NTRC links built-in thioredoxin to light and sucrose in regulating starch synthesis in chloroplasts and amyloplasts

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Plants have an unusual plastid-localized NADP-thioredoxin reductase C (NTRC) containing both an NADP-thioredoxin reductase (NTR) and a thioredoxin (Trx) domain in a single polypeptide. Although NTRC is known to supply reductant for detoxifying hydrogen peroxide in the dark, its other functions are unknown. We now report that NTRC plays a previously unrecognized role in the redox regulation of ADP-glucose pyrophosphorylase (AGPase), a central enzyme of starch synthesis. When supplied NADPH, NTRC activated AGPase in vitro in a redox reaction that required the active site cysteines of both domains of the enzyme. In leaves, AGPase was activated in planta either by light or external feeding of sucrose in the dark. Leaves of an *Arabidopsis* NTRC KO mutant showed a decrease both in the extent of redox activation of AGPase and in the enhancement of starch synthesis either in the light (by 40–60%) or in the dark after treatment with external sucrose (by almost 100%). The light-dependent activation of AGPase in isolated chloroplasts, by contrast, was unaffected. In nonphotosynthetic tissue (roots), KO of NTRC decreased redox activation of AGPase and starch synthesis in response to light or external sucrose by almost 90%. The results provide biochemical and genetic evidence for a role of NTRC in regulating starch synthesis in response to either light or sucrose. The data also suggest that the Trx domain of NTRC and, to a lesser extent, free Trxs linked to ferredoxin enable amyloplasts of distant sink tissues to sense light used in photosynthesis by leaf chloroplasts and adjust heterotrophic starch synthesis accordingly.

NADP-thioredoxin reductase | sugar sensing | redox regulation | ADP-glucose pyrophosphorylase

Since its discovery in chloroplasts 40 years ago, regulation of protein function by a change in thiol redox state has gained prominence in cellular processes throughout biology (1–3). In chloroplasts, the activity of numerous enzymes is linked to the redox status of the photosynthetic electron transport chain. Illumination promotes the reduction of ferredoxin (Fdx), which, in turn, leads to the sequential reduction of ferredoxin-thioredoxin reductase (FTR) and a family of thioredoxins (Trxs; *f*, *m*, *x*, and *y*). Trxs then reduce selected enzymes, including those of the Calvin-Benson cycle identified in the original studies. These regulatory changes are reversed in the dark when the Trx system is reoxidized, leading to deactivation of the affected enzymes.

Although well characterized in photosynthesis, relatively little is known about the role of redox in regulating other chloroplast processes and even less for amyloplasts. Several years ago, it was shown that Trx activates ADP-glucose pyrophosphorylase (AGPase) from potato tubers—a well-known regulatory enzyme of starch biosynthesis in plastids (4, 5). AGPase from potato tubers and pea leaf chloroplasts was activated by Trxs *f* and *m* in vitro. Activation was achieved by the Trx-dependent reduction of the intermolecular disulfide bridge of the Cys-82 residues joining the 2 small (B) subunits of the tetrameric holoenzyme. Following reduction, AGPase showed altered kinetic properties and electrophoretic mobility in nonreducing SDS gels. The small subunit of AGPase (AGPB) ran as a dimer in the oxidized enzyme and as a monomer following reductive activation.

Later work revealed that the posttranslational regulation of AGPase observed in vitro also occurs in vivo in photosynthetic as well as heterotrophic tissues. In leaves, AGPase is redox-activated via a light-dependent signal, reduced Trx, allowing the Calvin-Benson cycle and starch synthesis to be coordinately regulated (6). In addition to light, redox activation of AGPase is induced by sugars in illuminated as well as darkened leaves (6) and in nonphotosynthetic tubers (7). This capability allows starch synthesis to respond to signals reflecting the carbon status of the plant (8, 9). Although the reducing power for light-dependent activation can be provided by photoreduced Trx, the mechanism for the nocturnal activation of AGPase by sucrose is not known.

We have addressed this question in the current study and uncovered evidence for the involvement of the unique type of NADP-thioredoxin reductase C (NTRC), an enzyme of the chloroplast stroma identified in *Arabidopsis* and rice (*Oryza sativa*) (10). NTRC contains both an NADP-thioredoxin reductase (NTR) and a Trx domain in a single polypeptide chain and uses metabolically generated NADPH as an alternative to photoreduced Fdx in detoxifying hydrogen peroxide via 2-Cys peroxiredoxins (11, 12). Studies with a well-characterized *Arabidopsis* KO mutant showed that NTRC is important for protection against oxidative damage in low light and prolonged darkness (11).

