Thioredoxin f1 and NADPH-Dependent Thioredoxin Reductase C Have Overlapping Functions in Regulating Photosynthetic Metabolism and Plant Growth in Response to Varying Light Conditions

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Two different thiol redox systems exist in plant chloroplasts, the ferredoxin-thioredoxin (Trx) system, which depends on ferredoxin reduced by the photosynthetic electron transport chain and, thus, on light, and the NADPH-dependent Trx reductase C (NTRC) system, which relies on NADPH and thus may be linked to sugar metabolism in the dark. Previous studies suggested, therefore, that the two different systems may have different functions in plants. We now report that there is a previously unrecognized functional redundancy of Trx f1 and NTRC in regulating photosynthetic metabolism and growth. In Arabidopsis (Arabidopsis thaliana) mutants, combined, but not single, deficiencies of Trx f1 and NTRC led to severe growth inhibition and perturbed light acclimation, accompanied by strong impairments of Calvin-Benson cycle activity and starch accumulation. Light activation of key enzymes of these pathways, fructose-1,6-bisphosphatase and ADP-glucose pyrophosphorylase, was almost completely abolished. The subsequent increase in NADPH/NADP+ and ATP/ADP ratios led to increased nitrogen assimilation, NADP-malate dehydrogenase activation, and light vulnerability of photosystem I core proteins. In an additional approach, reporter studies show that Trx f1 and NTRC proteins are both relocated in the same chloroplast substructure. Results provide genetic evidence that light- and NADPH-dependent thiol redox systems interact at the level of Trx f1 and NTRC to coordinately participate in the regulation of the Calvin-Benson cycle, starch metabolism, and growth in response to varying light conditions.

Reversible disulfide bond formation between two Cys residues regulates structure and function of many proteins in diverse organisms (Cook and Hogg, 2013). Thiol disulfide exchange is controlled by thioredoxins (Trxs), which are small proteins containing a redox-active disulfide group in their active site (Holmgren, 1985; Baumann and Juttner, 2002). The latter can be reduced to a dithiol by Trx reductases using NADPH or ferredoxin (Fdx) as electron donors. Due to their low redox midpoint potential, reduced Trxs are able to reductively cleave disulfide bonds in many target proteins and, thus, modulate their functions.

Plants contain the most versatile Trx system found in all organisms with respect to the multiplicity of different isoforms and reduction systems (Buchanan and Balmer, 2005; Nikkanen and Rintamäki, 2014; Geigenberger and Fernie, 2014). The Arabidopsis (Arabidopsis thaliana) genome contains a complex family of Trxs, including up to 20 different isoforms grouped into seven subfamilies (Schürmann and Buchanan, 2008; Dietz and Pfannschmidt, 2011). Trxs f1-2, m1-4, x, y1-2, and z are located exclusively in the chloroplast, and Trx o is located exclusively in the mitochondria, while the eight Trx h representatives are distributed between the cytosol, nucleus, endoplasmic reticulum, and mitochondria (Meyer et al., 2012). The different Trxs can be reduced by two different redox systems, dependent on Fdx and Fdx-Trx reductase in the chloroplast or NADPH and NADPH-Trx reductase (NTRA and NTRB) in other cell
compartments (Buchanan and Balmer, 2005). More recently, a third type of NADPH-Trx reductase (NTRC) has been identified, which forms a separate Trx system in the chloroplast (Serrato et al., 2004; Pérez-Ruiz et al., 2006). NTRC is a bimodular enzyme containing both an NTR and Trx domain on a single polypeptide (Serrato et al., 2004). Its catalytic unit is a homodimer, transferring electrons from NTR to Trx domains via intersubunit pathways (Pérez-Ruiz and Cejudo, 2009). In vitro studies suggest that NTRC is a Trx with its own Trx reductase, because it has not been shown to interact with other free Trxs (Pérez-Ruiz et al., 2006; Bohrer et al., 2012).

In chloroplasts, Trxs are reduced via Fdx-Trx reductase in a light-dependent manner, using photosynthetic electrons provided by Fdx. The Fdx-Trx system with Trxs f and m was originally discovered as a mechanism for the regulation of the Calvin-Benson cycle, ATP synthesis, and NADPH export in response to light-dark changes (Buchanan et al., 1979; Buchanan, 1980). In numerous biochemical studies performed in vitro, the roles of Trxs f and m were extended to the regulation of many other chloroplast enzymes involved in various pathways of primary metabolism (Buchanan and Balmer, 2005; Meyer et al., 2012). In vitro experiments with purified proteins revealed differences in biochemical specificities to different types of Trxs. Enzymes of the Calvin-Benson cycle were found to be exclusively regulated by f-type Trxs (Collin et al., 2003; Michelet et al., 2013; Yoshida et al., 2015), while key enzymes involved in related pathways such as starch synthesis (Fu et al., 1998; Ballicora et al., 2000; Geigenberger et al., 2005; Thomählen et al., 2013), starch degradation (Mikkelsen et al., 2005; Valerio et al., 2011; Seung et al., 2013; Silver et al., 2013), fatty acid synthesis (Sasaki et al., 1997), amino acid synthesis (Lichter and Häberlein, 1998; Choi et al., 1999; Balmer et al., 2003), chlorophyll synthesis (Ikegami et al., 2007; Luo et al., 2012), NADPH export (Collin et al., 2003; Yoshida et al., 2015), and the oxidative pentose-phosphate pathway (Née et al., 2009) were found to be regulated by both Trxs f and m, with f-type being, in most cases, more effective than m-type. Other plastidial isoforms belonging to the x- and y-types were found to be essentially involved in antioxidant functions, being efficient electron donors to 2-Cys peroxiredoxins (Prxs) and Prx Q, respectively, but unable to activate carbon metabolism enzymes (Collin et al., 2003, 2004). The biochemical properties of Trx z are not fully resolved yet. While this new type of Trx has been identified to be part of the plastid-encoded RNA polymerase, implicating a role in the transcription of the plastome (Arsova et al., 2010), it has also been found to act as an electron donor for several antioxidant enzymes, indicating a role in plastid stress responses (Chibani et al., 2011).

While most of the results mentioned above are based on biochemical studies, little is known about the in vivo relevance and specificity of the different chloroplast Trxs isoforms in planta. Recent progress in this area was made by using reverse genetic studies, including Arabidopsis mutants and transgenic plants. Intriguingly, these genetic studies revealed specific roles of m-type Trxs in regulating photosynthetic electron transport and developmental processes rather than its expected roles in primary metabolism. Arabidopsis lines with combined underexpression of Trxs m1, m2, and m4 were defective in the biogenesis of PSII (Wang et al., 2013), and single mutants with deletions in Trx m4 were affected in alternative photosynthetic electron transport pathways (Courteille et al., 2013), while deletions in Trx m3 affected meristem development (Benitez-Alfonso et al., 2009). In addition to this, Arabidopsis mutants with deletions in Trx f1 leading to a more than 97% decrease in Trx f protein level showed alterations in diurnal starch accumulation rather than any changes in photosynthetic parameters and growth (Thomählen et al., 2013). This is surprising given the exclusive regulation of individual steps of the carbon fixation cycle by Trx f1 in vitro (Collin et al., 2003; Michelet et al., 2013).

Compared with the Fdx-Trx system, relatively little is known on the more recently identified chloroplast NADPH-dependent NTRC system, which uses NADPH as a source of electrons. So far, only a few targets have been identified to be regulated by NTRC, with 2-Cys Prxs involved in hydrogen peroxide detoxification (Pérez-Ruiz et al., 2006), ADP-glucose pyrophosphorylase (AGPase), the key enzyme of starch biosynthesis (Michalska et al., 2009; Lepistö et al., 2013), and enzymes of chlorophyll biosynthesis (Richter et al., 2013; Pérez-Ruiz et al., 2014) being the most elaborated ones. Regulation of these processes by NTRC was confirmed in planta by analyzing an insertional knockout mutant of NTRC, revealing (1) a decreased 2-Cys-Prx redox status and impaired hydrogen peroxide detoxification (Pérez-Ruiz et al., 2006), (2) an attenuation of redox activation of AGPase and starch accumulation (Michalska et al., 2009; Lepistö et al., 2013), and (3) impaired glutamyl-transfer RNA reductase1, MgP methyltransferase, and Mg chelatase activities together with decreased chlorophyll levels (Richter et al., 2013; Pérez-Ruiz et al., 2014). Because most of these effects were operational in the dark, this suggests a role of NTRC to regulate these pathways independently of light. In addition to the NADPH-dependent NTRC system, 2-Cys Prx and AGPase have also been found to be regulated by the light-dependent Fdx-Trx system with Trx x (Collin et al., 2003; Bohrer et al., 2012) and Trx f1 (Thomählen et al., 2013), respectively. However, little is known on the interrelation of light- and NADPH-dependent chloroplast redox systems in regulating these targets.

