Deletion of chloroplast NADPH-dependent thioredoxin reductase results in inability to regulate starch synthesis and causes stunted growth under short-day photoperiods

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Abstract

Plastid-localized NADPH-dependent thioredoxin reductase C (NTRC) is a unique NTR enzyme containing both reductase and thioredoxin domains in a single polypeptide. Arabidopsis thaliana NTRC knockout lines (ntrc) show retarded growth, especially under short-day (SD) photoperiods. This study identified chloroplast processes that accounted for growth reduction in SD-acclimated ntrc. The strongest reduction in ntrc growth occurred under photoperiods with nights longer than 14h, whereas knockout of the NTRC gene did not alter the circadian-clock-controlled growth of Arabidopsis. Lack of NTRC modulated chloroplast reactive oxygen species (ROS) metabolism, but oxidative stress was not the primary cause of retarded growth of SD-acclimated ntrc. Scarcity of starch accumulation made ntrc leaves particularly vulnerable to photoperiods with long nights. Direct interaction of NTRC and ADP-glucose pyrophosphorylase, a key enzyme in starch synthesis, was confirmed by yeast two-hybrid analysis. The ntrc line was not able to maximize starch synthesis during the light period, which was particularly detrimental under SD conditions. Acclimation of Arabidopsis to SD conditions also involved an inductive rise of ROS production in illuminated chloroplasts that was not counterbalanced by the activation of plastidial anti-oxidative systems. It is proposed that knockout of NTRC challenges redox regulation of starch synthesis, resulting in stunted growth of the mutant lines acclimated to the SD photoperiod.

Key words: ADP-glucose pyrophosphorylase; Arabidopsis thaliana, chloroplast, NTRC, ROS, starch, thioredoxins.

Introduction

Thioredoxins are small regulatory proteins containing redox-active cysteines. In the reduced state, thioredoxins control the function of cellular target proteins by reducing disulphide bridges in the redox-active sites of proteins. Subsequently, the oxidized thioredoxins are reduced by thioredoxin reductases, thereby constituting a thioredoxin system. Plants contain a particularly large number of thioredoxin isoforms in several cellular compartments, including the cytosol, nucleus, mitochondria, and chloroplasts (reviewed by Meyer et al., 2008). Unlike other cell organelles, chloroplasts have two different
systems to reduce thioredoxins. Ferredoxin-thioredoxin reductase links thioredoxins and light-dependent redox-active chloroplast proteins (reviewed by Schürmann and Buchanan, 2008). Ferredoxin-thioredoxin reductase is localized exclusively to plastids, while the isoforms of the NADPH-dependent thioredoxin reductase (NTR) are found in three cellular compartments. In Arabidopsis thaliana, the NTRA and NTRB enzymes are localized both to the cytosol and mitochondria (Reichheld et al., 2007), while the NTRC isoform is found exclusively in plastids (Serrato et al., 2004; Kirchsteiger et al., 2012). NTRC is a unique enzyme comprising both NTR and thioredoxin sequences within the polypeptide (Serrato et al., 2004). As NADPH can also be produced during the dark period by the pentose phosphate pathway (Neuhaus and Enmes, 2000), it has been suggested that the NTRC-dependent thioredoxin system plays an important role in chloroplasts during the night or under low light, when the level of reduced ferredoxin is low (Perez-Ruiz et al., 2006).

Photosynthetic carbon assimilation provides plants with energy and carbon skeletons for metabolism. In general, low light intensity, short photoperiods, and low CO₂ concentration decrease the vegetative growth of plants (Walters and Horton, 1995; Matt et al., 2001; Gibon et al., 2009). In the course of the photoperiod, sucrose is formed from the triose phosphates derived from the Calvin cycle and transported to the roots, developing leaves, and flowers that form sinks for photosynthates. In light, assimilated carbon is also transitorily stored in mesophyll chloroplasts as starch that is mobilized by degradation during the following night, providing plants with carbohydrates to support respiration and growth (recent reviews by Zeeman et al., 2007, 2010). The transitory formation of starch allows photosynthesis to continue in light, even after saturation of sucrose synthesis and transport. Starch turnover in leaf chloroplasts is critical for plant metabolism, and mutations affecting starch accumulation or remobilization result in a significant decrease in plant growth (Caspar et al., 1985; Schulze et al., 1991; Zeeman et al., 1998; Chia et al., 2004; Gibon et al., 2004; Nittylä et al., 2004).

The correlation between starch metabolism and growth is complex (Gibon et al., 2009; Sulpice et al., 2009). Metabolic profiling of Arabidopsis leaves acclimated to various photoperiods demonstrated that the starch degradation rate in the dark has the strongest correlation with relative growth rate of Arabidopsis (Gibon et al., 2009), indicating that efficient starch remobilization directly promotes growth. Accordingly, long photoperiods with short nights decrease the synthesis rate in light and increase the degradation rate of starch in darkness, respectively (Lu et al., 2005; Gibon et al., 2009). The primary molecular mechanisms controlling starch turnover rate in short-day (SD, 8 h light/16 h dark (8L/16D)) and long-day (LD, 16L/8D)-acclimated leaves remain unknown. Both the synthesis and degradation pathways comprise enzymes that are highly regulated by metabolite feedback control, reversible protein phosphorylation, and chloroplast redox state (Zeeman et al., 2007; Köttig et al., 2010). ADP-glucose pyrophosphorylase (AGPase) is the key enzyme in starch synthesis that controls the flux from carbon to starch (Stitt et al., 2010). It is redox activated in light by reduction of a disulphide bridge between the small subunits of the enzyme (Ballicora et al., 2000; Hendriks et al., 2003). Also, the enzymes involved in starch degradation, glucan, water dikinase, phosphoglucan phosphatase (DSP4), and β-amylase have been shown to be under a redox control (Mikkelsen et al., 2005; Sokolov et al., 2006; Sparla et al., 2006).