The present experiments extend the function of NTRC to a previously unrecognized arena—the synthesis of starch—through the regulation of AGPase. Results obtained by using a combination of biochemical and genetic approaches show that NTRC regulates the enzyme by complementing the light-dependent Fdx/Trx system. Moreover, NTRC appears to be essential for redox regulation of AGPase in darkened leaves as well as in roots in response to sugars. NTRC and, to a lesser extent, the Fdx/Trx system provide a means whereby light absorbed by chloroplasts is recognized by amyloplasts of heterotrophic tissues in the activation of AGPase. By a link to sucrose transported via the phloem, light processed via photosynthesis can be used to regulate metabolic processes taking place in sink tissues (13).

Results and Discussion

NTRC Catalyzes NADPH-Dependent Reduction of AGPase in Vitro. The earlier finding that NTRC acts in the reduction of peroxiredoxin

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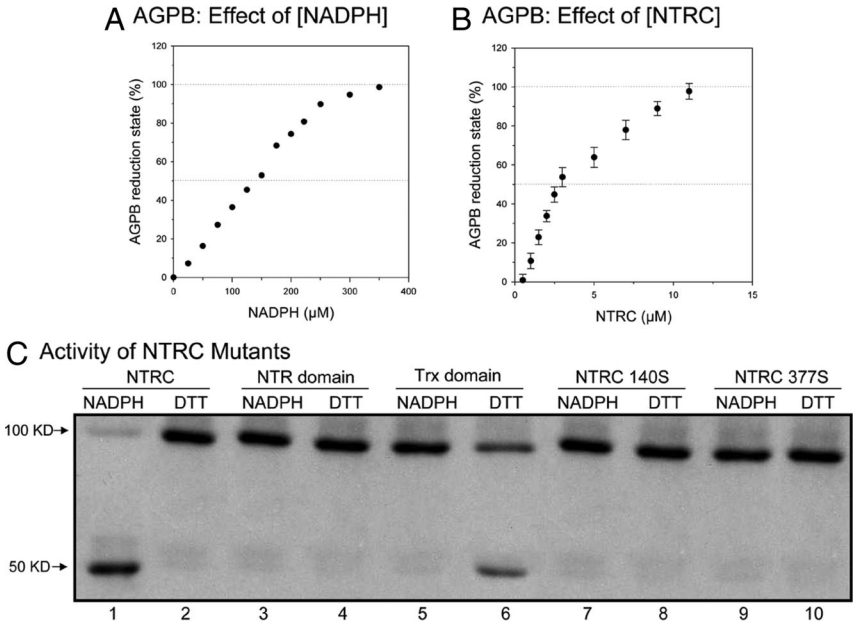

Fig. 1. NADPH-dependent reduction of AGPB by NTRC in vitro. (*A*) Effect of NADPH concentration on the redox state of AGPB. Purified recombinant potato tuber AGPB, 5 μM, and purified recombinant rice NTRC, 5 μM, were both incubated with the indicated concentrations of NADPH in the presence of 1 mM ATP and 1 mM 3PGA. Reduction was assessed by measuring the formation of AGPB monomers in the nonreducing SDS gel assay. (*B*) Effect of NTRC concentration on reduction of AGPB. Varying amounts of the recombinant rice NTRC were incubated with 5 μM purified recombinant AGPB in the presence of 350 μM NADPH, 1 mM ATP, and 1 mM 3PGA (analysis as in *A*). (*C*) Demonstration that the NTR and Trx domains of NTRC and their active sites are essential for transfer of reducing equivalents from NADPH to AGPB; incubation conditions were as in *A*, with either the WT or mutant NTRC enzymes [NTRC 140S (at the NTR module), NTRC 377S (at the Trx module)]. DTT, 500 μM, replaced NADPH, 350 μM, as indicated.

raised the question of a functional link to other chloroplast enzymes. Because of its unusual response to sugars in vivo, we considered AGPase to be an interesting candidate. We pursued this possibility initially by determining whether NTRC (purified recombinant enzyme from rice) could use NADPH to reduce AGPase (purified recombinant enzyme from potato) by monitoring the redox status of the regulatory small subunit dimer of the enzyme (AGPB) in nonreducing SDS/PAGE. In this assay, the oxidized less active form of AGPB migrates as a 100-kDa dimer that, on reduction, is activated and converted to a 50-kDa monomer. As seen in Fig. 1, NTRC converted AGPB from dimer to monomer form in a reaction dependent on the concentration of both NADPH cofactor (50% monomerization achieved at 140 μM NADPH) (Fig. 1*A*) and the enzyme itself (50% monomerization achieved at 2.8 μM NTRC) (Fig. 1*B*). The monomerization of AGPB catalyzed by NTRC was also dependent on the standard redox potential of the NADPH/NADP couple (midpoint potential E_0 of ≈ -330 mV) [supporting information (SI) Fig. S1]. Similar results were obtained when a partially purified AGPase preparation from *Arabidopsis* leaves was incubated with NTRC. Finally, it is noted that as for Trx (4–6), activation of AGPase by NTRC required the presence of the allosteric modifier 3-phosphoglycerate (3PGA).