In this report, the interrelation between Trx f1 and NTRC in regulating plant metabolism and growth was investigated by using a genetic approach. Analysis of an Arabidopsis trx f1 ntrc double mutant shows that combined inactivation of Trx f1 and NTRC leads to a strong inhibition in light activation of the Calvin-Benson cycle and related metabolic activities, resulting in a severe limitation of growth, while these responses were not or only weakly expressed in the
single mutants. Reporter studies show that both Trx f1 and NTRC are expressed in the same tissues during development and are colocalized in the same chloroplast substructure. This provides evidence for a previously unknown redundant function of Trx f1 and NTRC in regulating photosynthetic metabolism and growth in response to varying light conditions.

RESULTS

Combined Inactivation of Trx f1 and NTRC Leads to a Severe Growth Phenotype

To analyze the interrelation between Trx f1 and NTRC in regulating growth and metabolism of Arabidopsis plants, the well-characterized trxf1 (SALK_128365; Thommählen et al., 2013) and ntrc (SALK_012208; Serrato et al., 2004; Pérez-Ruiz et al., 2006) transfer DNA (T-DNA) insertion lines were crossed to generate a trxf1 ntrc homozygous line. Reporter studies show that both Trx f1 and NTRC are expressed in the same tissues during development and are colocalized in the same chloroplast substructure. This provides evidence for a previously unknown redundant function of Trx f1 and NTRC in regulating photosynthetic metabolism and growth in response to varying light conditions.

As previously reported, trxf1 (Thommählen et al., 2013) and ntrc single mutants (Pérez-Ruiz et al., 2006; Lepistö et al., 2013) showed no or moderate growth phenotypes, respectively, when grown in an 8-h photoperiod at 160 μmol photons m$^{-2}$ s$^{-1}$ light intensity (Fig. 2B; Supplemental Table S1). In contrast to this, growth of the trxf1 ntrc double mutant was very severely perturbed when compared with the wild type or the single mutants (Fig. 2B). The rosette fresh weights of the trxf1 ntrc double mutant decreased to below 2% of wild-type level, while those of the ntrc mutant decreased to 25% and those of the trxf1 mutant remained unaltered (Fig. 2H). Despite this very strong growth defect, trxf1 ntrc mutant plants were viable and produced seeds under these conditions (Fig. 2G). Interestingly, the extent of the growth phenotypes differed depending on the length of the photoperiod and the light intensity (Fig. 2, A–F). When the length of the photoperiod was decreased from 8- to 4-h light, rosette fresh weights decreased significantly to 80% and 15% of wild-type level in the trxf and ntrc single mutants, respectively, and to levels below the detection limit in the trxf1 ntrc double mutant (Fig. 2H). Conversely, an increase in the length of the photoperiod from 8 to 24 h led to a partial relief in the growth retardation of both the ntrc and the trxf1 ntrc mutant. In the ntrc mutant, rosette fresh weights increased from 25% to 50% of wild-type level in 16- and 24-h photoperiods compared with an 8-h photoperiod (Fig. 2H), in agreement with previous studies (Lepistö et al., 2009). The trxf1 ntrc double mutant showed no significant change in fresh weight when the photoperiod was increased from 8- to 16-h light, but there was an increase from 1% to 3% of wild-type level when the photoperiod was increased from 16- to 24-h light (Fig. 2H). Also, a change in the light intensity at an 8-h photoperiod affected the growth phenotype of the mutants. When the light intensity was decreased from 160 to 30 μmol photons m$^{-2}$ s$^{-1}$, the rosette fresh weights dropped significantly to 75% and 35% of wild-type level in the trxf1 and ntrc mutants, respectively, and to levels below the detection limit in the trxf1 ntrc double mutant (Fig. 2H). When the light intensity was increased from 160 to 950 μmol photons m$^{-2}$ s$^{-1}$, rosette fresh weights dropped severely in the trxf1 ntrc mutant and moderately in the ntrc mutant, while no effect was observed in the trxf1 mutant, compared with the wild type (Fig. 2H). Overall, the results show that knockout of Trx f1 leads to a severe growth inhibition in the ntrc background, but not in the wild-type background, suggesting a functional redundancy of both redox systems.

Combined Deficiency of Trx f1 and NTRC Leads to a Strong Impairment of Photosynthesis

To investigate whether the severe growth phenotype of the trxf1 ntrc mutant is due to an effect on
photosynthesis, CO₂ assimilation rates were measured in leaves of the different genotypes grown in an 8-h photoperiod at 160 μmol photons m⁻² s⁻¹ light intensity using an open gas exchange system. The light response curves at ambient CO₂ are shown in Figure 3A. At light intensities between 150 and 1,000 μmol photons m⁻² s⁻¹, CO₂ fixation rates were strongly decreased in the trxf1 ntrc mutant relative to the wild type, with the decrease being light intensity dependent: 80% at 150 μmol photons m⁻² s⁻¹, 60% at 200 to 300 μmol photons m⁻² s⁻¹, 50% at 400 to 600 μmol photons m⁻² s⁻¹, and 33% at 800 to 1,000 μmol photons m⁻² s⁻¹. At light intensities between 50 and 100 μmol photons m⁻² s⁻¹, CO₂ assimilation rates were below the respiration rate in the double mutant, but not in the wild type, with the light compensation point switching from 20 μmol photons m⁻² s⁻¹ in the wild type to 120 μmol photons m⁻² s⁻¹ in the double mutant. In the dark, CO₂ release rates were 4-fold higher in the double mutant compared with the wild type. In contrast to the double mutant, the single mutants showed no (trxf1) or only slight changes (ntrc) in CO₂ assimilation rates compared with the wild type.
Deletion of NTRC led to a slight decrease in CO2 assimilation rates at all light intensities, which was statistically significant at 50 and 200 μmol photons m\(^{-2}\) s\(^{-1}\) using the Student’s \(t\) test (Supplemental Table S2) and for all light intensities using the two-way ANOVA test (Fig. 3A), confirming previous studies (Lepistö et al., 2009). Overall, the results show that inactivation of Trx/f led to a strong decrease in CO2 fixation rates in the trxf background, but not in the wild-type background, lending further support to the proposal of a functional redundancy of both systems to regulate the CO2 fixation rate.

In Figure 3B, leaf transpiration rates are shown across different light intensities and genotypes. Compared with the wild type, there was a strong (up to 8-fold) increase in transpiration rates in the trxf ntrc mutant, while the single mutants behaved like the wild type at all light conditions tested. Similar results were observed for stomatal conductance (data not shown) and intercellular CO2 concentration (Fig. 3C), both parameters being strongly increased in the double mutant relative to the wild type. These results show that the lower rate of CO2 fixation caused by the combined deficiency of Trx/f and NTRC is not due to a restriction in CO2 uptake rates or a decrease in internal CO2 concentrations but most likely to a direct inhibition of the CO2 fixation cycle.

To investigate whether the inhibition of CO2 assimilation is accompanied by changes in photosynthetic light reactions, chlorophyll fluorescence parameters were measured by pulse amplitude modulation (PAM) fluorometry. A significant decrease of maximal quantum yield of PSII and effective quantum yield of PSII (\(\Phi_{\text{PSII}}\)) was observed in the double mutant relative to the single mutants or the wild type (Fig. 4, A and B), indicating that the combined deficiency of Trx/f and NTRC led to a strong impairment of PSII function and photosynthetic electron transport rates. Correspondingly, the quantum yield of regulated energy dissipation (\(\Phi_{\text{NPQ}}\); Fig. 4B) was strongly increased in the double mutant. Similar to previous studies (Lepistö et al., 2009; Thormählen et al., 2013), no changes in chlorophyll fluorescence parameters were found in the trxf1 mutant, while the ntrc mutant revealed moderate but significant changes in \(\Phi_{\text{PSII}}\) and \(\Phi_{\text{NPQ}}\) relative to the wild type (Fig. 4B).

