeasts to plants and animals. In heterotrophic organisms and nonphotosynthetic compartments of plant cells, the reducing power for Trx reduction is provided by NADPH in a reaction catalyzed by an NADPH-dependent Trx reductase (NTR; Jacquot et al., 2009). In contrast, redox regulation in plant chloroplasts has remarkable specific features. First, these organelles harbor a rather complex set of Trxs (Meyer et al., 2012; Balsera et al., 2014), while in heterotrophic organisms, Trx and NTR are encoded by small gene families. Moreover, the source of reducing

power for Trx reduction in chloroplasts is not NADPH, as in heterotrophic organisms, but reduced ferredoxin (Fdx); hence, chloroplasts are equipped with an Fdx-dependent Trx reductase (FTR; Schürmann and Buchanan, 2008), which is specific for these organelles. Based on the function of the FTR/Trx redox system, the classic view of the redox regulation of chloroplast metabolism states that metabolic pathways such as carbon assimilation by the Calvin-Benson cycle are active during the day due to the reductive activation of enzymes of the cycle, which relies on Fdx reduced by the photosynthetic electron transport chain, thus linking metabolic regulation to light (Michelet et al., 2013; Serrato et al., 2013).

The view of chloroplast redox regulation based on the action of the Fdx-dependent FTR/Trx system was modified after the discovery of a novel type of NTR with a joint Trx domain at the C terminus, which was termed NTRC (Serrato et al., 2004). NTRC is exclusive of organisms that perform oxygenic photosynthesis and shows plastid localization in plants (Kirchsteiger et al., 2012). Moreover, biochemical analyses revealed that NTRC is able to conjugate both NTR and Trx activities to efficiently reduce the hydrogen peroxide-scavenging enzyme 2-Cys peroxiredoxin (Moon et al., 2006; Pérez-Ruiz et al., 2006; Alkhalfioui et al., 2007). Arabidopsis (*Arabidopsis thaliana*) NTRC knockout mutants show a characteristic phenotype of retarded growth and pale green leaves, this phenotype being more severe under short-day conditions (Pérez-Ruiz et al., 2006; Lepistö et al., 2009). Based on these results, it was initially proposed that NTRC constitutes an NADPH-dependent redox system that might have a complementary function to the Fdx-dependent FTR/Trx system. Further evidence showed the participation of NTRC in the redox regulation of enzymes that were known previously to be Trx regulated. This is the case for ADP-Glc pyrophosphorylase (AGPase), a key enzyme of starch biosynthesis (Michalska et al., 2009; Lepistö et al., 2013) and of enzymes of the chlorophyll biosynthesis pathway (Richter et al., 2013; Pérez-Ruiz et al., 2014). These results suggest that chloroplast redox regulation depends on the cross talk of both Fdx/FTR/Trx and NTRC redox systems and, hence, is more complex than anticipated previously.

It was reported recently that a double mutant of Arabidopsis devoid of NTRC and Trx *f*1, which is the most abundant *f*-type Trx, shows a very severe growth inhibition phenotype and an almost complete abolishment of light activation of Fru-1,6-bisphosphatase (FBPase), a redox-regulated enzyme of the Calvin-Benson cycle (Thormählen et al., 2015). Furthermore, the Arabidopsis double mutant deficient in NTRC and FTR displays a lethal phenotype under autotrophic growth conditions (Yoshida and Hisabori, 2016), suggesting that NTRC and the Fdx/FTR/Trx systems act in concert in the redox regulation of common targets in planta. Bifluorescence complementation assays showing that NTRC interacts in vivo with well-established Trx-regulated enzymes (Nikkanen et al., 2016), further supporting this notion. The question arising is whether the effect of NTRC is exerted only on Trxs such as those of the *f* type,

which participate in the redox regulation of metabolic pathways, or also affects the activity of other types of Trxs. To address this issue, we analyzed the genetic interaction of NTRC with two functionally unrelated chloroplast Trxs: *f* type, as representative of Trxs involved in the redox regulation of metabolic pathways (Michelet et al., 2013), and *x* type, as representative of Trxs involved in antioxidant defense (Collin et al., 2003). The Arabidopsis *ntrc-trxx* double mutant and the *ntrc-trxf1f2* triple mutant were generated. While the Trx-deficient parental lines, *trxf1f2* and *trxx*, showed a visible phenotype similar to the wild-type plants when grown under long-day conditions, these deficiencies caused a severe growth inhibition phenotype when combined with the lack of NTRC. Moreover, the *ntrc-trxx* mutant and, to a higher extent, the *ntrc-trxf1f2* mutant showed a massive mortality at the seedling stage. These results reveal that NTRC is essential for the activity of unrelated Trxs, thus playing a pivotal role in chloroplast redox regulation, which is critical during early stages of plant development.

RESULTS

The Combined Deficiencies of NTRC and *f*- or *x*-Type Trxs Cause a Severe Growth Inhibition Phenotype and the Impairment of Light-Dependent Regulation of FBPase

In our attempt to establish the function of NTRC in chloroplast redox regulation, we studied the genetic interaction of NTRC with functionally divergent chloroplast Trxs, such as Trx *f* and Trx *x*. Previous results suggesting the concerted action of NTRC and Trx *f*1 were based on the severe growth-retarded phenotype of the Arabidopsis *ntrc-trxf1* double mutant (Thormählen et al., 2015). However, this mutant still contains Trx *f*2, which constitutes approximately 10% of the total content of *f*-type Trxs in wild-type plants (Thormählen et al., 2013). To avoid any effect of the residual Trx *f*2 in its relationship with NTRC, we generated the triple mutant *ntrc-trxf1f2* devoid of NTRC and Trxs *f*. In addition, the *ntrc* mutant was crossed with the *trxx* single mutant (Pulido et al., 2010) and the *ntrc-trxx* double mutant was obtained. These lines were effectively knockout mutants, as shown by the lack of transcripts of the corresponding genes in each of the mutants as determined by real-time quantitative PCR (RT-qPCR) analyses (Fig. 1A). As reported previously, the Trx-deficient lines *trxf1f2* (Naranjo et al., 2016a) and *trxx* (Pulido et al., 2010) showed a visible phenotype very similar to that of wild-type plants when grown under a long-day photoperiod (Fig. 1, B and C), while the *ntrc* mutant showed a growth-retarded and pale-green phenotype (Fig. 1, B and C), in agreement with previous results (Serrato et al., 2004; Lepistö et al., 2009). In contrast, the *ntrc-trxx* mutant, combining the deficiencies of NTRC and Trx *x*, showed a very severe growth-retarded phenotype, which was even more severe in the case of the *ntrc-trxf1f2* triple mutant (Fig. 1B). In line with their phenotypes, the *ntrc-trxf1f2* and *ntrc-trxx* mutants showed significantly