Reduction of AGPB Requires NTR and Trx Domains of NTRC and the Redox-Active Cys in Their Active Sites. To ascertain whether the native structure of NTRC is required for reduction of AGPB, truncated polypeptides containing either the NTR or Trx domain of NTRC (10) were analyzed with the nonreducing SDS/PAGE protocol. When assayed in the presence of NADPH, neither truncated polypeptide led to detectable monomerization of AGPB (Fig. 1*C*, lanes 3 and 5), whereas the untreated enzyme readily dissociated AGPB dimer (Fig. 1*C*, lane 1). Further, a mixture of both truncated polypeptides in the same assay failed to yield AGPB monomers under these conditions, confirming that the conformation of NTRC with both activities in a single polypeptide is required for AGPB reduction. The need for an intact polypeptide chain was supported by the observation that the Trx domain became accessible to DTT only after disruption of its link to the NTR domain (Fig. 1*C*, lane 6). Neither the complete protein (Fig. 1*C*, lane 2) nor the NTR domain (Fig. 1*C*, lane 4) responded to DTT under these conditions. It is therefore unlikely that free Trx interacts with NTRC. Overall, the results provide strong evidence that NTRC is a highly efficient redox system linking its bonded Trx domain to the regulation of AGPase.

The NTRC single-polypeptide chain, in effect, acts like the well-characterized NADP-Trx system known from cytosol and mitochondria in which NTR and Trx are present as separate proteins. Therefore, it would be expected that the active sites of both NTRC modules, NTR and Trx, participate in a thiol redox exchange reaction with AGPB. To test this possibility, a Cys residue of the active sites of both NTRC modules was replaced by Ser, producing mutants C140S (NTR domain) and C377S (Trx domain), respectively (11). As seen in Fig. 1*C* (lanes 7 and 9), neither of these mutant proteins supported detectable monomerization of AGPB with NADPH, confirming that the active sites of both NTRC modules are essential for the reduction of the AGPB regulatory disulfide leading to enzyme activation. Similarly, there was no effect of DTT with the mutant enzymes (Fig. 1*C*, lanes 8 and 10).

The biochemical studies reported above show that NTRC uses NADPH to activate AGPase by redox in a very efficient manner (Fig. 1 *A* and *B* and Fig. S1). Thus, half-maximal reduction of AGPB was achieved at an NTRC concentration of approximately 2.8 μM, which is lower than the $A_{0.5}$ for the activation of potato tuber AGPase by spinach leaf Trx *f* (4.6 μM) and *m* (8.7 μM; ref. 4) but higher than the more potent interaction of fructose-1,6-bisphosphatase with Trx *f* (0.9 μM; ref. 14). This property indicates that the Trx module forming part of NTRC has a comparable or even higher affinity for AGPB than free Trx.

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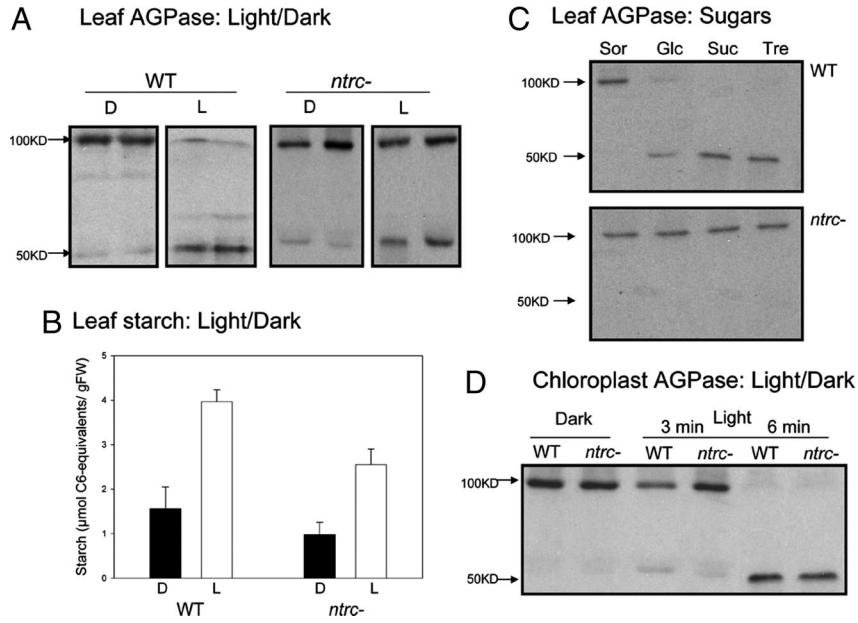