We further investigated whether impaired photosynthetic light reactions were accompanied by decreased abundance of proteins involved in photosynthetic electron transport (Fig. 5). Western-blot analyses showed that combined deficiency of Trx/f and NTRC led to a strong decrease in proteins of the PSI complex PsaA and PsaB down to approximately 25% of wild-type level and to more moderate decreases in proteins of PSII (PsbD), cytochrome \(b_{6}/f\) (PetC), light-harvesting (Lhca1 and Lhcb1), and ATP synthase (Atp\(\beta\)) complexes. Compared with this, the trxf1 and ntrc single mutants were only weakly affected. Chlorophyll content was slightly decreased in the trxf1 mutant and down to 55% and 45% of wild-type level in the ntrc single and trxf1 ntrc double mutant, respectively (Supplemental Fig. S1). This confirms previous studies showing chlorophyll levels to be decreased by 50% in the ntrc mutant relative to the wild type (Pérez-Ruiz et al., 2006; Lepistö et al., 2009), while a combined deficiency of Trx/f and NTRC only led to minor additional effects (Supplemental Fig. S1).
Combined Deficiency of Trx f1 and NTRC Affects NADP Reduction and Adenylate Energy States

The Calvin-Benson cycle uses most of the ATP and NADPH delivered by the photosynthetic light reactions (Michelet et al., 2013). To investigate the relationship between photosynthetic activity and the function of the Calvin-Benson cycle, the levels of NAD(P)H, NAD(P)+, ATP, and ADP were analyzed in leaves of the wild type and the different redox mutants (Fig. 6). In wild-type plants, the sum of NADPH and NADP+ increased at the end of the day relative to the end of the night (Fig. 6A), while the NADPH-NADP+ ratio decreased (Fig. 6B), confirming previous studies (Liu et al., 2008; Beeler et al., 2014; Lintala et al., 2014). The light-induced decrease in the NADPH-NADP+ ratio was probably attributable to the Calvin-Benson cycle being activated under these conditions. In the trxf1 ntrc mutant, the diurnal changes in the sum of NADPH and NADP+ levels were strongly attenuated (Fig. 6A), while there was a clear increase in the NADPH-NADP+ ratio at the end of the day (3-fold) and at the end of the night (2-fold) compared with the wild type (Fig. 6B). No changes were observed in the trxf1 mutant, while in the ntrc mutant, the NADPH-NADP+ ratio was slightly but significantly increased (Fig. 6, A and B). The wild type also showed diurnal changes in the sum of NADH and NAD+ (Fig. 6C) and in the NADH-NAD+ ratio (Fig. 6D), with the former decreasing and the latter increasing toward the end of the day. In the trxf1 ntrc mutant, the sum of NADH and NAD+ was further decreased, while the NADH-NAD+ ratio further increased compared with the wild type.

In the wild type, the diurnal changes in NADP redox state were accompanied by corresponding changes in the adenylate energy state, with ATP-ADP ratios being decreased at the end of the day compared with the end of the night (Fig. 6F). A similar decrease in the ATP-ADP ratio from 1.5 to 1.2 in response to light was found in previous studies with leaves of wild-type Arabidopsis plants (Carrari et al., 2005). This is most probably attributable to light activation of the Calvin-Benson cycle and other ATP-consuming biosynthetic processes. In the trxf1 ntrc mutant, diurnal changes in both

Figure 4. Changes in chlorophyll fluorescence parameters in leaves of trxf1, ntrc, and trxf1 ntrc Arabidopsis mutants compared with the wild type (WT). Plants growing in an 8-h photoperiod with 160 μmol photons m−2 s−1 were dark adapted for 10 min before exposure of a far-red light saturation pulse (5,000 μmol m−2 s−1 for 0.8 s) to single leaves. Afterward, the maximal chlorophyll a fluorescence was quenched by electron transport with an actinic red light of 166 μmol photons m−2 s−1. Within 10 min, the steady state was reached, and another saturation pulse was given. In the end, the maximal quantum yield of PSII (Fv/Fm; A) and ΦPSII, the quantum yield of nonregulated energy dissipation (ΦNO), and ΦNPQ (B) were calculated. Results are means ± se (n = 11 different plants). *, P < 0.05; **, P < 0.01; and ***, P < 0.001 (according to Student’s t test).

Figure 5. Changes in the levels of proteins involved in photosynthetic electron transport and ATP synthesis in leaves of trxf1, ntrc, and trxf1 ntrc Arabidopsis mutants compared with the wild type (WT). PsA, PsB, PsbA, PsbD, Lhca1, Lhcb1, and Atpβ proteins were detected using specific antibodies. Representative western blots are shown from 5-week-old plants growing in an 8-h day with 160 μmol photons m−2 s−1 light regime harvested 4 h into the light period. In the wild type, different amounts of samples were loaded (25%–100%) for comparison. Actin protein level is shown as control.
adenylate levels (Fig. 6E) and ATP-ADP ratios (Fig. 6F) were opposite to the wild type, being increased during the day relative to the night, while the *trxf1* and *ntrc* single mutants were largely similar to the wild type (Fig. 6, E and F).

Overall, these results show that a combined deficiency of Trx* f*1 and NTRC causes major alterations in both NADPH-NADP⁺ and ATP-ADP ratios during the day, indicating that the primary cause for the strong impairment of photosynthesis is an inhibition of the Calvin-Benson cycle rather than the light reactions.

### Combined Deficiency of Trx f1 and NTRC Strongly Impairs Redox Activation of FBPase while Having No Inhibitory Effect on Redox Activation of NADP-MDH

The above-described results show that the combined deficiency of Trx* f*1 and NTRC causes impairment of photosynthetic parameters, the diurnal oscillation of energy availability, and carbon fixation rate. We then analyzed whether this could be due to direct effects on enzymes of the Calvin-Benson cycle or NADPH export from the chloroplast. To this end, we focused on fructose-1,6-bisphosphatase (FBPase) and NADP-malate dehydrogenase (MDH), representing key regulatory steps of these processes and classical targets of Trx* f* and Trx* m*, respectively (Buchanan et al., 1979). Chloroplast FBPase (cpFBPase) is known to be subject to exquisite light activation via the Fdx-Trx* f* system, leading to reduction of an intramolecular disulfide that promotes activation of the enzyme (Zimmermann et al., 1976; Buchanan et al., 1979). To analyze the effect of a combined deletion of NTRC and Trx* f* on redox regulation of FBPase, the redox status of the chloroplast enzyme was analyzed in vivo by labeling of thiol groups with the alkylating agent methyl-maleimide-polyethylene glycol₂₄, which adds 1.5 kD per thiol, thus causing a switch of the electrophoretic mobility of the reduced form of the enzyme compared with the oxidized form. In the wild type, cpFBPase protein was completely oxidized at the end of the night, while more than 50% of the protein was in the reduced state at the end of the day (Fig. 7A), confirming light-induced reduction of its intramolecular disulfide in vivo. This response was strongly modified in the redox mutants. At the end of the day, the ratio of reduced to oxidized cpFBPase protein was completely oxidized at the end of the night, while more than 50% of the protein was in the reduced state at the end of the day (Fig. 7A), confirming light-induced reduction of its intramolecular disulfide in vivo. No changes were observed between the genotypes at the end of the night. Results from four independent experiments were quantified and are summarized in Figure 7B, showing...
that the ratio of reduced to oxidized cpFBPase protein decreased significantly by 40% in trxf1, 20% in ntrc, and 70% in trxf1 ntrc mutants relative to the wild type, indicating an additive effect in the double mutant. Finally, it was noticed that the content of cpFBPase protein in the double mutant was slightly decreased compared with the wild type or single mutants (Fig. 7A), suggesting minor effects on cpFBPase protein turnover in addition to posttranslational thiol disulfide modulation.

We then investigated whether a deficiency in Trx f1 and NTRC also affects FBPase enzyme activity. In leaves of the wild type, FBPase activity was very low at the end of the night and increased 20-fold toward the end of the day (Fig. 7C), confirming previous studies on the light activation of cpFBPase (Zimmermann et al.,...
1976; Chiadmi et al., 1999). This response was strongly attenuated in the redox mutants. At the end of the day, FBPase activity was progressively decreased down to 40%, 80%, and 7% of wild-type level in trxf1, ntrc, and trxf1 ntrc mutants, respectively, while there were no significant changes between these genotypes at the end of the night. In the double mutant, no significant changes between nocturnal and daytime FBPase activity were observed, indicating that light activation of FBPase has been abolished. The data also show a correlation between FBPase activity and FBPase redox state across different genotypes and day-night conditions (compare Fig. 7, B and C), confirming the major role of thiol disulfide modulation in regulating FBPase enzyme activity in vivo. It should be noted that the FBPase activity was measured in crude extracts, which will likely overestimate the residual chloroplastic activity in the dark, as the redox-insensitive cytosolic FBPase will contribute to the measured activity. However, analysis of Arabidopsis mutants lacking cytosolic FBPase show that, under these conditions, 80% of the FBPase activity in crude leaf extracts is due to chloroplastic cpFBPase (Rojas-González et al., 2015).