It has been shown previously that the growth of plants lacking NTRC is retarded particularly under short photoperiods (Perez-Ruiz et al., 2006; Lepistö et al., 2009). NTRC has been shown to control the redox state of chloroplast 2-Cys peroxiredoxins (2-Cys Prxs) that contribute to elimination of H₂O₂ in chloroplasts (Perez-Ruiz et al., 2006; Stenbaek et al., 2008; Kirchsteiger et al., 2009; Pulido et al., 2010). NTRC is also involved in the regulation of the enzymes in chlorophyll (Stenbaek et al., 2008; Richter et al., 2013) and starch (Michalska et al., 2009) synthesis. Furthermore, we have demonstrated that both wild-type chloroplasts and small plastids with poorly developed thylakoid membranes exist in a single Arabidopsis ntrc mesophyll cell (Lepistö and Rintamäki, 2012; Lepistö et al., 2012). Due to the variety of reported effects of NTRC, we raised the question of whether the slowest growth rate observed in SD-grown knockout ntrc plants primarily arises from defects in chloroplast biogenesis or increased reactive oxygen species (ROS) production, or whether the imbalance of the starch metabolism in chloroplasts impairs the biomass production in the absence of NTRC. We have shown recently that poorly developed chloroplasts are present in ntrc lines independently of the photoperiod (Lepistö et al., 2012) and that chlorophyll biosynthesis was reduced equally in plants acclimated to SD and LD conditions (Richter et al., 2013). Thus, the reduced growth of ntrc lines also observed in plants acclimated to LD conditions or to continuous light (Perez-Ruiz et al., 2006; Lepistö et al., 2009) is most likely due to the defects in chloroplast biogenesis in the absence of NTRC. Here, we report that acclimation of ntrc lines to photoperiods with nights longer than 14 h induced extra attenuation of ntrc plant growth. The detrimental effect of long nights on growth of ntrc plants was not primarily due to the rise in oxidative stress in chloroplasts but rather to retarded synthesis of starch in ntrc leaves. We also report that the acclimation of Arabidopsis Col-0 and ntrc to the photoperiods with long nights increased the capacity of light-dependent ROS production in chloroplasts that was not counterbalanced by the activation of antioxidant systems. We propose that thioredoxin-dependent redox control of starch synthesis is crucial for plants with a slow growth rate (e.g., SD-acclimated plants), in which photosynthates are largely allocated to starch during the light period.

Materials and methods

Materials and growth conditions

Wild-type Arabidopsis thaliana Col-0 and T-DNA insertion mutant (http://signal.salk.edu/; Alonso et al., 2003) of NTRC (At2g41680; SALK_096776, Lepistö et al., 2009) plants were grown on a mixture of soil and vermiculite (1:1) under 130 μmol of photons m⁻² s⁻¹ at 20 °C under light/dark (L/D) periods as indicated in the figures and tables. The age of the plants in each experiment is indicated separately in the results.
Measurements of rosette dry weight

Five rosettes of Col-0 and ntrc plants grown under each L/D period were excised and dried at 60 °C for 24 h and weighed. Statistical analysis of the data was performed by one-way analysis of variance (ANOVA) with Tukey’s HSD (P <0.05).

Analysis of sugar and starch content

Leaves (100 mg) were frozen in liquid nitrogen. Soluble sugars were extracted three times at 80 °C for 20 min each. The first extraction was done with 400 μL of 96% (v/v) ethanol;2 mM HEPES (pH 7.5) at a ratio of 4:1, the second extraction with 400 μL of 96% (v/v) ethanol;2 mM HEPES (pH 7.5) at 1:1, and the third extraction with 200 μL of 96% (v/v) ethanol;2 mM HEPES (pH 7.5) at 4:1. Supernatants were collected after each extraction and combined to measure soluble sugars. Sucrose, α-fructose and α-glucose were measured by spectrophotometry with a Sucrose/Fructose/α-Glucose Test kit (Megazyme). For measurement of starch content, the remaining leaf material was dried and ground in liquid nitrogen. Starch was enzymatically hydrolysed to glucose using a Total Starch Test kit (Megazyme) and the starch content was measured by spectrophotometry using the kit and expressed as glucose units per leaf fresh weight (FW).