Figure 1. Levels of NTRC, Trx *x*, Trx *f*1, and Trx *f*2 transcripts and growth phenotypes of wild-type (WT) and mutant plants. A, The contents of transcripts of the genes *NTRC, TRX x, TRX f1*, and *TRX f2* were determined by RT-qPCR from total RNA samples, which were extracted from leaves of Arabidopsis wild-type and mutant lines, as indicated. The pairs of oligonucleotides used for cDNA amplification are indicated in Supplemental Table S3. Transcript levels were normalized to *ACTIN* and the *AT5G25760* genes and referred to the level of each of the genes in wild-type plants. Determinations were performed three times, and mean values ± SE are represented. B, Plants of wild-type and mutant lines that were grown under long-day conditions (16 h of light/8 h of darkness, light intensity of 125 μE m^{-2} s^{-1}) during 22 d. C, Chlorophyll content was determined from leaf discs ($n = 6$), and average values ± SE are represented. Letters indicate significant differences by Student's *t* test and a confidence interval of 99%. FW, Fresh weight.
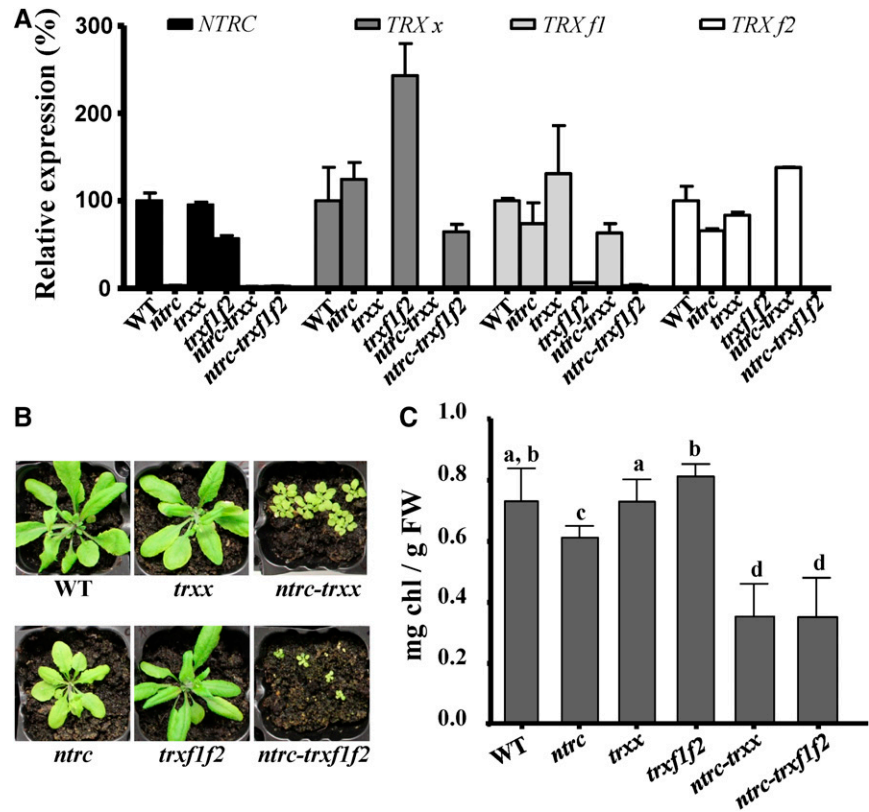
lower content of chlorophyll than their respective parent lines (Fig. 1C). It should be noted that, despite their severe growth inhibition, both the *ntrc-trxx* and *ntrc-trxf1f2* mutants were able to produce flowers and viable seeds when grown under a long-day photoperiod. Therefore, while the lack of Trxs *f* or Trx *x* has little effect on plant phenotype, when these deficiencies are combined with that of NTRC, the effects become very severe.

To establish the impact of the combined deficiencies of NTRC and Trxs *f* and *x* on the redox regulation of chloroplast metabolic pathways, we analyzed FBPase, a well-established redox-regulated enzyme of the Calvin-Benson cycle. First, activity assays showed slightly lower FBPase activity in leaves of the *ntrc, trxx,* and *trxf1f2* mutants than in the wild type, while the *ntrc-trxx* mutant showed lower activity, which was even lower in the *ntrc-trxf1f2* mutant (Fig. 2A). It should be taken into account that Arabidopsis contains three isoforms of FBPase, one localized in the cytosol (Cséke and Buchanan, 1986) and two in the chloroplast (Serrato et al., 2009); hence, the FBPase activity determined here reflects the contribution of these isoforms. However, only one of the chloroplast-localized FBPase isoforms is redox regulated, and Arabidopsis mutants lacking this isoform have a more severe dwarf phenotype than mutants lacking the cytosolic isoform (Rojas-González et al., 2015). Thus, to get more insight into the function of NTRC, Trxs *f*, and Trx *x* in chloroplast redox regulation, we analyzed the light-dependent changes of the redox state of the plastid-localized FBPase isoform,

which is known to be regulated by Trxs *f* but not by Trx *x* (Michelet et al., 2013). As reported previously (Thormählen et al., 2015; Naranjo et al., 2016a), thiol derivatization with the alkylating agent methyl-maleimide polyethylene glycol [MM(PEG)$_{24}$] showed that FBPase was fully oxidized in leaves from dark-adapted wild-type plants and became reduced upon illumination, the level of reduction being dependent on light intensity (Fig. 2, B and C). The light-dependent FBPase reduction was partially impaired in the *trxf1f2* mutant and, surprisingly, also in the *trxx* mutant (Fig. 2, B and C). Interestingly, the degree of FBPase photoreduction was even lower in the *ntrc* mutant and was almost abolished in mutants combining the deficiency of NTRC with those of Trx *x* or Trxs *f* (Fig. 2, B and C), in agreement with previous studies with *ntrc-trxf1* double mutants (Thormählen et al., 2015). Illumination with higher light intensity resulted in a higher level of enzyme reduction, although the pattern of FBPase reduction of the different lines was similar to that obtained under growth light (Fig. 2, B and C).

These results are surprising, since biochemical analyses have shown that FBPase is regulated by Trxs *f* but not by NTRC (Yoshida and Hisabori, 2016) or Trx *x* (Collin et al., 2003). Thus, to analyze further the role of these thiol redox systems in the regulation of FBPase, we performed in vitro assays with the purified enzymes. While almost full reduction of FBPase was accomplished with 0.5 μM concentration of either Trx *f*1 or Trx *f*2, the concentration of Trx *x* needed to obtain a similar level of
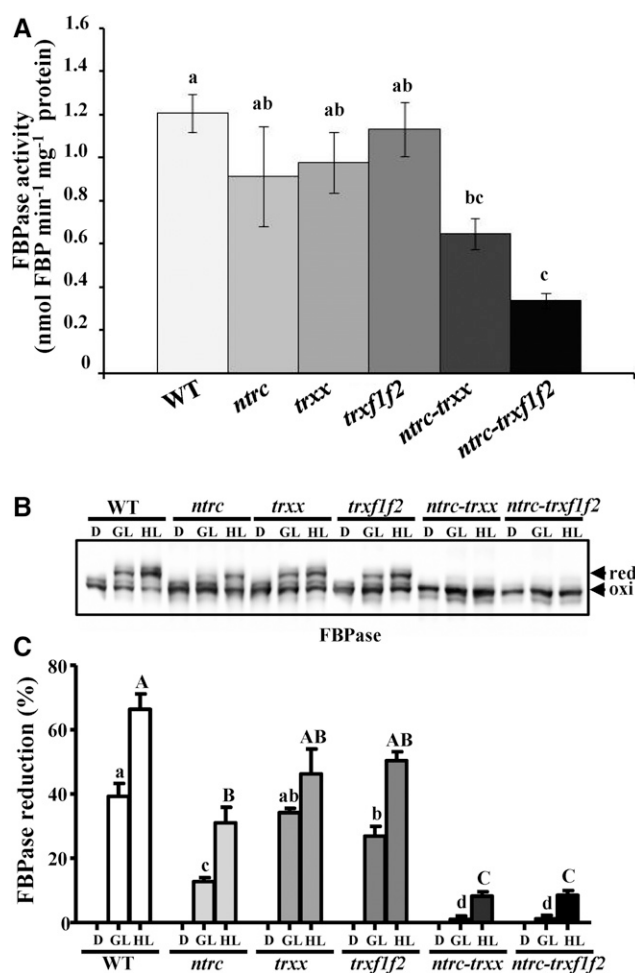
Figure 2. FBPase activity and in vivo redox state of FBPase in wild-type (WT) and mutant lines. A, FBPase activity was assayed in leaf extracts from plants grown for 4 weeks under a long-day photoperiod and harvested at 12 h of the day period. Results are means ± SE from three biological replicates. Different letters above each bar represent significant differences ($P < 0.05$) determined by one-way ANOVA followed by Tukey's posttest. B, The in vivo redox state of FBPase was determined by derivatization of thiols with the alkylating agent MM(PEG)$_{24}$, as indicated in "Materials and Methods." Plants of wild-type and mutant lines were grown under long-day conditions for 4 weeks. Samples were harvested at the end of the period of darkness (D) and then illuminated for 30 min with light intensities of 125 μE m^{-2} s^{-1} (growth light; GL) or 500 μE m^{-2} s^{-1} (high light; HL). Protein extracts from leaves were subjected to SDS-PAGE under nonreducing conditions, transferred onto nitrocellulose filters, and probed with an anti-FBPase antibody. red, Reduced; oxi, oxidized. C, Band intensities were quantified (ScionImage), and the percentage of reduction was calculated as the ratio between the shifted band (reduced form) and the sum of reduced and oxidized forms. Each value is the mean of three independent experiments ± SE, and letters indicate significant differences between mutants by Student's *t* test at the 95% confidence interval.