To investigate transient light activation of FBPase in a detailed time course, FBPase activity was analyzed in leaves 0, 2, 5, 10, 20, and 30 min after illumination. As shown in Figure 7D, light led to a rapid increase in FBPase activity, reaching half-maximal activity within 1 min after the start of illumination in the wild type. Compared with the wild type, the increase in FBPase activity was significantly delayed by approximately 50% in trxf1 and ntrc single mutants, reaching half-maximal activities 5 and 10 min after the start of illumination, respectively. Between 10 and 30 min, FBPase activity showed no further increase or increased only slightly, with trxf1 and ntrc mutants both saturating at approximately 50% of wild-type level. Intriguingly, combined deficiency of Trx f1 and NTRC led to a complete loss in light activation of FBPase, with the double mutant showing no significant increase in FBPase activity upon illumination. When FBPase activity was measured in the presence of 10 mM dithiothreitol (DTT) in the assay medium to fully reduce the regulatory disulfide of the enzyme, no significant changes were detected in the different genotypes and light conditions, except a slight decrease of the maximal FBPase activity in the trxf1 ntrc mutant compared with the wild type and single mutants (Fig. 7E). The ratio between the activities in the two assay conditions (minus DTT versus plus DTT) is shown as a calculated redox activation state (Fig. 7F). The changes in the estimated redox activation state followed similar curves as the initial activities measured without DTT (compare Fig. 7, D and F). The results show that knockout of Trx f1 led to a decreased efficiency in light activation of FBPase, which is in line with earlier studies showing that chloroplast FBPase is redox activated by f-type Trx in vitro (Collin et al., 2003). However, deletion of Trx f1 only led to a 50% inhibition in FBPase activation, which is similar to the degree of inhibition in the ntrc mutant, indicating that neither of these redox systems has an exclusive role in the redox regulation of FBPase in vivo. Moreover, the almost complete loss of light-dependent activation of FBPase in the trxf1 ntrc mutant strongly suggests the cooperative effect of Trx f and NTRC in FBPase redox regulation. A comparison between Figure 7, D and E, also documents that the redox sensitivity of the extracted FBPase protein itself was not compromised in the mutants relative to the wild type.

For comparative purposes, we also measured the activity of NADP-MDH, a chloroplast enzyme involved in the export of NADPH to the cytosol via the malate valve (Scheibe, 2004) and being subject to activation by Trxs f and m in vitro (Collin et al., 2003; Yoshida et al., 2015). In the wild type, NADP-MDH initial activity was higher at the end of the day compared with the end of the night (Fig. 8A), confirming previous studies on the light activation of this enzyme in the chloroplast stroma (Scheibe, 2004). Interestingly, this response was promoted rather than inhibited in the redox mutants. Compared with the wild type, trxf1, ntrc, and trxf1 ntrc mutants showed increased activation of NADP-MDH during the day, while there were no substantial changes observed in the night. When NADP-MDH activity was measured in the presence of 10 mM DTT in the assay medium to fully reduce the regulatory disulfides of the enzyme, no substantial changes were detected across the different genotypes and light conditions (Fig. 8B). The ratio between the activities in the two assay conditions (minus DTT versus plus DTT) is shown as a calculated redox activation state (Fig. 8C). It followed a similar curve as the initial activities measured without DTT (compare Fig. 8, A and C). In the mutants, increased activation of NADP-MDH is probably due to increased chloroplast NADPH-NADP+ ratios (Fig. 6), which promote NADP-MDH redox activation indirectly by acting on the redox potential of its regulatory disulfides (Faske et al., 1995).

Combined Deficiency of Trx f1 and NTRC Leads to Decreased Starch Accumulation and Decreased Redox Activation of AGPase

Following with our purpose of determining the function of Trx f1 and NTRC in redox regulation of different carbon metabolic pathways, we investigated the effect of the combined deficiency of Trx f1 and NTRC on the synthesis of photosynthetic end products starch and Suc. Wild-type leaves showed characteristic diurnal changes of starch (Fig. 9A) and Suc levels (Fig. 9B), which increased by 3- and 2-fold, respectively, toward the end of the day. These diurnal changes were attenuated in the redox mutants. At the end of the day, trxf1, ntrc, and trxf1 ntrc mutants showed a progressive decrease in starch accumulation down to 80%, 65%, and 25% of wild-type levels, respectively (Fig. 9A), confirming previous studies showing attenuation of starch accumulation in trxf1 (Thormählen et al., 2013) and ntrc.
single mutants (Michalska et al., 2009; Lepistö et al., 2013). The decrease in daytime starch content was additive in the double mutant (Fig. 9A), dropping to levels below those of the wild type at the end of the night. At any time, the starch content in the double mutant did not exceed nocturnal wild-type levels. At the end of the night, \textit{trxf1}, \textit{ntrc}, and \textit{trxf1 ntrc} mutants showed a further progressive decrease in the remaining starch content, reaching 35\%, 25\%, and less than 10\% of the nocturnal wild-type level, respectively (Fig. 9A), showing that starch reserves were exhausted in the double mutant. The \textit{trxf1 ntrc} mutant showed decreased accumulation of Suc, which at a lower level was also affected in the \textit{ntrc} mutant but not in the \textit{trxf1} mutant (Fig. 9B). All mutants under analysis showed a decrease of the starch-Suc ratio relative to the wild type, with the decrease being more pronounced in the \textit{trxf1 ntrc} double mutant, with 55\% at the end of the day and 80\% at the end of the night (Fig. 9C).

AGPase is a key enzyme of starch synthesis, which is rapidly activated upon illumination by reduction of an intermolecular disulfide bond between the Cys-81 residues joining the two small subunits (APS1) of this heterotetrameric enzyme (Hendriks et al., 2003; Kolbe et al., 2005; Hädrich et al., 2012). To investigate whether the inhibition of starch synthesis in the different genotypes is due to decreased redox activation of AGPase, monomerization of APS1 was analyzed in leaves harvested at the end of the night and at the end of the day. As seen in previous studies (Hendriks et al., 2003), wild-type leaves revealed a strong increase in the monomerization of APS1 during the day, while APS1 was almost completely dimerized in the night (Fig. 9D). Compared with the wild type, light-dependent monomerization of APS1 was attenuated in the \textit{trxf1} and \textit{ntrc} single mutants (Fig. 9D), confirming results from earlier studies (Michalska et al., 2009; Thormählen et al., 2013). Compared with the single mutants, there was an additional attenuation of APS1 monomerization in the \textit{trxf1 ntrc} double mutant (Fig. 9D), indicating Trx\textsubscript{f1} and NTRC to act additively on the reduction of APS1 in vivo.

Figure 8. Light-dependent redox activation of NADP-dependent MDH in leaves of \textit{trxf1}, \textit{ntrc}, and \textit{trxf1 ntrc} Arabidopsis mutants compared with the wild type (WT). Initial activity without DTT additions in the assay (A), maximal activity with 10 mM DTT included in the assay (B), and redox activation state (C; initial/maximal activity \times 100). Leaves were sampled at the end of night and end of day. Results are means ± se (\(n=24\) [wild type] or 12 [mutants] independent plant replicates). Plants were grown in an 8-h photoperiod with 160 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\). *, \(P<0.05\); **, \(P<0.01\); and ***, \(P<0.001\) (according to Student\'s t test). FW, Fresh weight.

Combined Deficiency of Trx\textsubscript{f1} and NTRC Causes Deep Effects on Metabolite Levels, Including an Increase in Amino Acids at the Expense of Organic Acids

The above data clearly indicate the combined action of Trx\textsubscript{f} and NTRC on redox regulation of different aspects of carbon metabolism. The impairment of the regulation of the Calvin-Benson cycle and attendant starch synthesis in the mutants under investigation is expected to provoke changes of in vivo metabolite levels indicative of regulatory steps in these pathways. In wild-type plants, the levels of 3-phosphoglycerate (3PGA; Fig. 10A), fructose-1,6-bisphosphate (FBP; Fig. 10B), and fructose-6-phosphate (F6P; Fig. 10C) showed strong diurnal alterations, with higher levels being observed at the end of the day, which is in line with the changes in Calvin-Benson cycle activity. These diurnal changes in metabolite levels were differentially modified in the \textit{trxf1 ntrc} mutant. Compared with the wild type, the daytime increase in the level of 3PGA, the first fixation product of Rubisco, was attenuated by 75\% in the \textit{trxf1 ntrc} mutant, while there were no changes in the \textit{trxf1} mutant and only a smaller decrease (by 35\%) in the \textit{ntrc} mutant (Fig. 10A). In contrast to this, the
daytime levels of FBP, the substrate of FBPase, were significantly increased by 50%, 125%, and 75% in trxf1, ntrc, and trxf1 ntrc mutants, respectively (Fig. 10B), while those of F6P, the product of FBPase, were only slightly increased in trxf1 and ntrc single mutants, or even decreased in the trxf1 ntrc double mutant, compared with the wild type (Fig. 10C). Concerning the ratio between product and substrate of FBPase (F6P−FBP), there was a significant and progressive decrease down to 80%, 55%, and 45% of wild-type level in trxf1, ntrc, and trxf1 ntrc mutants, respectively (Fig. 10D), indicating a progressive inhibition of plastidial FBPase in vivo and confirming the decrease in FBPase activity and cpFBPase reduction state (Fig. 7). It should be noted that whole-leaf levels of F6P and FBP were measured, which reflect the sum of the chloroplastic and cytosolic pools, not just the chloroplastic pool. However, studies with Arabidopsis mutants show that lack of chloroplastic FBPase leads to a decrease in the overall F6P−FBP metabolite ratio, while there was no change in the F6P−FBP ratio in response to a lack of cytosolic FBPase (Rojas-González et al., 2015). This demonstrates that a decrease in the overall F6P−FBP ratio is indicative for an inhibition of chloroplastic rather than cytosolic FBPase.