Isolation of thylakoid membranes and extraction of total and soluble leaf proteins, SDS-PAGE, and Western blotting

Isolation of thylakoid membranes and total and soluble leaf proteins was performed using the methods described by Lepistö et al. (2009). Total leaf protein extracts and protein extracts corresponding to 5–15 μg of protein were solubilized and separated by SDS-PAGE (Laemmli, 1970), using 15% (w/v) acrylamide and 6 M urea in the separation gel, and subsequently electroblotted onto a PVDF membrane (Millipore). After blocking with 1% (w/v) BSA (fatty acid free; Sigma-Aldrich), the polypeptides were immunodetected with protein-specific antibodies using a Phototope™-Star Detection kit (New England Biolabs). Protein-specific antibodies were purchased from Agrisera (GPX and SOD) and Innovagen (APX; Kangasjärvi et al., 2008) and kindly provided by Professor K.-J. Dietz from the Department for Biochemistry and Physiology of Plants, Bielefeld University, Germany (2-Cys Prx, PrxIIE and PrxQ). The analysis was repeated three times and representative Western blots are presented in Fig. 4.

In vivo detection of H$_2$O$_2$ and superoxide

Accumulation of H$_2$O$_2$ and superoxide (O$_2^-$) in the leaves was detected using diaminobenzidine (DAB; Sigma-Aldrich) and nitroblue tetrazolium (NBT; Sigma-Aldrich) substrates, respectively. Four rosettes were excised at the end of the light period and incubated on Petri dishes containing 0.1 mg ml$^{-1}$ solution of DAB (pH 3.8) or a 5 mg ml$^{-1}$ solution of NBT overnight in the dark. The next morning, the dishes were transferred to growth light (130 μmol of photons m$^{-2}$ s$^{-1}$ at 20 °C) for 1 h and thereafter the rosettes were incubated in 96% (v/v) ethanol until the chlorophyll was bleached, or the rosettes were incubated in ethanol directly after the dark period without light treatment. Finally, the most representative stained rosettes were photographed.

Determination of H$_2$O$_2$

An Amplex Red Hydrogen Peroxide/Peroxidase Assay kit (Invitrogen) was used to measure H$_2$O$_2$ production in 4-week-old (SD) and in 3-week-old (LD) plants. Leaves were frozen and ground in liquid nitrogen. A volume of 200 μL of phosphate buffer (20 mM K$_2$HPO$_4$, pH 6.5) was added to 30 mg of leaf powder. After centrifugation, the supernatant was incubated with horseradish peroxidase and Amplex Red reagent according to the Amplex Red Hydrogen Peroxide/Peroxidase Assay kit instructions. The fluorescence was quantified using an Infinite 200 Pro microplate reader (Tecan) with excitation at 545 nm and emission at 590 nm. Statistical analysis of the data was performed by one-way ANOVA with Tukey’s HSD (P <0.05).

Electron paramagnetic resonance (EPR) measurements

Spin trapping assays with N-(4-pyridylmethylene)-tert-butylnitroxide, N,N′-dioxide (4-POBN) were carried out using shocked chloroplasts at a concentration of 10 μg chloroplasts ml$^{-1}$. Samples were illuminated for 1 min with white light (1500 μmol of photons m$^{-2}$ s$^{-1}$) in the presence of 50 mM 4-POBN, 4% ethanol, 50 μM Fe-EDTA, and buffer (25 mM HEPES, pH 7.5, 5mM MgCl$_2$, 0.3 mM sorbitol). EPR spectra were recorded at room temperature in a standard quartz flat cell using an ESP-300 X-band (9.73 GHz) spectrometer (Bruker). The following parameters were used: microwave frequency, 9.73 GHz, modulation frequency, 100kHz, modulation amplitude, 1 G, microwave power, 6.3 mW, receiver gain, 2 × 10$^6$, time constant, 40.96 ms; number of scans, 4.

Results

Biomass production of Col-0 and ntrc grown under natural and artificial photoperiods

The ntrc and Col-0 plants were grown in continuous light and under various L/D regimes under natural 24 h cycles (T24) or under L/D cycles different from 24 h (T20, T28, and T32) to determine whether the lack of NTRC altered the circadian-clock-controlled growth of Arabidopsis (Fig. 1 and Supplementary Fig. S1 at JXB online). Rosettes were harvested either at identical developmental stages of Col-0 plants (before the induction of inflorescence) (Table 1) or at a stage when the plants were exposed to light for equal hours (Supplementary Table S1 at JXB online). The altered clock period length of T20 or T28 did not significantly diminish the biomass production of Col-0 rosettes, whereas growth under the clock period length of T32 with 16L/16D reduced the biomass production by more than 50% (see the plants grown under T20, T24, T28, and T32 with a total light period of 264 or 276 h in Table 1). The reduction in biomass production in the ntrc line corresponded to the pattern detected with Col-0, except that the growth of ntrc plants was reduced further in plants acclimated to the T24 and T32 clock period with a long night of 16 h. The percentage drop in ntrc biomass production in comparison with Col-0 plants was 43–76% in plants grown under photoperiods with nights of 14 h or shorter and more than 90% in plants grown under photoperiods with 16 h dark periods (Table 1). This additional drop in biomass production of ntrc leaves grown under long nights was independent of total light exposure of the plants during growth, as evidenced by comparison of the biomass production of plants with total exposure to light for ~170 h (16L/8D, 14L/14D, 12D/12D, and 8L/16D), or the plants with total illumination of ~270 h (12L/12D, 14L/14D and 16L/16D) (Table 1 and Supplementary Table S1). The large reduction in ntrc dry weight under photoperiods with long dark periods indicated that the long nights were detrimental to plants in the absence of chloroplast NTRC.
Steady-state levels of H$_2$O$_2$, superoxide, and antioxidant enzymes in Col-0 and ntrc plants grown under different light/dark rhythms