FBPase reduction was at least 10-fold higher (Fig. 3), which is in contrast with the similar level of impairment of light-dependent FBPase reduction in the *trxx* and *trxf1f2* mutants (Fig. 2, B and C). Moreover, NTRC did not show any capacity to reduce FBPase in vitro (Fig. 3), despite the fact that the degree of light-dependent

FBPase reduction was greatly decreased in the *ntrc* mutant (Fig. 2, B and C). The severe impairment of the light-dependent reduction of FBPase in the *ntrc-trxx* and *ntrc-trxf1f2* mutants demonstrates the concerted action of both NTRC and *f*- or *x*-type Trxs on FBPase redox regulation, although these results suggest that NTRC affects FBPase redox regulation by an indirect mechanism.

The Combined Deficiencies of NTRC and *f*- or *x*-Type Trxs Affect Photosynthetic Performance and Chloroplast Structure

The effects of the combined deficiencies of NTRC and Trxs *f* or Trx *x* on plant growth (Fig. 1) and the activity (Fig. 2A) and redox regulation (Fig. 2, B and C) of FBPase suggest that the impairment of these redox regulatory systems affects photosynthesis performance. Previous reports have shown that the deficiency of NTRC, but not of Trxs *f* or Trx *x*, provokes enhanced nonphotochemical quenching (NPQ) at low light intensity (Naranjo et al., 2016a, 2016b), hence causing a poor efficiency in the utilization of light energy by the *ntrc* mutant. Thus, as a first step to determine the effect of the combined deficiencies of NTRC and Trxs *f* or *x* on photosynthetic performance, we analyzed NPQ in the different lines under study. In agreement with previous results, the *ntrc* mutant showed enhanced NPQ at light intensity as low as 42 μmol quanta m^{-2} s^{-1}, while NPQ was enhanced only slightly in the *trxx* and *trxf1f2* mutants (Fig. 4A). Similar to the *ntrc* mutant, *ntrc-trxx* and *ntrc-trxf1f2* plants showed extended NPQ values during the whole illumination period; however, NPQ did not decay completely in the period of darkness as it did in *ntrc* plants (Fig. 4A), showing that light utilization in
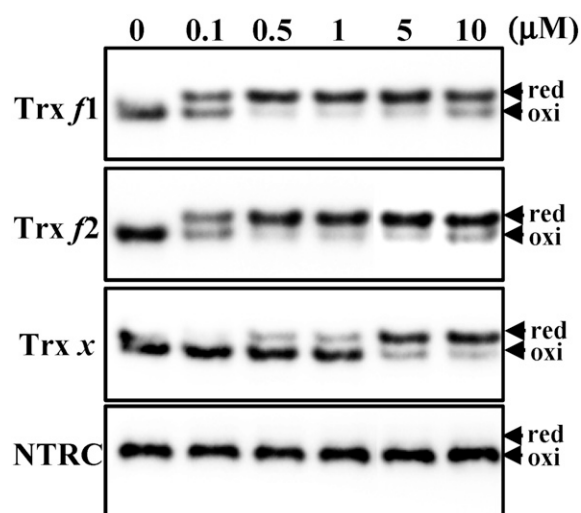

Figure 3. In vitro reduction of FBPase by NTRC and Trxs *x* or *f*. The in vitro FBPase reduction assay was carried out by incubating a fixed concentration of the purified enzyme (2.5 μM) in the presence of 0.1 mM DTT, with increasing concentrations, as indicated, of NTRC, Trx *f*1, Trx *f*2, and Trx *x*. red, Reduced; oxi, oxidized.
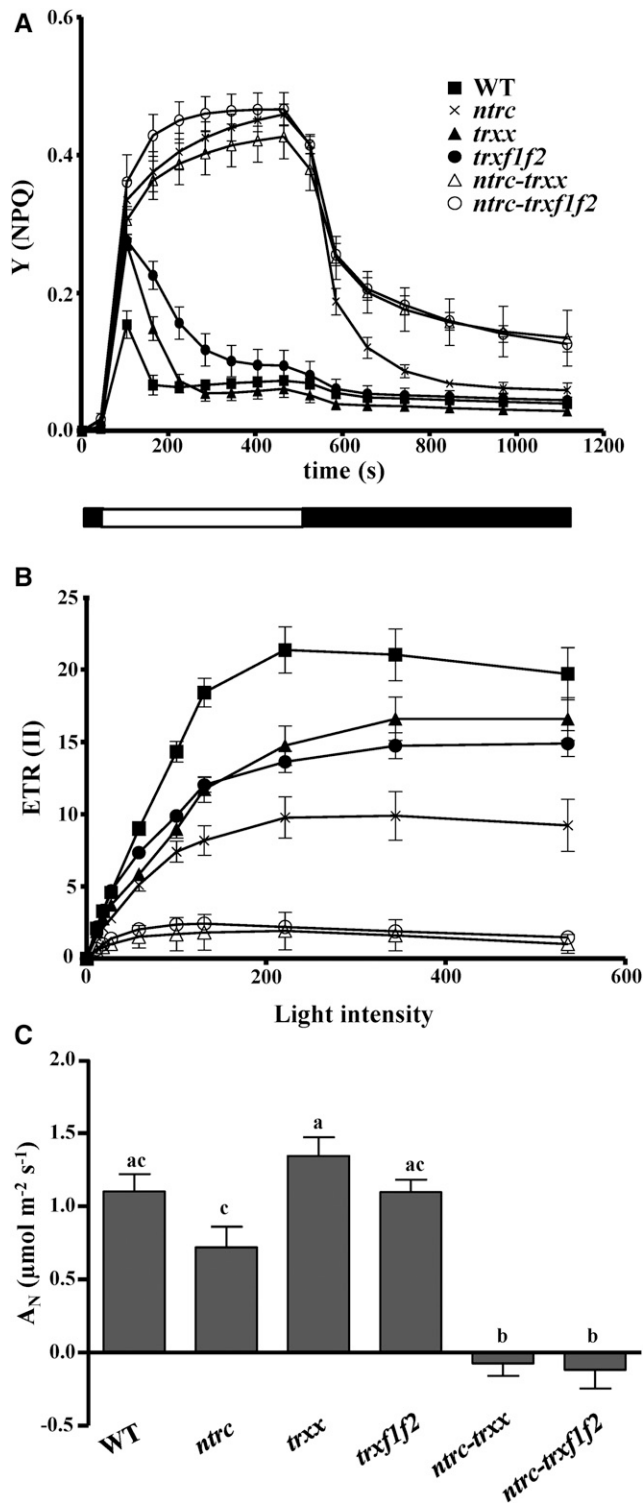
Figure 4. Light-dependent NPQ, linear photosynthetic electron transport rate (ETR), and net CO_2 assimilation rate (A_N) in wild-type (WT) and mutant lines. A and B, Quantum yields of NPQ [Y (NPQ); A] and relative linear ETRs of PSII [ETR (II); B] were measured in preilluminated attached leaves of plants grown at 125 μE m^{-2} s^{-1} under long-day conditions. Chlorophyll fluorescence of PSII was determined using a pulse-amplitude modulation fluorimeter. Determinations were performed at least five times, and each data point is the mean ± SE. White

ntrc-trxx and *ntrc-trxf1f2* mutants was even less efficient than in the *ntrc* mutant. In line with these results, the photosynthetic ETR also was impaired. The deficiency of NTRC affected the photosynthetic ETR more severely than the deficiency of Trxs *x* and *f* (Fig. 4B). Notably, both the *ntrc-trxx* and *ntrc-trxf1f2* mutants showed dramatically reduced photosynthetic ETR at any of the light intensities analyzed (Fig. 4B). Moreover, the stability of PSII, determined as the ratio of variable fluorescence to maximal fluorescence (F_v/F_m), which was affected slightly in the *ntrc* and *trxx* mutants, also was severely affected in the mutants combining the deficiencies of NTRC with those of *x*- or *f*-type Trxs (Table I). Finally, the net rate of CO_2 fixation (A_N) at growth light intensity (125 μE m^{-2} s^{-1}), which was lower in the *ntrc* mutant but unaffected in the *trxx* and *trxf1f2* mutants, compared with the wild type, showed negative values in the *ntrc-trxx* and *ntrc-trxf1f2* mutants (Fig. 4C), indicating that the rates of respiration and photorespiration were higher than the rate of CO_2 fixation in these plants. Moreover, the severe impairment of photosynthetic performance in mutants simultaneously devoid of NTRC and *f*- or *x*-type Trxs has a wider effect on chloroplast metabolism, as shown by the content of starch, which also was severely decreased in these mutants (Table I). Therefore, these results show that the simultaneous deficiency of NTRC and Trxs *f* or *x* has a severe effect on photosynthesis performance, in line with the severe growth inhibition phenotype of these mutants.