Compared with the wild type, Glc-1-P, the substrate of AGPase, remained unchanged or increased slightly in the different genotypes, suggesting that the inhibition of starch synthesis was unlikely to be due to a shortage of this substrate (Fig. 10F). At the end of the night, the levels of 3PGA (Fig. 10A) and hexose phosphates (Fig. 10, C, E, and F) all showed a progressive decrease in trxf1, ntrc, and trxf1 ntrc mutants relative to the wild type, with the decrease being specifically pronounced in the trxf1 ntrc double mutant. This is consistent with a progressive shortage of carbon in these mutants. Nocturnal levels of FBP were below the detection limit in all genotypes (Fig. 10B).

To gain a more in-depth insight into the global effects of Trx f1 and NTRC on redox regulation of metabolism, gas chromatography (GC)-mass spectrometry (MS)-based metabolite profiling was performed. Supplemental Table S4 and Figure 11A show significant changes in leaf metabolite levels at the end of the day in trxf1, ntrc, and trxf1 ntrc mutants relative to the wild type. In the trxf1 ntrc double mutant, sugars such as Glc and raffinose decreased by a factor of 2, while the levels of maltose and ribose were 3 times increased and the level of trehalose was 2 times increased. Similar or less strongly expressed changes in sugar levels were observed in the ntrc mutant, while sugar levels remained rather unchanged or increased slightly in the trxf1 mutant. Several organic acids showed a significant decrease in both ntrc and trxf1 ntrc mutants, including citrate, fumarate, glycerate, 2-oxoglutarate, shikimate, succinate, and threonate, while malate decreased only in the double mutant. The decrease in glycerate (3-fold) suggests possible effects on photorespiration. In contrast to this, organic acid levels were largely unchanged or showed only slight alterations in the trxf1 mutant. Also, amino acids showed large and significant alterations...
in the trxf1 ntrc mutant. With the exception of Gly and Ser, which were both 2-fold decreased, most other amino acids were increased in the double mutant, which is the case for Ala (1.4-fold), Asp (1.8-fold), Asn (2-fold), Ile (2.3-fold), Leu (2-fold), Met (1.4-fold), Phe (6.7-fold), Pro (7.2-fold), and Val (2.2-fold). Amino acids remained unchanged or showed only slight changes in the trxf1 and ntrc single mutants, with the exception of Gly and Ser, which both decreased by a similar degree in the ntrc single and ntrc trxf1 double mutants. The strong increase in most of the amino acids at the expense of organic acids indicates that combined deficiency of Trx f1 and NTRC has led to a stimulation of nitrogen assimilation, probably due to the increase in the NADP redox and adenylate energy states. The decrease in Gly and Ser is consistent with combined effects on photorespiration. The strong increase in ascorbate, which was observed in the trxf1 and ntrc single mutants, was strongly attenuated in the double mutant.

Metabolite levels were also determined at the end of the night, when sugars derive from the degradation of starch reserves (Fig. 11B; Supplemental Table S5). Compared with the wild type, trxf1, ntrc, and trxf1 ntrc mutants showed a further progressive decrease in the levels of various sugars, with the double mutant revealing a specifically strong decrease in maltose (down to 19% of wild-type level) and Suc (46% of wild-type level), consistent with an increased shortage of carbon under these conditions. There were significant decreases in the levels of various organic acids, which, in most cases, were more severe in the double mutant, compared with the single mutants, specifically fumarate, glycerate, pyruvate, shikimate, and succinate. Large and significant alterations were observed in the levels of various amino acids, which increased in the double mutant, compared with the wild type or the single mutants. While Gly decreased, there were increases in the levels of Ala (3-fold), Arg (7.5-fold), Asn (43.7-fold), Glu (1.9-fold), Ile (4-fold), Leu (3.3-fold), Lys (10.2-fold), Phe (10.3-fold), Pro (2.4-fold), Ser (2.3-fold), Thr (2-fold), and Val (2.6-fold). The more than 10-fold increase in Phe, while shikimate levels were 1.5-fold decreased, indicates aromatic amino acid synthesis to be strongly stimulated by the combined deficiency of Trx f1 and NTRC.

**Figure 10.** Changes in the in vivo levels of phosphorylated intermediates in leaves of trxf1, ntrc, and trxf1 ntrc Arabidopsis mutants compared with the wild type (WT). 3PGA level (A), FBP level (B), F6P level (C), F6P-FBP ratio (D), Glc-6-P level (E), and Glc-1-P level (F) were measured in leaves sampled at the end of the night and end of the day. Results are means ± se (n = 20–30 [wild type] or 10–15 [mutants] independent plant replicates growing in an 8-h photoperiod with 160 μmol photons m⁻² s⁻¹). *, P < 0.05; **, P < 0.01; and ***, P < 0.001 (according to Student’s t test). n.d., Not detectable (values were below the detection limit). FW, Fresh weight.

**Reportor Studies Provide Evidence for Colocalization of Trx f1 and NTRC in the Same Chloroplast Substructure and in the Same Tissues**

The above data clearly show combined functions of Trx f1 and NTRC in regulating photosynthetic metabolism. To investigate whether this is accompanied by a
possible colocalization of Trx f1 and NTRC, we analyzed the subcellular localization pattern of the two proteins. Constructs were generated containing the full-length complementary DNA of Trx f1 fused to yellow fluorescent protein (YFP) and NTRC fused to cyan fluorescent protein (CFP), respectively. Pairwise expression of these fusion proteins in tobacco (Nicotiana benthamiana) leaves resulted in YFP and CFP fluorescence signals that were induced in discrete regions inside the chloroplast (Fig. 12). As revealed by the merged picture, Trx f1:YFP and NTRC:CFP fluorescence patterns were congruent to each other, indicating that both Trx f1 and NTRC are colocalized in the same subchloroplast structure (Fig. 12). We also analyzed the expression pattern of Trx f1 at the tissue level. GUS analysis of Trx f1 expression (Supplemental Fig. S2) reveals a pattern similar to NTRC expression in Arabidopsis plants (Kirchsteiger et al., 2012). This indicates that Trx f1 and NTRC are both active in the same tissues, highlighting the biological relevance of their cooperative function.

DISCUSSION

Two different thiol redox systems exist in plant chloroplasts, the Fdx-Trx, which is dependent of Fdx reduced by the photosynthetic electron transport chain...
and, thus, of light, and the NADPH-NTRC system, which relies on NADPH and, thus, may be operative also during the night. Previous studies led to the view that the two different systems may have different functions in plants. However, the possibility remains that both systems might act cooperatively. In this work, we have tested this possibility by using a genetic approach. Results provide evidence that light- and NADPH-dependent redox systems interact at the level of Trx$f_1$ and NTRC to coordinately participate in the regulation of photosynthetic carbon metabolism and growth in response to changes in light conditions.