Fig. 2. Effect of KO of NTRC gene on redox regulation of chloroplast AGPase and attendant synthesis of starch in leaves of *Arabidopsis* plants. (*A*) Redox status of AGPase in leaves of WT and NTRC KO (*ntrc*-) *Arabidopsis* plants sampled at the end of the night (D) and end of the day (L). AGPB monomerization was measured as in Fig. 1*A*. (*B*) Content of starch in WT and *ntrc*- plants. Conditions and designations were as in *A*. (*C*) Effect of sugars on redox status of AGPase. Leaf discs were fed for 3 h after the end of the night; AGPB monomerization was then measured as in *A*. Leaves were fed 100 mM sorbitol (Sor, osmotic control), 100 mM glucose (Glc), 100 mM sucrose (Suc), or 100 mM trehalose (Tre). (*D*) AGPase redox status in chloroplasts isolated from WT and NTRC KO (*ntrc*-) plants. Chloroplasts were isolated and subsequently incubated under light or dark conditions in standard assay solution at pH 7.8 supplemented with 0.67 mM $NaPP_i$, 1 mM 3PGA, and 1 mM ATP as described previously (6). These conditions allow high CO_2 fixation rates, leading to a relatively small increase in the NADPH/NADP ratio on illumination (6). Error bars represent SE of 3 different samples.

With respect to NADPH concentration, half-maximal reduction of AGPB was achieved at approximately 140 μM, which is well within the range of stromal NADPH concentrations in vivo (15). Half-maximal activation of AGPB was observed at ≈ -330 mV (Fig. S1), a value in the range of the ratio of chloroplast NADPH/NADP observed in vivo (15).

An *Arabidopsis* KO Mutant of NTRC Shows Decreased Redox Activation of AGPase and Lower Starch Synthesis in Leaves During the Day. The in vitro results described above suggest that NTRC provides an alternate mechanism for the utilization of reducing power in the redox activation of AGPase and the attendant increase in starch synthesis. To test its functional significance in vivo, we characterized the metabolic phenotype of an *Arabidopsis* KO mutant deficient in NTRC (10). The experiments revealed that leaves of WT plants showed strong diurnal changes in the monomerization of AGPB, increasing more than 5-fold at the end of the day compared with the end of the night (Fig. 2*A*, WT D vs. L, and Table S1). Parallel measurements showed that WT starch levels were approximately 2.7-fold higher at the end of the day compared with the nocturnal counterpart (Fig. 2*B*, L vs. D). This observation confirms earlier studies in documenting that changes in monomerization of AGPB are linked to the level of AGPase activity in leaves (6, 9). The results also revealed that the monomerization of AGPB was similar for mutant and WT at the end of the night (Fig. 2*A*). By contrast, the NTRC KO showed a 40–60% decrease in monomerization of leaf AGPB in the corresponding light phase compared with WT (Fig. 2*A*, *ntrc*-, and Table S1). This difference in AGPase monomerization (activation) was reflected by commensurate changes in the diurnal and nocturnal levels of starch (Fig. 2*B*).

The above results demonstrate that KO of NTRC dampens diurnal changes in redox activation of AGPase in leaves. It is unlikely that this change is attributable to indirect effects of the mutation such as impairment of the light reactions or accumulation of oxygen radicals. First, intact chloroplasts isolated from KO plants were not altered in their overall capacity for light-dependent activation of AGPase (Fig. 2*D*). Second, KO of NTRC did not lead to accumulation of reactive oxygen species such as superoxide or hydrogen peroxide in leaves during the day (11, 16). We therefore propose that NTRC complements the light-dependent Fdx/Trx system in redox activation of AGPase by using photochemically generated NADPH as an alternate source of reducing power. The capability to link reduced Fdx to either Trx or NADP enhances flexibility and enables AGPase to respond to dynamic changes in the level of reduction of both activators. This behavior is in keeping with previous studies showing that light activation of AGPase increased in isolated intact chloroplasts when NADPH accumulated as the result of inhibition of carbon assimilation (6).