NTRC provides electrons to chloroplast ROS detoxification systems, particularly to 2-Cys Prxs (Perez-Ruiz et al., 2006; Stenbaek et al., 2008; Kirchsteiger et al., 2009). It has been suggested that NTRC is a particularly critical mediator of reducing power in chloroplasts during the night, when the light-dependent antioxidant systems are unavailable (Spinola et al., 2008). The measurement of H$_2$O$_2$ content in a whole rosette demonstrated that ntrc leaves acclimated to SDs accumulated slightly higher amounts of H$_2$O$_2$ both in dark-adapted and illuminated leaves than Col-0 rosettes, but the differences were not statistically significant (Supplementary Fig. S2 at JXB online). We further studied the accumulation of H$_2$O$_2$ and O$_2^-$ in vivo in leaves that were exposed directly to light by staining the rosettes with DAB and NBT, respectively (Fig. 2). Illumination of the rosettes grown under a SD, LD, or 16L/16D rhythm for 1 h after the dark period induced higher accumulation of H$_2$O$_2$ in Col-0 and ntrc rosettes acclimated to the L/D rhythms with long nights than in LD-acclimated leaves. The differences in the DAB staining intensity were highest in younger, photosynthetically active leaves, whereas the old rosette leaves of both SD- and LD-acclimated plants accumulated H$_2$O$_2$ under illumination. The ntrc leaves did not accumulate higher amounts of H$_2$O$_2$ when the DAB staining intensity was compared with the respective Col-0 plants. Staining of leaves with NBT demonstrated that no significant differences in staining intensities were observed between Col-0 and ntrc leaves.

Measurement of the total H$_2$O$_2$ content in rosette leaves did not reveal the origin of ROS in the plant cell. As NTRC is a plastid redox regulator and the photosynthetic electron transport chain is the main source of ROS in chloroplasts (Gill and Tuteja, 2010), the light-induced formation of ROS in isolated thylakoids was investigated by EPR spectroscopy using ethanol/4-POBN as the spin traps. Performing 4-POBN

### Table 1. Rosette dry weights in Arabidopsis thaliana Col-0 and ntrc grown under various light-dark rhythms and harvested at an equal developmental stage

Rosette age (d) is the number of T24 cycles from germination to harvesting. Biomass data are means ± standard error ($n=5$ in all experiments). For phenotype of plants, see Fig. 1. Statistically significant differences (ANOVA, Tukey’s HSD; $P<0.05$) between biomasses under different L/D rhythms in each line are indicated with letters (a–d). DW, dry weight.

| L/D rhythm | Total h (L/D h on day of harvest) | Rosette age (d) | Rosette biomass (DW; mg) | $ntrc$ biomass (% Col-0) | Biomass (% 24L) |
|------------|---------------------------------|-----------------|-------------------------|-------------------------|----------------|
| 24L        | 384 (384/0)                     | 16              | Col-0                   | 18.8 ± 1.9$^a$          | 100            |
|            |                                 | ntrc            | 6.6 ± 0.6$^b$           | 35                      | 100            |
| 16L/8D     | 504 (336/168)                   | 21              | Col-0                   | 16.0 ± 1.1$^d$          | 85             |
|            |                                 | ntrc            | 5.4 ± 0.6$^d$           | 34                      | 82             |
| 10L/10D=T20| 528 (264/264)                   | 22              | Col-0                   | 20.1 ± 0.8$^d$          | 107            |
|            |                                 | ntrc            | 4.7 ± 0.5$^d$           | 34                      | 82             |
| 12L/12D    | 552 (276/276)                   | 23              | Col-0                   | 22.3 ± 3.2$^d$          | 119            |
|            |                                 | ntrc            | 5.3 ± 0.5$^d$           | 24                      | 80             |
| 14L/14D=T28| 528 (264/264)                   | 22              | Col-0                   | 17.4 ± 1.3$^d$          | 93             |
|            |                                 | ntrc            | 4.9 ± 0.2$^d$           | 28                      | 74             |
| 8L/16D     | 672 (224/448)                   | 28              | Col-0                   | 20.1 ± 2.0$^d$          | 107            |
|            |                                 | ntrc            | 1.8 ± 0.2$^d$           | 9                       | 27             |
| 16L/16D=T32| 552 (276/276)                   | 23              | Col-0                   | 8.5 ± 0.4$^d$           | 45             |
|            |                                 | ntrc            | 0.7 ± 0.1$^d$           | 8                       | 11             |