Figure 11. Overview of changes in metabolite profiles from leaves of trxf1, ntrc, and trxf1 ntrc Arabidopsis mutants compared with the wild type. Results from leaves sampled at the end of day (A) and end of night (B) are visualized using VANTED diagrams. Metabolite levels that are significantly different from the wild type according to the Student’s t test (P < 0.05) are indicated in blue (increase) or red (decrease) color, while black color indicates no significant difference from the wild type. The order of the squares from left to right is trxf1, ntrc, and trxf1 ntrc mutants being in first, second, and third position, respectively. Data are taken from Supplemental Tables S4 to S7. BPGA, Bisphosphoglycerate; CoA, coenzyme A; DHAP, dihydroxyacetone phosphate; F1,6-BP, Fru 1,6-bisphosphate; F6-P, Fru 6-phosphate; G1-P, Glc 1-phosphate; G6-P, Glc 6-phosphate; Gaba, γ-aminobutyric acid; GAP, glyceraldehyde 3-phosphate; P, phosphate; PEP, phosphoenolpyruvate; PGA, phosphoglycerate; R1,5-BP, ribulose 1,5-bisphosphate; T6-P, trehalose 6-phosphate; TCA, tricarboxylic acid; Tre, trehalose.
Trx \( f \) and NTRC Cooperatively Participate in Light Regulation of the Calvin-Benson Cycle and Growth while Having No Effect on the Redox Regulation of the Malate Valve

Our results show that single knockouts of Trx \( f \) or NTRC cause no or only slight impairment of photosynthesis (Figs. 3 and 4), respectively, confirming previous studies (Lepistö et al., 2009; Thormählen et al., 2013). Interestingly, the combined deficiency of both thiol redox regulators led to a more severe inhibition of photosynthetic CO₂ assimilation (Fig. 3), electron transport rates (Fig. 4), and growth (Fig. 2) than in both single knockouts. This was accompanied by an increase in both the NADPH-NAD⁺ and ATP-ADP ratios (Fig. 6), indicating that the primary cause for the strong impairment of photosynthesis is an inhibition of the Calvin-Benson cycle rather than the light reactions. Direct measurements of FBPase, a key enzyme of the Calvin-Benson cycle, confirm this interpretation (Fig. 7). Light activation of FBPase was attenuated by up to 50% in the Trx \( f \) and NTRC single mutants, while it was almost completely abolished in the double mutant. A similar picture emerged when the redox state of the regulatory disulfide of chloroplast FBPase was directly analyzed using gel-shift assays in vivo. Inhibition of FBPase is also indicated at the metabolite level, because it was accompanied by a decrease in F6P-FBP metabolite ratios in vivo (Fig. 10D). This shows that combined knockout of Trx \( f \) and NTRC strongly impedes light-dependent changes in FBPase redox transition (Fig. 7) and hence Calvin-Benson cycle activity (Fig. 3) and plant growth (Fig. 2). Results are in line with previous studies on transgenic plants (Koßmann et al., 1994) or Arabidopsis mutants (Livingston et al., 2010; Rojas-González et al., 2015), with knockdown of chloroplast FBPase showing that a decrease in chloroplast FBPase activity below 36% of wild-type level impairs F6P-FBP metabolite ratios, photosynthetic CO₂ assimilation, starch accumulation, and growth.

In textbooks, Trx \( f \) is proposed to act as the exclusive thiol redox regulator of FBPase and the Calvin-Benson cycle, a scenario that is based on pioneering in vitro experiments of Buchanan et al. (1979) and Buchanan (1980) and subsequent comparative studies using a large set of different recombinant purified Trx isoforms in vitro (Collin et al., 2003). Our in vivo studies show that this textbook view cannot be transferred to the more complex situation in planta, where an almost complete deficiency of Trx \( f \) has been found to be not sufficient enough to decrease light-dependent redox activation of FBPase by more than 50% (compare Figs. 1 and 7). The latter requires a combined deficiency of Trx \( f \) and NTRC, indicating that both proteins have redundant functions in light regulation of FBPase and the Calvin-Benson cycle activity in planta. The involvement of NTRC in redox regulation of the Calvin-Benson cycle allows carbon assimilation to be linked to the NADP redox state as an additional input, which is influenced by light via ferredoxin-NADP reductase and by metabolic parameters.

In contrast to the strong effects on FBPase, single and combined knockouts of Trx \( f \) and NTRC did not lead to any inhibition in the redox activation of NADP-MDH (Fig. 8), a key enzyme in the export of excess-reducing equivalents from the chloroplast to the cytosol via the malate valve (Scheibe, 2004). This indicates that neither Trx \( f \) nor NTRC affect NADP-MDH redox activation in planta, suggesting that the latter is regulated by other Trx isoforms, such as \( m \)-type Trxs. This regulatory feature could be important to prevent an imbalance of the chloroplast NADP redox state. If Trx \( f \) and NTRC would activate Calvin-Benson cycle and the malate valve simultaneously, this could lead to a strong depletion in chloroplast NADPH levels, with adverse effects on the operation of the Calvin-Benson cycle. Our results therefore show that the predominant role of Trx \( f \) in redox regulation of NADP-MDH, which was proposed on the basis of in vitro studies (Collin et al., 2003; Yoshida et al., 2015), cannot be translated to the situation in planta.

Trx \( f \) and NTRC Cooperatively Participate in Light Regulation of Starch Metabolism and Balancing of Carbon and Nitrogen Metabolism

Combined deficiency of Trx \( f \) and NTRC led to a nearly complete inhibition of starch accumulation. This was accompanied by an additive decrease in light...
activation of the key enzyme of starch synthesis, AGPase (Fig. 9). The effect is most likely attributable to both Trx f1 and NTRC being able to reduce the small subunit of AGPase with similar efficiencies, as observed by previous in vitro studies (Thormählen et al., 2013). In the trxf1 ntrc double mutant, the additive decrease in redox activation of AGPase will partly contribute to the strong decrease in starch accumulation (compare Fig. 9, A and D), while there will be an additional contribution from the inhibition of the Calvin-Benson cycle (Fig. 3A) and the subsequent decrease in the level of its first fixation product, 3PGA (Fig. 10A), which is a strong allosteric activator of AGPase (Preiss 1988). Changes in the redox (Hädrich et al., 2012) and allosteric properties of AGPase (Obana et al., 2006; Mugford et al., 2014) have been found to substantially alter diurnal starch turnover in leaves of Arabidopsis plants.

Interestingly, combined, but not single, deficiencies of Trx f1 and NTRC led to a strong and unexpected increase in the level of the starch degradation product, maltose, during the light period (Fig. 11A; Supplemental Table S4). This could indicate that Trx f1 and NTRC may participate in the diurnal regulation of starch degradation in addition to starch synthesis. This is in line with previous in vitro studies reporting that several enzymes in the pathway of starch degradation are subject to thiol redox regulation (Valerio et al., 2011; Glaring et al., 2012), including a plastid-targeted β-amylase, which has been shown to be regulated by both Trx f1 and NTRC (Valerio et al., 2011). It remains to be determined whether a similar mechanism is operational in vivo.

Unexpectedly, combined deletion of Trx f1 and NTRC resulted in a strong increase in the levels of most amino acids, while sugars and organic acids were mainly decreased (Fig. 11), indicating an induction of nitrogen assimilation in the face of a decreased carbon assimilation. It is unlikely that this is due to direct effects of Trx f1 and NTRC deficiencies on redox activation of enzymes involved in nitrogen assimilation, such as nitrate and nitrite reductase, Glu:oxoglutarate amidotransferase, or Gln synthetase, because this would have led to a decrease rather than an increase in the activities of these enzymes (Lichter und Häberlein, 1998; Choi et al., 1999). However, there will be indirect effects due to the elevated NAD and NADP reduction and adenylate energy states as a consequence of the inhibition of carbon assimilation (Fig. 6), which will promote nitrogen assimilation by increasing the levels of its cofactors. This is accompanied by a decrease in the levels of organic acids, which are used as substrates to provide carbon skeletons for amino acid synthesis. These results suggest a combined role of Trx f1 and NTRC in balancing carbon and nitrogen assimilation as well as the levels of sugars and amino acids to avoid an overreduction of PSI and osmotic imbalances, respectively (Fernie et al., 2002; Faix et al., 2012). Alternatively, the increase in amino acids may also be attributable to (1) the overall attenuation of protein synthesis in the cell because of the strongly reduced growth rate of the double mutant or (2) a specific stimulation of protein degradation to mobilize additional carbon reserves when sugars are limiting. However, in both of these scenarios, organic acid levels would increase rather than decrease.

Trx f1 and NTRC Cooperatively Participate in Light Acclimation of PSI

Impaired photosynthetic light reactions in the trxf1 ntrc mutant were most likely a consequence of the inhibition of the Calvin-Benson cycle and the subsequent increase in NADPH-NADP⁺ ratios (Figs. 3, 4, and 6B). Combined knockout of Trx f1 and NTRC led to a strong decrease in the abundance of PSI core proteins (Fig. 5), which was most likely due to an inhibition of electron transfer at the acceptor side of PSI, leading to its over-reduction. As shown by previous studies, PSI is very sensitive to excess electrons delivered from PSII due to its limited capacity of regeneration by protein turnover (Suorsa et al., 2012; Tikkanen and Aro, 2014). The moderate decreases in the abundance of PSII core proteins (Fig. 5) and in chlorophyll levels (Supplemental Fig. S1) are most likely part of an adaptive response to relieve the electron pressure on PSI and to protect PSI from photodamage (Grieco et al., 2012; Suorsa et al., 2012; Tikkanen et al., 2014). The decreased ability of the trxf1 ntrc mutant to adapt to high-light conditions (Fig. 2F) provides further evidence for a role of Trx f1 and NTRC in light acclimation of PSI. Proteins involved in cyclic electron transport around PSI may also be part of an adaptive response to preserve PSI, although their specific functions remain to be clarified (Livingston et al., 2010; Suorsa et al., 2012; Hertle et al., 2013). Interestingly, an Arabidopsis mutant deficient in chloroplast FBPase showed impaired linear electron transport and increased cyclic electron flow (Livingston et al., 2010). More studies are needed to resolve the roles of the stromal redox regulators Trx f1 and NTRC in the regulatory network of plant thylakoid energy transduction.