Such a defective photosynthetic performance of the *ntrc-trxx* and *ntrc-trxf1f2* mutants suggested as well alterations in chloroplast morphology. To test this possibility, the chloroplast structure in the different lines under study was analyzed by transmission electron microscopy. The *ntrc* mutant contains chloroplasts with different levels of ultrastructural alterations, as reported previously (Pérez-Ruiz et al., 2006; Lepistö et al., 2009), while chloroplasts in the *trxx* and *trxf1f2* mutants showed similar morphology to those in the wild type (Fig. 5). In contrast, the *ntrc-trxx* and, to a higher extent, the *ntrc-trxf1f2* mutants showed heterogenous chloroplast ultrastructure, with a number of chloroplasts showing clear alterations (Fig. 5). The most remarkable characteristic of chloroplasts from both the *ntrc-trxx* and *ntrc-trxf1f2* mutants was the increased number and size of plastoglobules (Fig. 5), which is indicative of oxidative stress and senescence (Austin et al., 2006).

As an additional approach to determine the effect of the combined deficiencies of NTRC and Trxs *f* or Trx *x* on chloroplast function, selected proteins indicative of the different chloroplast compartments were analyzed by western blot. The *ntrc-trxx* and *ntrc-trxf1f2* mutants showed decreased levels of D1, a core component of PSII, PsaC, a core component of PSI, lhca1, a component of the

and black bars indicate light and dark periods. C, A_N was measured using an open gas-exchange system in leaves of plants grown during 4 weeks under a long-day photoperiod, dark adapted, and then illuminated with a photosynthetically active radiation of 125 μE m^{-2} s^{-1}. Six leaves were measured per line, and means ± SE are represented. Letters indicate significant differences by Tukey's test and a confidence interval of 99.9%.

Table I. *Effects of the combined deficiencies of NTRC and Trxs f or Trx x on PSII stability and starch content*

The maximum PSII quantum yield was determined as F_v/F_m in dark-adapted leaves of plants grown under long-day conditions. The F_v/F_m values ± SD are averages of at least 10 measurements. The content of starch was determined from rosette leaves of plants grown under long-day conditions for 31 d and harvested after 12 h of the light period. Values represent means ± SD of three independent samples. Letters indicate significant differences by Student's *t* test (F_v/F_m) or Tukey's LSD test (starch content) and a confidence interval of 95%.

Parameter	Wild Type	*ntrc*	Plant Line *trxx*	*trxf1f2*	*ntrc-trxx*	*ntrc-trxf1f2*
F_v/F_m	0.78 ± 0.03 a	0.76 ± 0.02 b	0.75 ± 0.03 b	0.79 ± 0.02 a	0.62 ± 0.11 c	0.52 ± 0.10 d
Starch (μg mg^{-1} fresh wt)	8.11 ± 2.06 a	2.25 ± 0.49 c,d	4.36 ± 0.81 b	3.57 ± 1.65 b,c	0.82 ± 0.34 d	1.59 ± 0.51 d

PSI antenna, and the α- and γ-subunits of the ATPase (Fig. 6). However, no significant differences in the content of lhcb1, a component of the PSII antenna, and the stromal Rubisco large subunit were detected (Fig. 6). Altogether, these results show that the combined deficiency of NTRC and *x*- or *f*-type Trxs affects chloroplast structure and key components of the chloroplast photosynthetic electron transport chain, which is in agreement with the low photosynthetic performance and severe growth-retarded phenotype of these plants. The increased number and size

of plastoglobules in these chloroplasts suggest that the deficiency of the redox regulatory network of these organelles causes oxidative stress.

The Combined Deficiencies of NTRC and *f*- or *x*-Type Trxs Cause High Mortality at the Seedling Stage

A remarkable feature of the *ntrc-trxx* and *ntrc-trxf1f2* mutants was the low number of individuals that reached
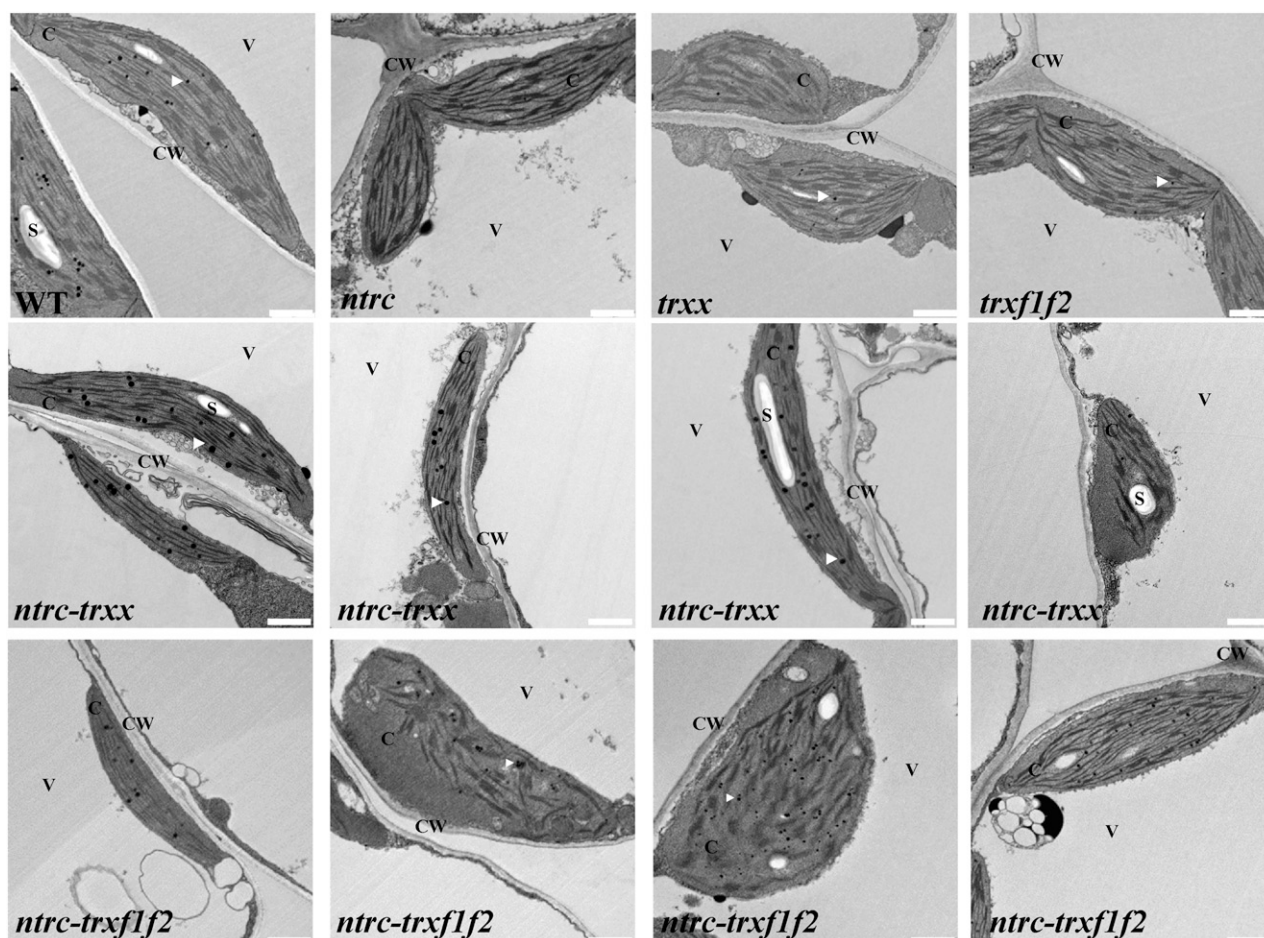
Figure 5. Electron transmission microscopy analysis of chloroplast structure from wild-type (WT) and mutant lines. Leaves of plants cultured under a 16-h-light/8-h-dark regime were collected after the first symptoms of bolting. Increases in plastoglobule (white arrowheads) number, size, and association are observed in *ntrc-trxx* and *ntrc-trxf1f2* mutant plants compared with wild-type and single mutant plants. C, Chloroplasts; CW, cell wall; S, starch granules; V, vacuoles. Bars = 1 μm.