**Fig. 1.** Phenotypes of A. thaliana Col-0 and ntrc rosettes grown under various photoperiods. Bar, 2 cm.
spin trapping in the presence of ethanol and Fe-EDTA is a procedure to demonstrate indirectly the formation of O$_2^–$ and H$_2$O$_2$ via the detection of HO$^•$ through its secondary 4-POBN/$\alpha$-hydroxyethyl spin adduct (Mubarakshina et al., 2010). In the absence of other electron acceptors, O$_2$ is reduced to O$_2^–$ at the acceptor side of photosystem I. O$_2^–$ disproportionates spontaneously or by superoxide dismutase (SOD) catalysis to H$_2$O$_2$, which generates HO$^•$ radicals in the presence of Fe(II) in the so-called Fenton reaction. Whereas no signal was observed when samples were maintained in the dark in the presence of ethanol/4-POBN, illumination of thylakoids resulted in strong EPR signals as sextets of lines ($a_{N}=15.61$ G; $a_{H,\beta}=2.55$ G) characteristic of 4-POBN/$\alpha$-hydroxyethyl aminoxyl. Representative spectra are shown in Fig. 3. After 1 min of illumination with 1500 μmol quanta m$^{-2}$ s$^{-1}$ of white light, thylakoids from Col-0 grown in SD conditions produced approximately 50% more ROS than those from Col-0 grown in LD conditions (Supplementary Table S2 at JXB online). Thylakoids from ntrc plants produced a signal that was of comparable size to the signal obtained in SD Col-0 plants independent of the light regime (Supplementary Table S2). Addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), an inhibitor of electron transfer in photosystem II, completely inhibited the spin adduct formation. Addition of dibromothymoquinone (DBMIB), an inhibitor of the cytochrome b6/f complex, also inhibited the spin addduct formation. When the generation of singlet oxygen was measured using the spin trap 2,2,6,6-tetramethyl-4-piperidone (TEMPD), no significant difference between Col-0 and ntrc was observed, independent of the light regime (data not shown). Taken together, this showed that OH radicals were generated by the reduction of O$_2$ to O$_2^–$ at the acceptor side of photosystem I and not by excitation of chlorophylls in disordered reaction centres or antenna systems. We concluded that the L/D rhythm with long nights raises a potential to generate ROS in chloroplasts. This increase, however, was not significantly higher in SD-grown ntrc plants than in SD-Col-0 plants.

We next analysed whether the higher tendency to produce ROS in SD-acclimated chloroplasts altered the levels of antioxidant enzymes in chloroplasts. We estimated by immunodetection the relative amounts of a superoxide-detoxifying enzyme [chloroplastic Cu/Zn superoxide dismutase (SOD)] and H$_2$O$_2$-detoxifying enzymes; peroxiredoxins E and Q (PrxIE and PrxQ), 2-Cys Prx A and B, glutathione peroxidase (GPX) and ascorbate peroxidases [thylakoid-bound ascorbate peroxidase (tAPX), stromal ascorbate peroxidase (sAPX), and cytoplasmic APX (cAPX)] in Col-0 and ntrc plants grown under SD and LD photoperiods (Fig. 4). Neither knockout of NTRC nor the photoperiod induced substantial changes in the level of the cAPX. Acclimation to LD increased only the amount of PrxIIE in Col-0 plants. In comparison with Col-0, the amounts of SOD and sAPX were increased in both SD- and LD-grown ntrc plants. No distinct differences in the amounts of PrxIIE, PrxQ, or GPX were detected in Col-0 and ntrc grown under SD conditions,
whereas under LD conditions the amounts of these antioxidative proteins were decreased slightly in ntrc in comparison with Col-0 plants. The amount of 2-Cys Prxs was decreased in both SD- and LD-grown ntrc plants compared with Col-0 plants. For the amounts of tAPX, no distinct differences were observed. We concluded that, in spite of a higher capacity to produce ROS, growth of Arabidopsis under SD conditions does not significantly upregulate the enzymatic components of ROS scavenging pathways in chloroplasts. Furthermore, knockout of NTRC reduces the amount of 2-Cys Prxs that is compensated for by an increase in the amount of water–water cycle enzymes in ntrc chloroplasts.

Diurnal carbohydrate contents in ntrc plants grown under SD and LD photoperiods

It has been reported that the diurnal leaf growth rhythm follows the fluctuations in endogenous carbohydrate content (Wiese et al., 2007). Compared with metabolites, the nocturnal degradation rate of starch in Arabidopsis leaves shows the strongest correlation with the relative growth rate (Gibon et al., 2009). Therefore, we next analysed the diurnal content of soluble sugars and starch in Col-0 and ntrc leaves grown under SD and LD conditions. The samples for the analyses of soluble sugars were taken at three different time points during the course of 24 h (Supplementary Fig. S3 at JXB online). Starch accumulation and degradation in SD- and LD-grown plants was followed from samples taken at several time points during the course of 24 h (Fig. 5).

In Col-0 plants, sucrose accumulated at the same rate under both SD and LD photoperiods, the residual amounts after long dark period being lower under SD conditions (Supplementary Fig. S3). After the dark period, the sucrose content of the LD ntrc leaves was equal to that of LD Col-0, but while it increased in the light period in LD Col-0 leaves, no net accumulation of sucrose occurred in the light period in LD ntrc plants. This suggested that sucrose was either rapidly consumed or was transported from the LD-grown

**Fig. 3.** Light-induced hydroxyl radical formation in A. thaliana Col-0 and ntrc thylakoids. Generation of H$_2$O$_2$-derived hydroxyl radicals was shown by indirect spin trapping with 4-POBN/ethanol in the presence of Fe-EDTA. Typical spectra of the 4-POBN/α-hydroxyethyl adduct are shown. Samples were illuminated for 1 min at high white light (1500 μmol of quanta m$^{-2}$ s$^{-1}$).