Alternatively, our data could indicate more specific effects of Trx f1 and NTRC on chloroplast protein synthesis. It has been shown in previous studies that light plays a crucial role in regulating chloroplast protein translation, which most likely involves the Fdx-Trx system as one of the underlying signaling pathways (Pfannschmidt and Liere, 2005). More recently, a role of NTRC was proposed to regulate translation of the D2 protein of PSII by thiol disulfide modulation of chloroplast translation factors in Chlamydomonas reinhardtii (Schwarz et al., 2012). However, in vivo evidence to support this conclusion is lacking at the moment.

Trx f1 and NTRC Cooperatively Participate in Growth Acclimation to Varying Light Conditions

Combined, but not single, deficiencies of Trx f1 and NTRC severely affected growth acclimation to varying
light conditions, leading to strongly impaired acclimation of plant growth to a decrease in the length of the photoperiod or changes in light intensities over a wide range of conditions (Fig. 2H). The ability to acclimate to a 4-h photoperiod or to low light intensity (30 μmol photons m$^{-2}$ s$^{-1}$) was almost completely lost, as well as the ability to acclimate to high-light conditions (950 μmol m$^{-2}$ s$^{-1}$). The vulnerability of the trxf1 ntrc double mutant to low light conditions is most likely due to its strongly impaired ability to activate the Calvin-Benson cycle in response to light. Decreased photoperiods, but also low light intensities, require efficient mechanisms to fully activate the Calvin-Benson cycle when light becomes available. The decreased ability of the trxf1 ntrc double mutant to acclimate to high light is most likely attributable to the increased sensitivity of PSI to photodamage (see Fig. 5 and discussion above). The strong depletion in soluble sugars prevailing in the double mutant (Fig. 11) may also have contributed to the decrease in its ability for high-light acclimation. As shown in previous studies, soluble sugars act as signals in the high-light response of Arabidopsis plants, while high-light acclimation is impaired when soluble sugar levels are decreased (Schmitz et al., 2014). Overall, these results suggest a combined role of Trx f1 and NTRC in growth acclimation to varying light conditions.

**Trx f1 and NTRC Colocalize in the Same Chloroplast Substructure**

The Trx f1:YFP and NTRC:CFP fusion proteins were found to be colocalized in discrete regions within the chloroplast stroma (Fig. 12). The pattern is similar to that previously reported for the interaction of NTRC with 2-Cys Prx (Bernal-Bayard et al., 2014) or the chelatase subunit I of the Mg-chelatase complex (Pérez-Ruiz et al., 2014) and for immunogold labeling of NTRC (Pérez-Ruiz et al., 2009). The colocalization of these clusters with specific chloroplast structures is not clear from these studies. Our preliminary results indicate that the substructure where Trx f1 and NTRC are colocalized does not correlate with chloroplast nucleoids but was found to be associated to starch granules (data not shown). The vicinity of these clusters to starch granules may be related to the combined function of Trx f1 and NTRC in regulating carbon assimilation and storage.

**MATERIALS AND METHODS**

**Plant Material and Growth Conditions**

*Arabidopsis (Arabidopsis thaliana)* T-DNA insertion lines trxf1 (SALK_128565; Thormählen et al., 2013), ntrc (SALK_012208; Pérez-Ruiz et al., 2006), the double mutant trxf1 ntrc, generated by cross breeding, and the respective ecotype Columbia (Col-0) wild types were grown for 5 weeks on potting soil (Stender) in a growth chamber with an 8-h photoperiod, 160 μmol photons m$^{-2}$ s$^{-1}$, at 20°C/16°C, and 60%/75% humidity (day/night), if not indicated otherwise in the figure legends. For rosette fresh weight determination, plants were grown for the first week under the conditions indicated above, before they were transferred to 16- or 24-h photoperiods for additional 3 weeks or to a 4-h photoperiod and 30 or 950 μmol photons m$^{-2}$ s$^{-1}$ for additional 4 weeks using a growth chamber at 21°C.

**Homogenization of Plant Material**

For all the metabolite, DNA, or protein extractions described below, leaves were shock frozen directly into liquid nitrogen and subsequently homogenized to a fine powder using a liquid nitrogen-cooled ball mill (MM 400, Retsch GmbH).

**Selection and Molecular Characterization of the Knockout Lines**

The trxf1 ntrc mutant was selected after crossing the well-characterized homozygous parental lines carrying T-DNA insertions in Trx f1 (Thormählen et al., 2013) and NTRC genes (Pérez-Ruiz et al., 2006). The selection of a homozygous line with insertion in both alleles was performed by PCR analyses of genomic DNA using gene-specific primers for the Trx f1 (AT5G02730; 5'-GTCACTTGCTGGAGAGCAG-3' and 5'-GAAGACCATCAACACACTTG-3') and NTRC (AT2G41680; 5'-TATTGAGCAACACCAAAAGGCGA-3' and 5'-CAT- AATTCCAGCTGCTTCAGC-3') genes or oligonucleotides of the T-DNA (5'-ATTTGGCCGATTCGGAAC-3'). PCR products were fractionated on 1% (w/v) agarose gels and visualized by ethidium bromide staining. Detection of Trx f and NTRC proteins was done by western-blot analysis (Laemmli, 1970) using antibodies raised against pea (*Pisum sativum*) Trx f1 (Hodges et al., 1994) and rice (*Oryza sativa*) NTRC (Serrato et al., 2004). To do this, frozen leaf powder was extracted with 2-fold Laemmli buffer (Laemmli, 1970), including 20 mM DTT instead of β-mercaptoethanol. After shaking the extract for 3 min at 90°C, each lane of the polyacrylamide gels was loaded with sample corresponding to 1-μg fresh weight for each genotype. The pea Trx f antibody used in these experiments has been found in previous studies to recognize Arabidopsis Trx f1 and Trx f2 recombinant proteins with comparable efficiencies (Thormählen et al., 2013).

**Gas Exchange Measurements**

Photosynthesis-related gas exchange parameters were determined for 4- to 5-week-old plants with the portable GFS-3000 system (Heinz Walz GmbH). The control unit 3000-C with the measuring head 3010-S was used by adapting the cuvette to Arabidopsis Chamber 3010-A. The conditions within the cuvette were 22°C, 60% relative humidity, and ambient CO2 concentrations, while the impeller speed was set to 7 and the flow rate to 750 μmol m$^{-2}$ s$^{-1}$. The monitoring of the light curve was started with darkened rosettes. When the CO2 and water system parameters were stabilized, the light was switched on and changed in the following order: 50, 100, 150, 200, 300, 400, 600, 800, and 1,000 μmol m$^{-2}$ s$^{-1}$. The parameters of CO2 assimilation rate, transpiration rate, and intercellular CO2 mole fraction were calculated by the software GFS-Win V3.50b (Heinz Walz GmbH).

**Chlorophyll Fluorescence Analysis**

For the in vivo chlorophyll a fluorescence measurement and the calculation of standard photosynthesis parameters of PSI, a dual PAM fluorometer (Dual-PAM 100, Walz GmbH) was used as described previously (Thormählen et al., 2013).

**Analysis of Chlorophyll Content**

The chlorophyll level was determined and calculated as described in Porra et al. (1989). Twenty-five milligrams of frozen leaf material was extracted twice with 1 mL of 80% acetone. For each extraction step, the samples were vortexed for 2 min, incubated for 10 min in darkness on ice, and centrifuged for 10 min at 4°C. The collected supernatants were pooled, and the light absorption measured at 663, 645, and 750 nm (absorption at 750 nm was subtracted from the values at 665 and 652 nm) with an ultraviolet-visible spectrophotometer (Ultraspex 3100 pro, GE Healthcare Europe GmbH).