Figure 6. Levels of representative chloroplast proteins in wild-type (WT) and mutant plants. Western-blot analysis is shown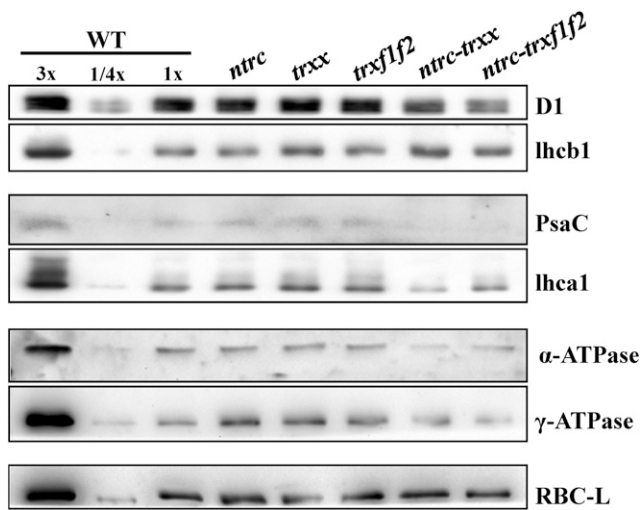 for the content of representative components of photosynthetic electron transport (D1, Lhcb1, PsaC, and Lhca1), ATP synthesis (α-ATPase and γ-ATPase), and carbon assimilation (RBC-L) in the lines under study. Chloroplasts of the indicated lines were isolated from 5-week-old long-day-grown plants and fractionated into thylakoid and stromal fractions as described in "Materials and Methods." Protein samples corresponding to thylakoids (0.5 μg of chlorophyll) or stroma (1 μg of protein) were subjected to SDS-PAGE under reducing conditions, transferred to nitrocellulose filters, and probed with antibodies against thylakoid (D1, Lhcb1, PsaC, Lhca1, α-ATPase, and γ-ATPase) and stromal (RBC-L) proteins. Additional amounts (3$\times$ and 1/4$\times$) of wild-type extracts were loaded for comparison. The experiment was performed twice with similar results.

the adult phase, indicating that the combined deficiency of these plastidial redox systems might be critical at early developmental stages. To investigate this possibility, we analyzed embryonic (seed germination) and postembryonic (production of roots and true leaves) growth and development. Of the Arabidopsis lines under study, only the *ntrc-trxf1f2* triple mutant showed decreased seed germination capacity (Supplemental Fig. S1). At 5 d after germination, all lines had produced cotyledons, although these were slightly smaller and showed paler green color in the case of the *ntrc-trxf1f2* mutant (Fig. 7A). At a later stage, 8 d after germination, seedlings of the wild type, as well as those of the *ntrc*, *trxf1f2*, and *trxx* mutants, produced true leaves, a process that was delayed in the *ntrc-trxx* and, to a higher extent, *ntrc-trxf1f2* mutants (Fig. 7, A and B). This delay was coincident with a sharp decrease of seedling viability in the case of the *ntrc-trxf1f2* triple mutant, so that up to 95% of the seedlings of this line did not reach this stage of development (Fig. 7, A and C). Although not as dramatic, the *ntrc-trxx* double mutant also showed a high decrease of viability at this stage of development (Fig. 7, A and C). Seedlings of the *ntrc-trxx* and *ntrc-trxf1f2* mutants that survived the true leaf stage were able to continue development, although with impaired growth rate, which was more severe for the *ntrc-trxf1f2* triple mutant, as shown above (Fig. 1B).

The high mortality of the *ntrc-trxx* and *ntrc-trxf1f2* mutants at the transition from seedling to true leaves suggests that photosynthesis performed by cotyledon chloroplasts is critical for the development of vegetative organs. However, these assays were performed in soil and, thus, the nutritional conditions could not be strictly controlled. Therefore, we analyzed in more detail the contribution of photosynthesis in cotyledons to seedling development. To this end, the generation of roots as a sink organ was studied in synthetic medium supplemented or not with Suc under continuous light. Like the wild type, seedlings of the *ntrc*, *trxx*, and *trxf1f2* mutants produced roots regardless of the presence of Suc in the medium (Fig. 8). In contrast, root formation was completely abolished or severely impaired in seedlings of the *ntrc-trxf1f2* or *ntrc-trxx* mutants, respectively (Fig. 8, A and B). Root elongation was restored by the addition of external Suc, although the rate of root growth was still decreased in the *ntrc-trxx* and *ntrc-trxf1f2* mutants, as compared with the other lines under analysis (Fig. 8, A and C). These results show the relevance of redox regulation in the photosynthetic activity of cotyledon chloroplasts for early plant development.

DISCUSSION

NTRC Acts in Concert with Different Types of Trxs to Sustain Chloroplast Function

Chloroplasts play important roles in plant metabolism and development. A key component of the rapid adjustment of chloroplast metabolism to ever-fluctuating light conditions is the redox regulation of enzyme activity, which relies on two thiol redox systems, the NADPH-dependent NTRC and the Fdx-dependent FTR/Trx pathways (Spínola et al., 2008; Cejudo et al., 2012). Thus, a central issue in understanding chloroplast redox regulation is to establish the functional relationship between these two redox systems and the relevance of chloroplast redox regulation to plant development. In this work, we addressed this issue by performing a comparative analysis of Arabidopsis mutants combining the deficiency of NTRC with that of two Trxs proposed previously to have unrelated functions, such as Trxs *f*, which participate in the redox regulation of metabolic pathways (Michelet et al., 2013), and Trx *x*, which was proposed to have antioxidant function (Collin et al., 2003). Based on our results, we propose that NTRC exerts a pivotal role in chloroplast redox regulation. Moreover, the high mortality of *ntrc-trxx* and, to a higher extent, *ntrc-trxf1f2* seedlings uncovers the essential function of cotyledon chloroplast redox regulation for early stages of plant development.