**Fig. 4.** Steady-state levels of antioxidant enzymes in A. thaliana Col-0 and ntrc leaves grown under SD and LD. Below each band, the relative amount of protein in each sample is indicated. SD-grown Col-0 was given a value of 100. Plants were illuminated for 4 h under growth light (130 μmol of photons m$^{-2}$ s$^{-1}$) before sampling. Three biological replicates were analysed by SDS-PAGE and typical blots are presented.
ntrc leaves in light. In the light, the sucrose content of the SD-grown ntrc leaves was low, being only 60% of the amount of SD Col-0 leaves after the dark period. In summary, sucrose content in leaves correlated with the growth capacity of plants, being highest in LD Col-0 plants and lowest in SD ntrc plants. The content of fructose and glucose increased during the first 4 h of the light period in Col-0 and ntrc leaves but stayed rather stable or decreased until the end of the day. Both SD- and LD-grown ntrc plants accumulated fewer hexoses when compared with Col-0 leaves (Supplementary Fig. S3).

The amount of starch in SD-grown plants, both Col-0 and ntrc, began to rise directly after the onset of the light, and the accumulation of starch was further accelerated towards the end of the light period (Fig. 5), indicating that starch is an important storage form of photosynthates in the course of the entire SD photoperiod. In contrast, no accumulation of starch occurred in LD-grown plants in the first 4 h of the light period, and the synthesis accelerated significantly just at the end of the photoperiod (Fig. 5). The ntrc plants accumulated less starch than Col-0 plants, with a reduction of about 50% at the end of both photoperiods. The largest distinction in starch content between Col-0 and ntrc plants was that ntrc plants were not able to accelerate starch formation like the wild-type plants throughout the photoperiod in SD-grown plants and in the last 4 h of the photoperiod in LD-grown plants (Fig. 5). The approximate accumulation rate of starch in the last 4 h of the photoperiod was 1.0 μmol h⁻¹ g⁻¹ FW in SD Col-0 plants and 2.3 μmol h⁻¹ g⁻¹ FW in LD Col-0 plants, respectively, whereas the approximate maximal rates were 0.6 and 0.7 μmol h⁻¹ g⁻¹ FW for SD- and LD-grown ntrc leaves, respectively (Fig. 5). The low starch synthesis rate became especially critical for SD-grown ntrc leaves because of the short photoperiod (Fig. 5). The LD-grown ntrc plants were capable of accumulating as much starch in a 16 h photoperiod as SD Col-0 plants in an 8 h photoperiod. The reduced ability to synthesize starch could also be seen in electron micrographs of ntrc chloroplasts. In contrast to Col-0, no starch granules were present in chloroplasts of SD-grown ntrc leaves illuminated for 1 h (Supplementary Fig. S4 at JXB online).

The initial rate of starch remobilization after transition of plants to the dark correlated with the amount of starch in leaves in the end of the photoperiod and was independent of the knockout of NTRC (Fig. 5 and Supplementary Fig. S5 at JXB online). Because of the reduced amount of starch in the end of the photoperiod, only a minor amount of starch was left in SD-grown ntrc leaves after 5 h in the dark (Fig. 5). No significant differences between ntrc and Col-0 plants were recorded in the amount of starch left in the leaves after the dark period.

Thioredoxin-regulated proteins in starch and ROS metabolisms

AGPase and DSP4 are crucial thioredoxin-regulated enzymes in the starch synthesis and degradation pathways, respectively (Neuhauß and Stitt, 1990; Ballicora et al., 2000; Sokolov et al., 2006). Furthermore, NTRC has been shown to activate AGPase by inducing the monomerization of the small subunit of AGPase both in vitro and in vivo (Michalska et al., 2009). To analyse whether NTRC is capable of regulating both the synthesis and degradation pathways of starch, we tested the direct interaction of thioredoxin reductase and the thioredoxin domains of NTRC with the small subunit of AGPase both in vitro and in vivo (Michalska et al., 2009). In the test, the cDNA encoding the chloroplast 2-Cys PrxB was used as an additional positive control. The polypeptides of the small subunit of AGPase interacted with the thioredoxin domain of NTRC (Supplementary Fig. S6 at JXB online), but the interaction was weaker than with 2-Cys PrxB. No interaction between NTRC domains and DSP4 was detected in the yeast two-hybrid test.