**Enzyme-Coupled Analysis of Metabolite Levels by Spectrophotometry**

For metabolite analysis, leaf samples were directly frozen in liquid nitrogen without any shading, allowing rapid quenching of metabolism. Extraction and
analysis of the pyridine nucleotides NAD, NADP, NADH, and NADPH as performed as described previously (Lintal et al., 2014). In brief, 25 mg of frozen leaf powder was resuspended in 250 μL of 0.1 M HClO4 (NAD’ and NADP’) or 250 μL of 0.1 M KOH (for NADH and NADPH, respectively) and incubated for 10 min on ice. Samples were centrifuged at 20,000 × g for 10 min at 4°C, and the supernatant was heated to 95°C for 2 min. The pH was adjusted to between 8.0 and 8.5 by addition of an equal volume of 0.2% Tris (pH 8.4) or 0.1% KOH or 0.2% Tris (pH 8.4) and 0.3% HClO4, respectively. The final extraction mix for NAD(H) contained 100 μL Tricine/ KOH (pH 9), 4 μL EDTA, 500 μL 100 mM Tris, 0.1 mM phenazine ethosulfate, 0.6 mM methylthiazolyldiphenyl-tetrazolium bromide, and 6 units mL⁻¹ alcohol dehydrogenase. For NADPH, the final mix consisted of 100 μL Tricine/KOH (pH 9), 4 μL EDTA, 3 μL Glc-6-P, 0.1 mM phenazine ethosulfate, 0.6 mM methylthiazolyldiphenyl-tetrazolium bromide, and 6 units mL⁻¹ Glc-6-P dehydrogenase. Absorption was monitored at 570 nm at 30°C in a microplate reader (HT3, Anthos Mikrosystems GmbH). To validate the method, small representative amounts (2-3 fold the endogenous content) of NAD’, NADP’, NADH, and NADPH were added to the plant material in the mixing solution of HClO4 or KOH. The recoveries of these metabolites from Arabidopsis leaves during extraction and assay were (as measured by the amount of added): NAD’, 92%; NADP’, 98%; NADH, 79%; and NADPH, 111%. The extraction of ATP and ADP was performed according to procedures previously described at previous studies, where also the validity of this method has been documented (Jellito et al., 1992). In brief, 50 mg of frozen leaf powder was extracted with ice-cold 16% (w/v) TCA and 5 mM EGTA by vortexing 1 h at 4°C. After centrifugation at 20,000 × g and 8.5 by addition of an equal volume 0.2M Tris (pH 8.4) and 0.1M KOH (for NADH and NADPH, respectively) and incubated for 10 min on ice. Samples were centrifuged at 20,000 × g for 10 min at 4°C, and the supernatant was heated to 95°C for 2 min. The pH was adjusted to between 8.0 and 8.5 by addition of an equal volume of 0.2% Tris (pH 8.4) or 0.1% KOH or 0.2% Tris (pH 8.4) and 0.3% HClO4, respectively. The final extraction mix for NAD(H) contained 100 μL Tricine/KOH (pH 9), 4 μL EDTA, 500 μL 100 mM Tris, 0.1 mM phenazine ethosulfate, 0.6 mM methylthiazolyldiphenyl-tetrazolium bromide, and 6 units mL⁻¹ alcohol dehydrogenase. For NADPH, the final mix consisted of 100 μL Tricine/KOH (pH 9), 4 μL EDTA, 3 μL Glc-6-P, 0.1 mM phenazine ethosulfate, 0.6 mM methylthiazolyldiphenyl-tetrazolium bromide, and 6 units mL⁻¹ Glc-6-P dehydrogenase. Absorption was monitored at 570 nm at 30°C in a microplate reader (HT3, Anthos Mikrosystems GmbH). To validate the method, small representative amounts (2-3 fold the endogenous content) of NAD’, NADP’, NADH, and NADPH were added to the plant material in the mixing solution of HClO4 or KOH. The recoveries of these metabolites from Arabidopsis leaves during extraction and assay were (as measured by the amount of added): NAD’, 92%; NADP’, 98%; NADH, 79%; and NADPH, 111%. The extraction of ATP and ADP was performed according to procedures previously described at previous studies, where also the validity of this method has been documented (Jellito et al., 1992). In brief, 50 mg of frozen leaf powder was extracted with ice-cold 16% (w/v) TCA and 5 mM EGTA by vortexing 1 h at 4°C. After centrifugation at 20,000 × g for 10 min at 4°C, the supernatant was shortly mixed with 4 mL of ice-cold, water-saturated diethyl ether and centrifuged again at 3,200 × g at 4°C for 5 min. The upper ether phase was discarded to repeat the washing step. The pH adjustment of the remaining water phase was done with 5 μL KOH, 1 μL triethanolamine, or 1 μL HCl until a pH of 6 to 7 was reached. The remaining diethyl ether in the extract evaporated under the hood for 1 h on ice. Directly after the extraction, ATP and ADP levels were measured enzymatically as described previously (Stitt et al., 1989), with the exception that the change in NAD (P)H levels was measured by fluorescence spectroscopy in 96-well microplates at 360 nm with a FilterMax F5 Microplate Reader (Molecular Devices). Starch and Suc were measured photometrically by NADPH absorption, hexose phosphates, FBP, triose phosphates, and 3PGA by NAD(P)H fluorescence as described previously (Thornmalm et al., 2013). Each individual plant sample was measured with at least two analytical replicates.

**GC-time-of-flight-MS Analysis of Polar Primary Metabolites**

GC-time-of-flight-MS-based analysis of primary metabolites was performed exactly as described previously (Thornmalm et al., 2013). For each biological replicate, three analytical replicates were measured. To visualize the metabolite changes within an overview, we used the open-source software VANTED version 2.1.0 (https://immersive-analytics.introlab.montana.edu/vanted/).

**Immunodetection of Photosynthesis and Starch-Related Proteins**

Proteins involved in photosynthetic electron transport (PsaA, PsaB, PsbA, PsbD, Lhca1, Lhcb1, PetC, and Atpf) ribulose 1,5-biphosphate carboxylase/oxygenase large subunit, and actin were detected by western blotting using specific antibodies (Agrisera) according to company instructions. Frozen leaf powder was extracted with 2-fold Laemmli buffer (Laemmli, 1970), including 0.05% [v/v] Triton X-100, 100 μg Tris [pH 8.0], and 1 mM β-mercaptoethanol. After shaking the extract for 3 min at 90°C, each lane of the polyacrylamide gels was loaded with sample corresponding to 1 mg of fresh weight (100%) for each genotype. Immunodetection of APS1 and detection and quantification of enhanced chemiluminescence signals were performed as described previously (Thornmalm et al., 2013).

**FBPase Gel-Shift Assays**

For FBPase gel-shift assays, proteins from leaves of wild-type and mutant plants, harvested at the end of the night or the day, were extracted in the presence of 10% (v/v) TCA, and protein thiols were alkylated with 10 mM iodoacetamide and 5 mM ethanedithiol. After shaking the extract for 3 min at 90°C, each lane of the polyacrylamide gels was loaded with sample corresponding to 1 mg of fresh weight (100%) for each genotype. Immunodetection of APS1 and detection and quantification of enhanced chemiluminescence signals were performed as described previously (Thornmalm et al., 2013).
performing an F-test in the beginning). When western-blot signals were quantified, the paired Student’s t test was used.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers NTRC (AT2G41680) and Trx f (AT3G02730).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Changes in chlorophyll content in leaves of trx f, ntrc, and trx f ntrc Arabidopsis mutants compared with wild type.

Supplemental Figure S2. Histochemical localization of GUS expression in Arabidopsis plants transformed with a Trx f pGUS reporter gene.

Supplemental Table S1. Statistical analysis for rosette fresh weights of trx f, ntrc, and trx f ntrc Arabidopsis mutants growing in different light conditions, compared with wild type.

Supplemental Table S2. Statistical analysis for gas exchange parameters of trx f, ntrc, and trx f ntrc Arabidopsis mutants dependent on different light intensities, compared with wild type.

Supplemental Table S3. Statistical analysis for the time course of Fru-1,6-bisphosphatase light activation in leaves of trx f, ntrc, and trx f ntrc Arabidopsis mutants, compared with wild type.

Supplemental Table S4. Changes in GC-MS based metabolite profiles in leaves of trx f, ntrc, and trx f ntrc Arabidopsis mutants compared with wild type (end of the day).

Supplemental Table S5. Changes in GC-MS based metabolite profiles in leaves of trx f, ntrc, and trx f ntrc Arabidopsis mutants compared with wild type (end of the night).

Supplemental Table S6. Changes in the levels of phosphorylated intermediates and starch in leaves of trx f, ntrc, and trx f ntrc Arabidopsis mutants compared with wild type, based on spectrophotometric measurements (end of the day).

Supplemental Table S7. Changes in the levels of phosphorylated intermediates and starch in leaves of trx f, ntrc, and trx f ntrc Arabidopsis mutants compared with wild type, based on spectrophotometric measurements (end of the night).

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