Chloroplasts harbor a complex set of different Trxs (Meyer et al., 2012; Balsera et al., 2014). Based on extensive biochemical analyses, it was established that Trxs of types *m* and *f* are relevant in the redox regulation of metabolic pathways (Buchanan and Balmer, 2005; Michelet et al., 2013; Okegawa and Motohashi, 2015), while those of types *x* and *y* were proposed to perform antioxidant
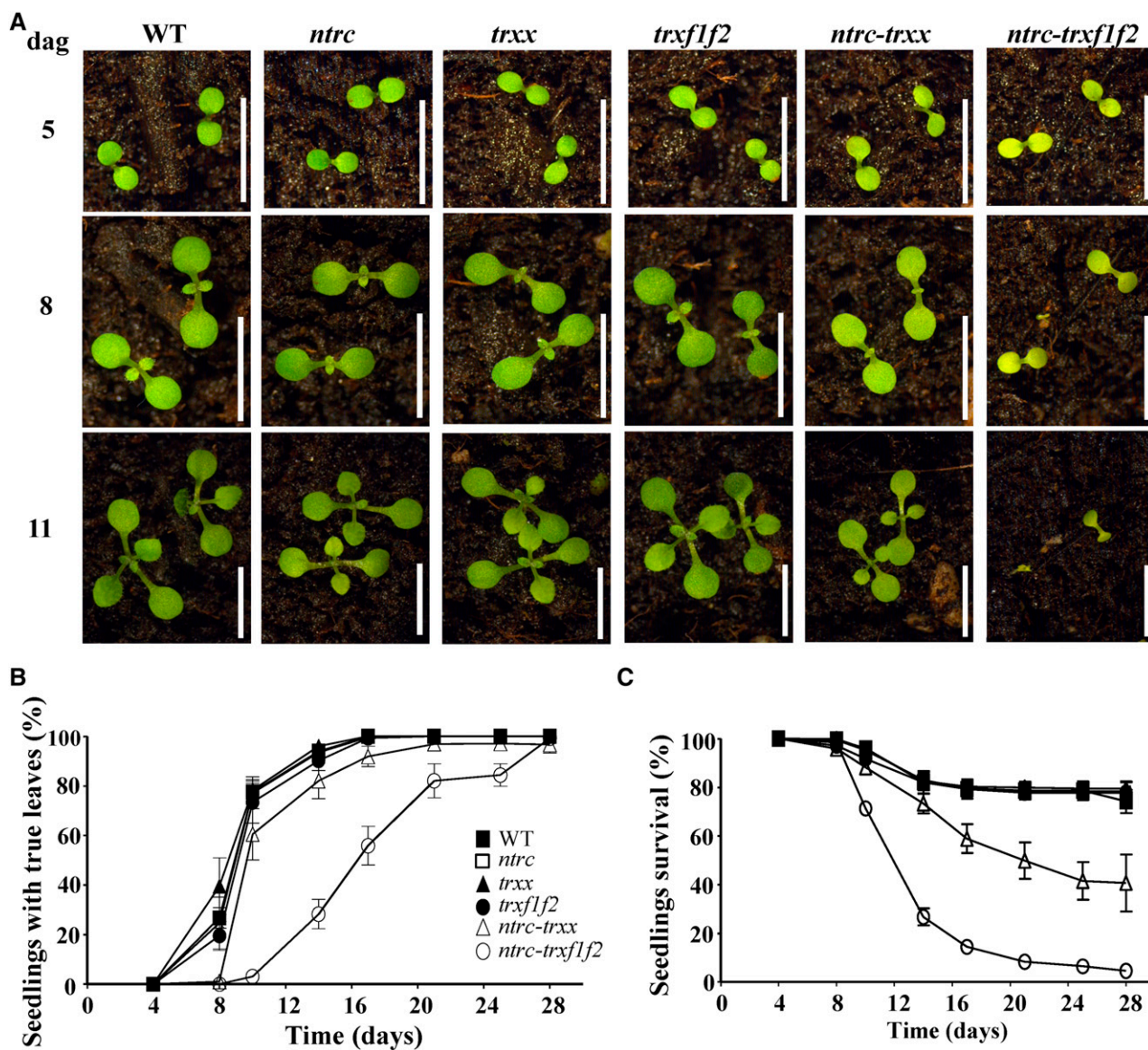
Figure 7. Redox regulation impairment provokes high mortality at the seedling stage. A, Seeds of wild-type (WT) and mutant lines, as indicated, were allowed to germinate on soil, and representative seedlings at 5, 8, and 11 d after germination (dag) are shown. Bars = 0.5 cm. B and C, For each line, the percentage of seedlings with emerging true leaves (B) and the percentage of seedlings that remained alive (C) at the indicated days after germination were determined and represented. The experiment was performed twice, and mean values ± SE from three replicates of each experiment are represented.

activity (Collin et al., 2003, 2004). However, despite the major function proposed for Trxs *f* in the redox regulation of central metabolic pathways, such as the Calvin-Benson cycle, Arabidopsis mutants devoid of Trx *f*1 (Thormählen et al., 2013) or Trx *f*1 and *f*2 (Yoshida et al., 2015; Naranjo et al., 2016a) show a visible phenotype very similar to that of the wild type, as does an Arabidopsis mutant devoid of Trx *x* (Pulido et al., 2010). These results indicate that alternative redox systems, either additional chloroplast Trxs or NTRC, might compensate for the deficiency of these Trxs. In contrast, when the deficiency of Trx *f*1 (Thormählen et al., 2015), Trxs *f*1 and *f*2, or Trx *x* is combined with the deficiency of NTRC, a dramatic effect on plant phenotype is produced (Fig. 1, B and C). As reported previously (Thormählen et al., 2015; Naranjo et al., 2016a, 2016b), the deficiency of NTRC causes more severe impairment on photosynthesis performance than the deficiency of type-*f* Trxs, as shown by the extensive NPQ at low light intensity, the lower photosynthetic ETR, and the rate of carbon fixation (Fig. 4) in the *ntrc* mutant. Remarkably, these parameters were affected dramatically in both the *ntrc-trxx* and *ntrc-trxf1f2* mutants, thus showing the poor efficiency of light energy utilization by these mutants, which has a wide effect on chloroplast metabolism, as indicated by the lower content of starch in these mutants (Table I).

Figure 8. Effects of Suc on root production from seedlings of wild-type (WT) and mutant lines. Seedlings were grown on Murashige and Skoog (MS) synthetic medium supplemented or not with 0.5% (w/v) Suc. A, Seedlings of the different lines, as indicated, after 7 d of growth under continuous light (125 μE m^{-2} s^{-1}). B and C, Root growth in the absence (B) or presence (C) of added Suc was monitored, and mean values ± SE from three replicates are represented.
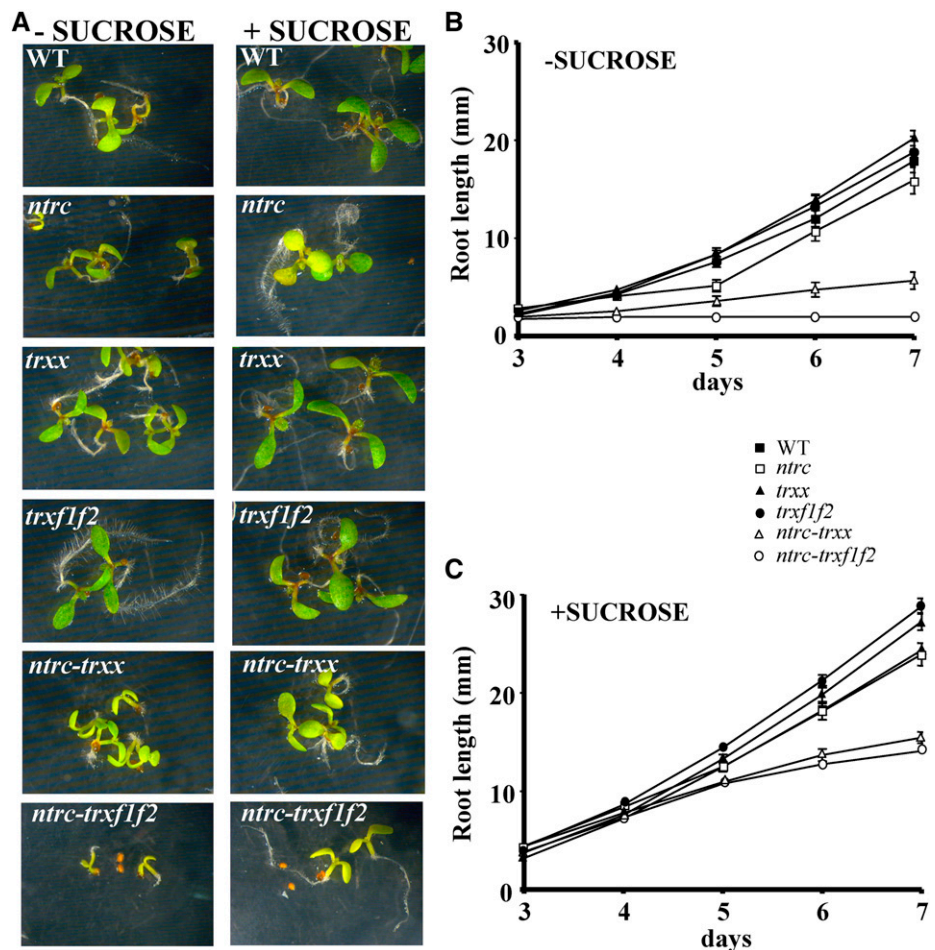
The results discussed above provide an explanation for the severe growth inhibition phenotype of the *ntrc-trxx* and *ntrc-trxf1f2* mutants and suggest the concerted action of both systems in the redox regulation of common targets in vivo, a notion further supported by the finding that NTRC interacts with well-established Trx targets (Nikkanen et al., 2016). Indeed, NTRC has been shown to participate in the regulation of enzymes such as AGPase (Michalska et al., 2009) and different enzymes of the pathway of tetrapyrrole synthesis (Richter et al., 2013; Pérez-Ruiz et al., 2014; Yoshida and Hisabori, 2016), which were identified previously as Trx regulated (Ballicora et al., 2000; Geigenberger et al., 2005; Ikegami et al., 2007). Here, we addressed this issue by analyzing FBPase, a well-established redox-regulated enzyme of the Calvin-Benson cycle. The impairment of redox regulation caused by the combined deficiencies of NTRC and Trxs *f* or *x* affected FBPase, as shown by the lower level of activity of this enzyme in leaves of the *ntrc-trxx* and *ntrc-trxf1f2* mutants (Fig. 2A). Interestingly, the in vivo level of FBPase reduction in response to light was lower in the *ntrc* mutant than in the *trxf1f2* and *trxx* mutants (Fig. 2B). However, in vitro assays showed that Trxs *f*1 and *f*2 are more efficient reductants of FBPase than Trx *x*, while NTRC is unable

to reduce the enzyme (Fig. 3). These results strongly support the notion that the in vivo effect of NTRC on the redox regulation of FBPase is indirect. The deficiency of NTRC may cause an imbalance of the redox state of the chloroplast, which, in turn, affects the regulatory function of Trxs *f* with respect of its targets.