KO of NTRC Impedes Sucrose-Mediated Redox Activation of AGPase in Leaves in the Dark. Earlier studies showed that in addition to its photochemical regulation as seen above, AGPase can be reductively activated by sugars in the dark (6). To test directly whether KO of NTRC affects the sugar-dependent reduction of AGPase, leaf discs were harvested at the end of the night period and incubated in the dark in solutions supplemented with different sugars. In confirmation of earlier studies, external feeding of glucose, sucrose, or trehalose led to almost complete monomerization of AGPB in the WT (Fig. 2*C Upper*) (6, 9). This effect was, however, almost completely abolished in the NTRC KO, indicating that the enzyme is indispensable for the nocturnal reduction of AGPase dependent on sugar (Fig. 2*C Lower*).

These observations highlight the role of NTRC as an alternative to the classical Fdx/Trx system in the regulation of AGPase. In the light, NTRC is linked to photoreduced Fdx via ferredoxin-NADP reductase (FNR) and NADPH. In the dark or under conditions in which the light reactions are impaired (17), NTRC is linked to sugar oxidation via the initial reactions of the

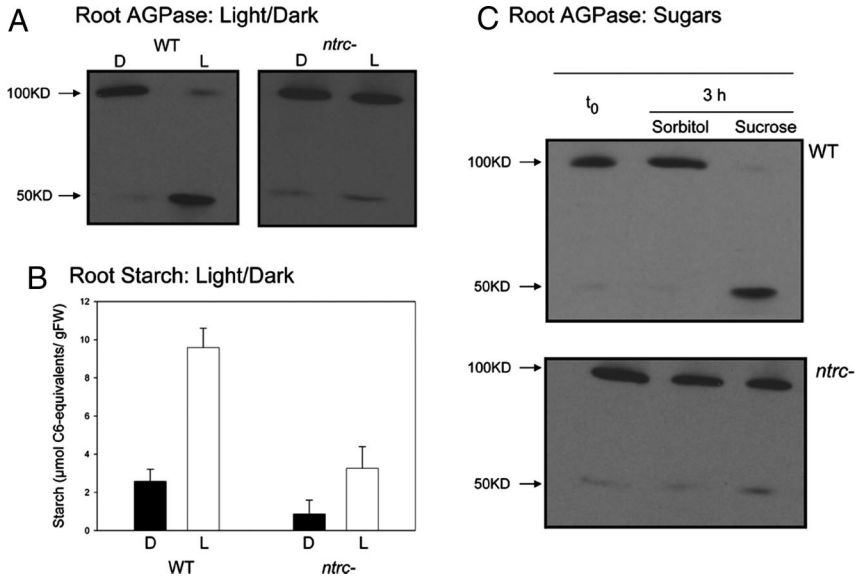

Fig. 3. Effect of KO of NTRC gene on redox activation of AGPase and starch synthesis in roots during the diurnal cycle and in response to sucrose feeding. AGPase redox status (*A*) and starch content (*B*) of roots of WT and NTRC KO (*ntrc-*) *Arabidopsis* plants. Roots were sampled at the end of the night (D) and end of the day (L). AGPB monomerization and starch were measured as in Fig. 2 *A* and *B*, respectively. Error bars represent SE of 3 different samples. (*C*) AGPase redox status in roots after feeding. Roots were fed 100 mM sorbitol (osmotic control) or 100 mM sucrose for 3 h at the end of the night. AGPB monomerization was measured as in *A*.

oxidative pentose-phosphate pathway (OPP) that regulate AGPase independently of the Fdx/Trx system. In *Arabidopsis* mutants lacking the major form of Fdx, NTRC and glucose-6-P dehydrogenase are both strongly up-regulated to compensate for decreased electron flow via Fdx (17). The evidence above shows that NTRC is indispensable for the redox activation of leaf AGPase by sugars in the dark when Fdx would be fully oxidized. Future work will focus on assessing the contribution of the different pathways of electron transfer to the redox regulation of AGPase under different leaf environments.

KO of NTRC Dramatically Decreases Light-Dependent Changes in AGPase Redox Transition and Starch Synthesis in Roots. The results presented in Fig. 2*C* suggest that NTRC plays a primary role in the nocturnal sugar-dependent redox activation of AGPase in leaves. To assess the importance of the enzyme in nonphotosynthetic tissue, the monomerization of AGPB and content of starch and sugar were measured at 2 different diurnal time points in roots of NTRC mutant and WT plants: the end of the night and the end of the day. The experiments uncovered a previously unknown response to light (i.e., recognition by roots of a redox signal generated by light absorbed and processed by the reactions of photosynthesis in leaves). This phenomenon was evidenced by the strong light-dependent monomerization of AGPB in WT roots (Fig. 3*A Left* and Table S2) and was accompanied by the expected buildup of starch in roots (ref. 18; Fig. 3*B*). To our knowledge, light absorbed by leaves has not previously been shown to generate a signal functional in redox regulation in nonphotosynthetic sink tissue. The light-induced changes in AGPB monomerization (Fig. 3*A Right*) and starch content (Fig. 3*B*) were markedly reduced but not fully eliminated in the NTRC mutant. This residual activity of approximately 10% of WT level (Table S2) appears to reflect a limited but significant contribution from the amyloplast Fdx/Trx system in redox regulation of root AGPase (13). On the other hand, KO of NTRC did not significantly alter the levels of soluble sugars in roots that showed clear diurnal changes (Table 1). This observation indicates that unlike AGPase redox status and starch content, amyloplast NTRC has no influence on the import of sugars from the leaf to the root, and thus no effect on the redox signal transmitted from the leaf to the root via sucrose.