Fig. 5. Accumulation and degradation of starch in A. thaliana Col-0 and ntrc under SD (A) and LD (B). Open and filled bars below the graphs indicate light and dark periods, respectively. Numbers indicate the apparent rates of starch synthesis and degradation (μmol glucose g⁻¹ FW h⁻¹) during distinct phases of the light and dark periods (SD: 0–4 h, 4–8 h, 8–13 h; LD: 0–4 h, 4–12 h, 12–16 h, 16–20 h). Data are means ± standard error, n=5 in all experiments.
Discussion

Characterization of knockout lines has indicated that photoperiodic conditions have an impact on the development of the ntrc phenotype (Perez-Ruiz et al., 2006; Lepistö et al., 2009). NTRC controls multiple processes in chloroplasts, including chlorophyll biosynthesis, ROS metabolism, and the biosynthetic pathways of starch and amino acids (Perez-Ruiz et al., 2006; Stenbaek et al., 2008; Lepistö et al., 2009; Michalska et al., 2009; Pulido et al., 2010; Lepistö and Rintamäki, 2012). The molecular process(es) that drastically weaken growth of knockout ntrc lines under a SD photoperiod, however, have remained elusive.

As the length of the photoperiod influences both the vegetative growth and the timing of flowering in Arabidopsis, comparison of biomass production between plants grown under different L/D cycles is difficult. Therefore, we aimed to measure the rosette biomass both at an equal developmental stage and at a stage when the plants had been exposed to light for an equal length of time. It has been reported that the growth of Arabidopsis under shorter (T20) or longer (T28) L/D cycles than the standard T24 results in reduced vegetative yield (Dodd et al., 2005; Graf et al., 2010). In our experiments, the L/D cycle of T28 slightly and the rhythm of T32 strongly reduced the biomass production. The reduction of Col-0 biomass production under extended day length did not depend on the total exposure time of plants to light before harvesting (Table 1 and Supplementary Table S1). This supports the hypothesis that the lower productivity under altered T-cycles depends primarily on the functions controlled by the circadian clock rather than the duration of the photosynthetically active period (Graf and Smith, 2011). The growth of ntrc rosettes was reduced under all studied light regimes (Table 1), which is probably primarily due to impairment of chloroplast biogenesis in mutant lines (Lepistö et al., 2012; Richter et al., 2013). The biomass production of ntrc lines was equally diminished in natural and unnatural photoperiods, suggesting that the lack of NTRC does not alter the circadian-clock-controlled growth of Arabidopsis. The highest retardation of vegetative growth in ntrc lines was observed under L/D rhythms with nights longer than 14h (Table 1). This extra retardation of ntrc growth depended neither on the total number of hours plants were exposed to light during growth nor on the length of the daily light period. This suggests that NTRC-dependent redox regulation in chloroplasts becomes increasingly important under the L/D rhythms with uninterrupted long nights.

Knockout of NTRC modifies the antioxidant pattern in chloroplast but does not cause severe oxidative stress

It has been established that NTRC is a primary reductant for plastid 2-Cys Prxs that eliminate H$_2$O$_2$ in chloroplasts (Perez-Ruiz et al., 2006; Kirchsteiger et al., 2009; Pulido et al., 2010). Thus, imbalanced ROS metabolism may account for the reduced growth of ntrc lines grown under long nights. However, neither SD- nor LD-grown ntrc leaves accumulated significantly higher amounts of H$_2$O$_2$ or superoxide in comparison with Col-0 leaves (Fig. 2), suggesting that oxidative stress is not a primary reason for the deteriorated growth of the ntrc line. The amounts of 2-Cys Prxs were significantly decreased both in SD- and LD-grown ntrc leaves, indicating chronic oxidation of 2-Cys Prxs pool in the absence of NTRC (Fig. 4) (Pulido et al., 2010). On the other hand, the levels of both chloroplast Cu/Zn-SOD and stromal APX were significantly elevated in ntrc leaves. These results are in line with recent reports showing an increase in the activity of Cu/Zn-SOD and a higher pool size of ascorbate in ntrc leaves compared with wild-type leaves (Pulido et al., 2010). Correspondingly, knockout of stromal and thylakoid APXs induced higher accumulation of 2-Cys Prxs (Kangasjärvi et al., 2008), indicating a close interlinkage between the ascorbate-based antioxidant and the NTRC/2-Cys Prx system in the scavenging of chloroplast H$_2$O$_2$. We concluded that knockout of NTRC modulates ROS homeostasis in Arabidopsis leaves, while the oxidative stress does not primarily account for the extra retardation of vegetative growth in the ntrc lines under the L/D rhythms with long nights. This conclusion is in accordance with the phenotype of the double-knockout mutant of chloroplast 2-Cys Prxs, which shows no visible differences in comparison with wild type (Pulido et al., 2010).

ROS metabolism may, however, be indirectly linked to the retarded growth of ntrc plants under SD conditions. Thylakoids isolated from SD-grown leaves have a higher capacity to produce ROS than the thylakoids isolated from LD-grown leaves (Fig. 3 and Supplementary Table S2) (Michelet and Krieger-Liszkay, 2011). This is consistent with the increased intensity in DAB staining of the photosynthetically active Col-0 leaves, which indicates higher accumulation of H$_2$O$_2$ in illuminated Arabidopsis leaves acclimated to a photoperiod with long nights (Fig. 2). Accordingly, acclimation to SDs increases expression of H$_2$O$_2$ marker genes as indication of the elevated steady-state content of H$_2$O$_2$ (Queval et al., 2007). However, a higher capacity to produce ROS in SD-grown chloroplasts was not associated either with the increase in steady-state expression of plastid antioxidative genes (Lepistö and Rintamäki, 2012) or with the higher level of antioxidative enzymes in chloroplasts (Fig. 4).