Trxs *f* and *x* interact with different targets (Collin et al., 2003; Yoshida et al., 2015) and, hence, are considered to have different functions in chloroplast redox regulation (Meyer et al., 2012). Thus, an additional issue addressed in this work was to establish whether NTRC is required specifically for the activity of Trxs involved in the regulation of metabolic pathways, such as Trxs *f*, or is also required for Trxs involved in antioxidant defense, such as Trx *x*. The *trxx* mutant shows a phenotype indistinguishable from the wild-type plants (Pulido et al., 2010), whereas the *ntrc-trxx* double mutant shows a severe growth-retarded phenotype (Fig. 1, B and C) and impairment of photosynthetic performance (Fig. 4; Table I), thus indicating that the function of Trx *x* is dispensable in plants containing NTRC but is not properly exerted in the absence of NTRC. It should be noted that the level of light-dependent FBPase reduction was affected similarly in the *trxx* and *trxf1f2* mutants (Fig. 2, B and C). However, Trxs *f*, which are

about 4-fold more abundant than Trx *x* in Arabidopsis chloroplasts (Okegawa and Motohashi, 2015), are more efficient reductants of FBPase in vitro than Trx *x* (Fig. 3). The phenotype of the *ntrc-trxf1f2* mutant is more severe than that of the *ntrc-trxx* mutant at the stage of adult plant (Fig. 1B), seed germination capacity (Supplemental Fig. S1), or seedling viability (Fig. 7), suggesting that the functions of Trxs *f* are more relevant for plant growth than those of Trx *x*.

The deficiency of the redox regulatory network in the *ntrc-trxx* and *ntrc-trxf1f2* mutants has a dramatic impact on photosynthetic performance, as shown by the high levels of NPQ at low light intensities (Fig. 4A), the corresponding decrease of the photosynthetic ETR (Fig. 4B), PSII activity based on the F_v/F_m (Table I), and the rate of net photosynthesis (Fig. 4C). Previous reports have shown that the deficiency of NTRC causes the impairment of light utilization and slightly affects the redox state of the γ-subunit of ATPase, hence affecting the qE component of NPQ (Carrillo et al., 2016; Naranjo et al., 2016b). Therefore, the combined deficiency of NTRC and *f*- or *x*-type Trxs affects the redox regulation of chloroplast metabolic pathways such as the Calvin-Benson cycle but also the efficiency of light utilization, which ultimately might be the reason for the severe growth-retarded phenotype shown by the *ntrc-trxx* and *ntrc-trxf1f2* mutants. These results reveal the pivotal role of NTRC in the thiol redox network of the chloroplast stroma, integrating the feedback control of photochemical reactions and the regulation of metabolic pathways. Moreover, a combined deficiency in NADPH- and Fdx-dependent chloroplast redox networks has severe effects on the structure of these organelles, as shown by the presence of chloroplasts with aberrant ultrastructure in both the *ntrc-trxx* and *ntrc-trxf1f2* mutants (Fig. 5) and the lower content of representative components of PSI, PSII, and ATPase (Fig. 6).

Therefore, the severe growth phenotype of the *ntrc-trxx* and *ntrc-trxf1f2* mutants is the result of the effect of these mutations on a wide variety of chloroplast processes from photochemical reactions to carbon metabolism. Our results indicate that the regulation of these processes is coordinated and that NTRC exerts a central function in this coordination. The question arising is the mechanistic basis of this central function of NTRC in chloroplast redox regulation. A possibility is that the lower efficiency of light energy utilization caused by the lack of NTRC (Fig. 4) affects the availability of reducing equivalents for Trxs, hence impairing the light-dependent redox regulation of Calvin-Benson enzymes such as FBPase. This would provide an explanation for the lower level of reduction of FBPase in NTRC-deficient plants (Fig. 2), despite the fact that NTRC is unable to reduce FBPase in vitro (Fig. 3). An additional aspect to be taken into account is that the deficiency of NTRC may perturb chloroplast redox balance affecting the Trx activity. In this regard, it should be noted that the chloroplasts of the *ntrc-trxx* and *ntrc-trxf1f2* mutants show higher number and size of plastoglobules (Fig. 5), which are indicative of oxidative stress (Austin et al.,

2006), hence suggesting that the severe alteration of the redox regulatory network in these mutants also has important effects on the antioxidant machinery of the chloroplast. Indeed, NTRC is an efficient reductant of the peroxide-scavenging enzyme 2-Cys peroxiredoxin (Pérez-Ruiz et al., 2006; Pulido et al., 2010).

The Photosynthetic Activity of Cotyledon Chloroplasts Is Essential for Early Plant Development

Most studies on chloroplast redox regulation have been performed at the adult plant stage, hence when leaves are fully developed. However, besides the severe growth inhibition of the *ntrc-trxx* and *ntrc-trxf1f2* mutants (Fig. 1, B and C), a remarkable feature of the phenotype of these mutants was the low number of individuals that reached the adult phase (Fig. 7), suggesting that the photosynthetic function becomes critical at earlier stages of plant development. It is known that embryonic photosynthesis affects seed vigor (Allorent et al., 2015); thus, the lower germination capacity of seeds of the *ntrc-trxf1f2* mutant (Supplemental Fig. S1) suggests the relevance of plastid redox regulation in seed germination. It should be noted that the *ntrc-trxx* mutant shows germination capacity similar to that of the wild type (Supplemental Fig. S1), which is in line with the other phenotypic parameters analyzed here, most of them being more affected in the *ntrc-trxf1f2* mutant than in the *ntrc-trxx* mutant.

A critical stage in the plant life cycle occurs after seed germination, when the growing seedling depends on the heterotrophic use of seed storage compounds until autotrophic growth is possible (Kircher and Schopfer, 2012). Once germinated, the cotyledons of the *ntrc-trxx* and *ntrc-trxf1f2* mutants were slightly smaller and showed yellowish green color, compared with cotyledons from the wild type or the *ntrc*, *trxx*, and *trxf1f2* mutants (Fig. 7A). This indicates that the developmental program of photomorphogenesis is not compromised by the impairment of chloroplast redox regulation at this early stage of development. This notion was further supported by growth tests in synthetic medium supplemented or not with Suc (Fig. 8A). In contrast with the wild type and the *ntrc*, *trxx*, and *trxf1f2* mutants, which produced roots in the absence of Suc, root production was severely defective in the *ntrc-trxx* mutant and abolished completely in the *ntrc-trxf1f2* mutant unless Suc was added to the medium to compensate for decreased photosynthesis (Fig. 8). These results provide further evidence that the impairment of redox regulation caused by the combined deficiency in NTRC and Trxs *f* or Trx *x* severely affects the photosynthetic performance of cotyledon chloroplasts. However, while in adult plants this failure causes growth retardation but is not lethal, in the case of cotyledons it produces a delay in the generation of new organs such as roots and true leaves, which provokes increased seedling mortality (Fig. 7). Photomorphogenesis, the reprograming of seedling growth promoted by light, has received much attention,

and a complex set of light perception and signaling components has been identified (Huang et al., 2014; Wu, 2014; Pogson et al., 2015). Less attention has been given to the contribution of photosynthesis to this developmental process. The reduced viability of the *ntrc-trxx* and, to a higher extent, the *ntrc-trxf1f2* seedlings uncovers the essential function of the redox regulation of photosynthesis at this developmental stage.