In sum, Fig. 3 *A* and *B* extends the function of NTRC to nonphotosynthetic tissue. KO of NTRC dramatically decreased diurnal changes in both redox activation of AGPase and starch synthesis in roots in response to sucrose delivery via the phloem. This finding pinpoints NTRC as the predominant redox system transferring reducing equivalents to AGPase in sink tissue, thereby allowing starch synthesis to be regulated in response to light-dependent changes in sucrose flux via the phloem. Results also show that redox regulation of AGPase is central to the regulation of starch synthesis in nonphotosynthetic tissues.

KO of NTRC Strongly Decreases Redox Activation of Root AGPase in Response to External Feeding of Sucrose. The present results provide evidence that KO of NTRC lowers the light-dependent redox activation of AGPase and the attendant accumulation of starch in roots by interrupting the link between sugars supplied by the phloem and AGPase in the amyloplast. To obtain additional support for this conclusion, we analyzed the redox status of AGPB after external feeding of sucrose to roots of WT and mutant plants at the end of the night. In the WT, sucrose feeding led to near-complete monomerization of AGPB in roots within 3 h, a change that was greatly decreased in the NTRC KO mutant (Fig. 3*C Upper* vs. *Lower*). However, unlike leaves, monomerization of AGPB was not completely abolished in the root supplied with sucrose (Figs. 2*C Lower* and 3*C Lower*). This difference may lie in the unique properties of the Fdx/Trx system

Table 1. Diurnal changes of soluble sugars in roots

Plants	Dark	Light
WT	6.33 ± 0.66	13.75 ± 1.55
ntrc-	7.03 ± 0.83	11.02 ± 1.05

At the end of the night (dark) and at the end of the day (light), soluble sugars (sum of sucrose, glucose, and fructose) were measured in roots of WT and NTRC-KO (*ntrc-*) plants. Results represent the mean ± SE ($n = 3$) and are given as μmol gFW^{-1} (gram fresh weight^{-1}).

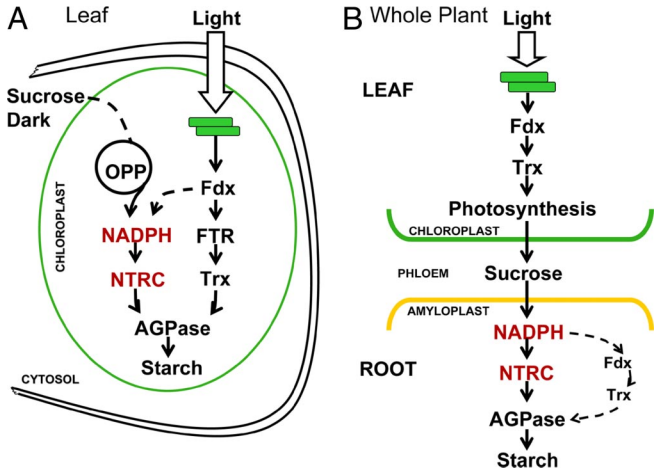

Fig. 4. Proposed role of the Fdx/Trx system and NTRC in linking photosynthesis and sucrose to starch synthesis. (*A*) NTRC serves as an alternate system for transferring reducing equivalents to AGPase in leaves, thereby enhancing storage starch synthesis. In the light, NTRC is mainly linked to photoreduced Fdx via FNR (identified with the dashed arrow) and complements the FTR/Trx system in activating AGPase. In the dark or under conditions in which light reactions are impaired, NTRC is primarily linked to sugar oxidation via the initial reactions of the OPP and regulates AGPase in this way independently of the Fdx/Trx system. (*B*) Fdx/Trx and NTRC mechanisms link light and chloroplasts in leaves to starch synthesis in root amyloplasts. When the light is on, Fdx reduced by the photosynthetic electron transport chain serves as a source of reducing power for FTR and Trx, thereby enhancing photosynthetic CO_2 assimilation and sucrose synthesis. The increase in leaf sucrose in the light (Table 1) leads to activation of AGPase in roots via NTRC and, to a lesser extent, the Fdx/Trx system (modified from ref. 13).

of leaves vs. roots. As discussed earlier, the more oxidizing potential of Fdx in amyloplasts enables free Trx to respond to NADPH, a reductant formed from imported sucrose (13). In chloroplasts, by contrast, because of the strong reducing potential of Fdx, the reduction of free Trx is strictly dependent on light, a feature that ensures efficient regulation of the Calvin-Benson cycle and related pathways (1, 2). Thus, it appears that free Trx linked to Fdx can partly replace the Trx module of NTRC in linking sugar to the activation of AGPase in amyloplasts but not in chloroplasts. Additional studies are needed to provide direct evidence for this conclusion.