MATERIALS AND METHODS

Biological Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) wild-type (ecotype Columbia) and mutant plants were routinely grown in soil in growth chambers under long-day conditions (16 h of light/8 h of darkness) at 22°C during the light and 20°C during the dark periods and a light intensity of 125 μE m^{-2} s^{-1}. For in vitro culture experiments, seeds were surface sterilized using chlorine gas for 16 h, plated on MS medium (Duchefa), pH 5.8, containing 0.35% (w/v) Gelrite (Duchefa) in the presence or absence of 0.5% (w/v) Suc, and stratified at 4°C for 2 to 3 d. Mutant plants *ntrc*, *trxx*, and *trxf1f2* were described previously (Serrato et al., 2004; Pulido et al., 2010; Naranjo et al., 2016a; Supplemental Table S1). The single *ntrc* mutant was crossed manually with the single *trxx* mutant and the double *trxf1f2* mutant. Seeds resulting from these crosses were checked for heterozygosity of the T-DNA insertions in the *NTRC*, *TRX x*, *TRX f1*, and *TRX f2* genes, respectively. Plants were then self-pollinated, and the *ntrc-trxx* and *ntrc-trxf1f2* mutants were identified in the progeny by PCR analysis of genomic DNA. Primer sequences for PCR are listed in Supplemental Table S2.

RNA Extraction and RT-qPCR Analysis

Total RNA was extracted using Trizol reagent (Invitrogen). cDNA synthesis was performed with 2 μg of total RNA using the Maxima first-strand cDNA synthesis kit (Fermentas) according to the manufacturer's instructions. RT-qPCR was performed using an IQ5 real-time PCR detection system (Bio-Rad). A standard thermal profile (95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s) was used for all reactions. After the PCR, a melting-curve analysis (55°C–94°C at 0.5°C per 30 s) was performed to confirm the specificity of the amplicon and to exclude primers-dimers or nonspecific amplification. Oligonucleotides used for the RT-qPCR analyses are listed in Supplemental Table S3. Expression levels were normalized using two reference genes: *ACTIN* and *AT5G25760* (Czechowski et al., 2005).

Protein Extraction, FBPase Activity, Alkylation Assays, and Western-Blot Analysis

Plant tissues were ground with a mortar and pestle under liquid nitrogen. FBPase activity was determined as reported previously (Rojas-González et al., 2015) from leaves of plants grown for 4 weeks under a long-day photoperiod and harvested at 12 h of the day period. For determination of the redox state of thiol-regulated enzymes in vivo, alkylation assays using MM(PEG)$_{24}$ (Thermo Scientific) were performed as described previously (Naranjo et al., 2016a). To study chloroplast protein levels, the Minute Chloroplast Isolation Kit (Invent Biotechnologies) was used following the manufacturer's instructions. Intact chloroplasts were isolated from leaves of plants adapted to the dark during 30 min, and total protein content was quantified using the Bradford reagent (Bio-Rad).

Protein samples were subjected to SDS-PAGE under reducing or nonreducing conditions using various acrylamide gel concentrations, as stated in the figure legends, and transferred onto nitrocellulose membranes, which were probed with the indicated antibodies. Specific anti-NTRC antibodies were raised previously in our laboratory (Serrato et al., 2004). Other antibodies (D1, lhcb1, PsaC, lhca1, α-ATPase, γ-ATPase, and RBC-L) were purchased from Agrisera.

In Vitro FBPase Reduction Assay

Arabidopsis chloroplast FBPase (2.5 μM) was incubated for 30 min at 22°C with increasing concentrations (0–10 μM) of NTRC, Trxs *f1* and *f2*, or Trx *x* in reaction mixtures containing 100 mM Tris-HCl, pH 8, and 0.1 mM DTT. In order

to prevent further oxidations, free thiol were alkylated by incubation in the dark for 30 min at 37°C in the presence of equal volumes of a solution containing 60 mM iodoacetamide, 2% CHAPS, and 100 mM Tris-HCl, pH 8.

Determination of Chlorophylls, Starch, and FBPase Activity

Leaf discs were weighed and frozen in liquid nitrogen. After extraction in 1 mL of methanol for 16 h at 4°C, chlorophyll levels were measured spectrophotometrically, as described by Porra et al. (1989), and normalized to fresh weight. Starch contents were determined as reported previously (Rojas-González et al., 2015) from rosette leaves of plants grown for 4 weeks under a long-day photoperiod and harvested at 12 h of the day period.

Measurements of Chlorophyll a Fluorescence and A_N

Room temperature chlorophyll fluorescence was measured using a pulse-amplitude modulation fluorimeter (DUAL-PAM-100; Walz). The maximum quantum yield of PSII was assayed after incubation of plants in the dark for 30 min by calculating F_v/F_m. Induction-recovery curves were performed using red (635 nm) actinic light at 42 μE m^{-2} s^{-1} for 8 min. Saturating pulses of red light at 10,000 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 NPQ, were calculated by the DUAL-PAM-100 software according to the equations of Kramer et al. (2004). Relative linear ETRs were measured in leaves of preilluminated plants by applying stepwise increasing actinic light intensities up to 2,000 μE m^{-2} s^{-1}.

A_N was measured in leaves from wild-type and mutant plants grown under long-day conditions for 4 weeks using an open gas-exchange system (Li-6400) equipped with the chamber head (Li-6400-40). Leaves were dark adapted and then illuminated with photosynthetically active radiation of 125 μE m^{-2} s^{-1} until the rate of CO_2 assimilation was stabilized. Six leaves were measured per line. Measurements were performed by the Service for Photosynthesis, Instituto de Recursos Naturales y Agrobiología, Sevilla, Spain.

Determination of Seedling Development and Survival

The growth and development of the lines under study were monitored based on two parameters: the formation of true (postembryonic) leaves and seedling mortality. For each line, three sets of at least 60 seeds were sown on soil and grown during 28 d under long-day conditions. Seedling survival and true leaf generation were scored at 3- to 4-d intervals. Seedlings that were still green/greenish, considered as survivors, were quantified to calculate the survival rate. Seedlings that exhibited true leaves were quantified to determine the rate of seedlings reaching the true leaf stage.

Root Growth and Germination Capacity Measurements

For root growth assays, at least 30 seedlings were grown for 7 d on vertically oriented MS plates in the presence or absence of 0.5% (w/v) Suc. Root elongation was scored at 1-d intervals from day 3 until day 7.

For germination studies, five sets of at least 100 seeds were sown on horizontally oriented MS plates, and the percentage of germinated seeds was recorded at day 2 and day 7.

Transmission Electron Microscopy Analysis

Fully expanded leaves from plants cultured under a long-day photoperiod were collected at bolting time, after approximately 28 d of growth, just when the first floral bud was visible. Since the growth of *ntrc-trxx* and *ntrc-trxf1f2* plants is delayed, the leaves of these mutants were collected after approximately 60 d of growth. Small pieces (2 mm^2) of leaves were cut with a razor blade and immediately fixed in 4% glutaraldehyde in 0.1 M sodium-cacodylate buffer, pH 7.4 (3 h at 4°C, under vacuum). After fixation, samples were rinsed three times for 30 min at 4°C with the same buffer. Samples were postfixed in 1% (w/v) osmium tetraoxide in cacodylate buffer (0.1 M, pH 7.4) for 1 h at 4°C. After washing, samples were immersed in 2% (w/v) uranyl acetate, dehydrated through a gradient acetone series (50%, 70%, 90%, and 100%), and embedded in Spurr's resin. Semithin sections (300 nm thickness) were obtained with a glass

knife and stained with 1% (v/v) Toluidine Blue for cell localization and reorientation using a conventional optic microscope. Once a suitable block face of the selected area was trimmed, several ultrathin sections (70 nm) were obtained using an ultramicrotome (Leica UC7) equipped with a diamond knife (Diatome) and collected on 200-mesh copper grids. Sections were examined with a Zeiss Libra 120 transmission electron microscope and digitized (2,048 × 2,048 × 16 bits) using an axis-mounted TRS camera. Sample postfixation and cutting were performed by the Microscopy Service at the Centro de Investigación, Tecnología, e Innovación from the University of Seville.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Effect of the deficiency in NTRC and *x*- or *f*-type Trxs on germination capacity.

Supplemental Table S1. Arabidopsis mutants used in this study.

Supplemental Table S2. Oligonucleotides used for the determination of Arabidopsis mutant genotypes.

Supplemental Table S3. Oligonucleotides used for RT-qPCR analyses.

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