Concluding Remarks. The present study has unveiled a previously unrecognized function of NTRC in regulating AGPase and the attendant synthesis of starch in both chloroplasts (Fig. 4*A*) and amyloplasts (Fig. 4*B*), thereby extending its role beyond the detoxification of reactive oxygen species via reduction of 2-Cys peroxiredoxins as envisioned earlier (11, 12, 19). This finding has implications for our general understanding of redox regulation in photosynthetic and nonphotosynthetic tissues and also raises a number of questions.

First, it provides evidence that two different forms of Trx are involved in redox regulation of plastid metabolism, one free-linked to Fdx– and the other bonded to NTRC. When viewed in this manner, it becomes timely to know which of the 40 established or potential Trx-linked target proteins (13, 20–23) are more effective with bonded vs. free Trx and which interact with both forms in amyloplasts as well as chloroplasts. Significantly, it was shown recently that KO of NTRC does not affect redox regulation of fructose 1,6-bisphosphatase or NADP-malate dehydrogenase in leaf chloroplasts, indicating that enzymes such as those of the Calvin-Benson cycle react only with free Trx via Fdx to avoid regulatory problems (16). This point also raises structural questions regarding the different target enzymes that interact with free and/or bonded Trx. The latter may be more specific for intermolecular disulfide bonds owing to its efficiency in reducing the regulatory disulfide joining the two subunits of 2-Cys peroxiredoxin. These issues will be resolved when additional structures are determined and other candidates are tested with the NTRC system.

Second, because of the properties of free and bonded Trx, each redox system can act independently. This observation is supported by the finding that DTT does not reduce the Trx domain of intact NTRC in AGPase reduction (Fig. 1*C*) and the inability of NTRC to transfer electrons to free Trx of the *f*- or *m*-type (10). Also, from theoretical considerations, it will be important to keep the NTRC and Fdx/Trx systems independent of each other in the regulation of photosynthesis and metabolic events in chloroplasts. The separation of the two systems allows bonded Trx (and certain metabolic processes) to respond to NADPH in the presence of highly reduced Fdx in the light and prevents free Trx from responding to metabolically generated NADPH in the dark, permitting the Calvin-Benson cycle and photosynthetic processes to remain inactive under these conditions.

Third, the two redox systems differ in their importance for starch synthesis in photosynthetic and nonphotosynthetic tissues, with Fdx/Trx being predominant in chloroplasts and NTRC in amyloplasts. NTRC may be better suited to amyloplasts on thermodynamic grounds: NADPH generated from sucrose can be used directly by NTRC without going via Fdx and Trx, even with the more oxidizing Fdx of amyloplasts. The Fdx/Trx is ideal for chloroplasts for the same reason: light recognition and irreversibility. However, NTRC also seems to play an important role in chloroplasts via its link to photoreduced Fdx. In the light, Fdx has two options for the activation of AGPase and the associated increase in starch synthesis: FTR/Trx and FNR/NADP. This versatility enables AGPase to respond to dynamic changes in the level of reduction of both activators and chloroplasts to adjust to changes in a wider variety of conditions such as metabolite flux and reactive oxygen species.

Fourth, unlike the Fdx/Trx system, Trx bonded to NTRC provides an efficient redox link between sugars and AGPase in plastids. In this context, it becomes timely to investigate the mode of formation of NADPH in these organelles. The OPP likely functions in this capacity, independently of light in nonphotosynthetic tissues and primarily in the dark in photosynthetic tissues. The plastid form of glucose-6-phosphate dehydrogenase, the relevant enzyme, is composed of 2 different isoforms, P1 and P2, which are preferentially expressed in photosynthetic and nonphotosynthetic tissues, respectively. Although the enzyme actively generates NADPH in both organelles, current evidence indicates that the amyloplast P2 form is much less susceptible to deactivation by reduced Trx and end product inhibition by NADPH than the P1 chloroplast counterpart (24). These features are consistent with a role for the enzyme in providing the substrate levels of NADPH required for heterotrophic sink metabolism. The picture is, however, not fully clear. For example, although transgenic experiments in which glucose-6-phosphate dehydrogenase was overexpressed in tobacco leaves indicate that the enzyme plays a role in determining the ratio of starch to soluble sugars (25), it is not known whether this contribution extends to amyloplasts.

A related point needing clarification concerns the contribution of different sugars to the regulation of AGPase. Unlike glucose, which leads to an increase in both the NADPH/NADP ratio and redox-linked activation of AGPase, sucrose and trehalose act independently of NADPH in increasing activity of this enzyme (9). Rather, these disaccharide sugars are linked to trehalose-6-phosphate, a signal metabolite that promotes redox activation of AGPase, most likely by modifying its interaction with Trxs (9, 26). Moreover, other metabolites such as enzyme substrates and 3PGA, an allosteric modifier, are required for full

activation of AGPase by Trx (4–6). Although details need to be worked out, the interactions among these metabolites are likely required for fine redox control of AGPase as the plant encounters changing environmental conditions.

Finally, in addition to uncovering a link between NTRC and AGPase, the present work adds biochemical and genetic support to the view that thiol signals link photosynthesis to metabolic processes in amyloplasts of sink tissue (7, 13). The data suggest that light is initially recognized as a thiol signal in chloroplasts, then as a sugar signal during transit to the sink, and then again as a thiol signal in amyloplasts for coordinating the regulation of resident metabolic processes with photosynthesis. Although the thiol signal in chloroplasts, Trx, is linked to Fdx, the present data highlight NTRC as the major relevant thiol signal in roots (Fig. 4*B*). The Fdx/Trx system apparently plays a less prominent role, at least with respect to AGPase. Future research will determine whether this hierarchy is a general principle or whether other thiol-modulated amyloplast enzymes of roots react more actively with free Trx in responding to light. Future research should also address the related question of whether one or both of these systems allow other sink tissues (e.g., seeds) to sense and use light to adjust metabolism.

Materials and Methods

Plant Material. *Arabidopsis* var. col 0 WT and the well-characterized NTRC KO mutant (10, 11) were grown in a growth chamber with a 16-h day under 180 μE, 21/19 °C (day/night), and 50% humidity as previously described (6). Two-week-old plants were used for the experiments.

Preparation of Antibody Against *Escherichia coli* Trx. Trx was purified to homogeneity from WT *E. coli* cells by using the procedure described previously (27). An antibody against the pure protein was raised in rabbits (28).

Incubation Experiments with Plant Material. Incubation experiments with *Arabidopsis* leaf discs were performed as previously described (9). Discs cut from leaves at the end of the dark period were floated on buffer solution containing 2 mM 2-(N-morpholino)ethane sulfonic acid (Mes)-KOH (pH 6.5), supplemented with sugars, and incubated 3 h, as indicated in the figure legends, before they were frozen in liquid nitrogen. Chloroplasts were prepared from *Arabidopsis* leaves purified with Percoll (Sigma-Aldrich) and incubated as previously described (6). Intactness of chloroplasts was assessed routinely by microscopy, and chlorophyll content was determined as previously described (6). For incubation experiments with roots, *Arabidopsis* plants were grown in hydroponic culture for 2 weeks. At the end of the night, sucrose or mannitol was added to the hydroponic medium to a final concentration of 100 mM. After incubation in the dark for 3 h, roots were sampled, rinsed quickly with water, immediately blotted with tissue paper, and frozen in liquid nitrogen for further analysis.

In Vitro Assays with Purified Recombinant Proteins. Purified recombinant rice NTRC was produced as previously described (11). Control experiments with antibodies against *E. coli* Trx (see above) revealed no detectable contamination by bacterial Trx in the different NTRC preparations (Fig. S2). Potato AGPB was heterologously overexpressed in *E. coli* and purified as previously described (7). Rice NTRC and potato AGPB proteins, at concentrations between 0.5 and 12 μM, were assayed in buffer solution containing 50 mM Hepes (pH 7.8), 3 mM $MgCl_2$, 1 mM ATP, and 1 mM 3PGA for 10 min in the presence of various NADPH concentrations as indicated in the figure legends. In the indicated incubations, stromal extracts from isolated *Arabidopsis* chloroplasts replaced AGPB as previously described (5).

Analysis of AGPase Redox Activation. Redox activation of AGPase was assessed by analyzing the degree of AGPB monomerization under nonreducing assay conditions (6). Previous studies with different tissues have shown that the degree of monomerization of AGPB is indicative of change in the activation state of AGPase (4–9, 29).

Metabolite Analysis. The levels of starch and soluble sugars were quantitatively determined in ethanol extracts by enzymatic analysis (30).

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