The elevated ROS production in SD-thylakoids has been assigned to increased activity of pseudocyclic electron transport in Photosystem I (PSI) (Michelet and Krieger-Liszkay, 2011). It has been hypothesized that the increased production of ROS by PSI in SD-chloroplast is controlled by an unknown protein (Michelet and Krieger-Liszkay, 2011), suggesting that the ROS production in SD-chloroplast is inducible. This hypothetical inducible change in the redox state of chloroplast may operate in adjusting the chloroplast metabolism to daily light/dark rhythm.

Adjustment of the rate of starch synthesis is critical for the growth of Arabidopsis in SD photoperiods

Starch content in the leaves at the end of the light period positively correlates with the length of the photoperiod (Fig. 5) (Gibon et al., 2004, 2009; Lu et al., 2005; Smith and Stitt,
Starch accumulation in chloroplasts of Arabidopsis leaves (Fig. 5) (Michalska et al., 2009). The starch degradation rate correlated linearly with the content of starch at the end of the photoperiod, whereas only minor changes were observed in the apparent accumulation rate of starch during the light period in ntrc leaves (Fig. 5). This result suggests that Arabidopsis leaves lacking NTRC are not capable of accelerating starch synthesis in light. Flux control analysis has shown that AGPase has a large impact on the flux of hexoses to starch (Stitt et al., 2010). The rise in the levels of 3-phosphoglyceric acid and P1 activates and inhibits leaf-type AGPase activity (Crevillon et al., 2003), respectively. This regulation couples the activity of AGPase to the light reactions, to the flux of the Calvin cycle, and to transport of triose phosphates from the chloroplast to the cytosol. AGPase is also redox modulated by thioredoxins (Supplementary Fig. S6) (Hendriks et al., 2003; Gibon et al., 2004, Michalska et al., 2009; Thormählen et al., 2013). Reduction of the intermolecular cysteine bridge between small subunits (APS1) of the enzyme by chloroplast thioredoxins increases the enzyme activity by fourfold (Ballicora et al., 2000). Reduced AGPase has a higher affinity to ATP and a higher sensitivity to the allosteric activator 3-phosphoglyceric acid (Ballicora et al., 2000), suggesting that the chloroplast redox state controls the fine-tuning of the AGPase activity in vivo. We propose that ntrc lines are incapable of fine-tuning of AGPase activity and consequently, SD-grown ntrc leaves in particular suffer a shortage of energy during long nights resulting in reduced growth (Table 1).

The role of NTRC in starch synthesis and the impact of the redox regulation of AGPase in vivo have, however, recently been under discussion. Li et al. (2012) reported that the growth of ntrc lines in dim light under LD conditions did not cause any growth retardation or any decline in the accumulation of starch in comparison with Col-0 leaves. They concluded that retarded growth of ntrc plants under moderate light intensity is due to oxidative stress caused by the imbalanced antioxidative systems in ntrc leaves and that the redox regulation of AGPase has no physiological impact on starch synthesis (Li et al., 2012). In contrast, Hädrich et al. (2011) showed with transgenic lines expressing permanently reduced AGPase that the redox regulation of AGPase controls the flux of hexoses to starch, and that the redox control of AGPase was more crucial for plants acclimated to SD conditions. We have shown that the chloroplasts of SD-grown plants have a higher tendency to produce ROS in light (Fig. 3) (Michel et al. and Krieger-Liszkay, 2011), which may challenge the redox control of thioredoxin-dependent enzymes. Previously, thioredoxins and ROS have been shown to oppositely influence the activity of redox-regulated enzymes (Rintamäki et al., 2000; Martinsuo et al., 2003). We propose that the need for redox regulation of starch synthesis, and consequently the extent of growth retardation in ntrc lines, is conditional, depending on the activities of carbon fixation and utilization of sugars in the sinks. We have reported previously that, in constant light intensity, the relative growth of ntrc plants in comparison with Col-0 plants was less retarded at 10 °C than at 20 °C under a SD photoperiod (Lepistö et al., 2009). Thus, the ntrc phenotype is less severe under conditions demanding less rapid starch synthesis either due to a low activity of the Calvin cycle (under low light or low temperature; Li et al., 2012; Lepistö et al., 2009) or due to a high sink activity (high growth rate of LD-acclimated plants; Gibon et al., 2009). We presume that under such conditions other chloroplast thioredoxins, probably thioredoxin f (Thormählen et al., 2013), are able to maintain reasonable redox stages of AGPase in ntrc chloroplasts to support starch synthesis. We conclude that NTRC is a significant redox regulator of AGPase activity under conditions where the fine-tuning of starch synthesis during the photoperiod is essential for survival of plants.

Supplementary data
Supplementary data are available at JXB online.
Supplementary Fig. S1. Phenotypes of A. thaliana Col-0 and ntrc rosettes after growth for equal hours of light under various photoperiods.
Supplementary Fig. S2. Accumulation of H2O2 in A. thaliana Col-0 and ntrc under SD and LD photoperiods.
Supplementary Fig. S3. Accumulation of soluble sugars in A. thaliana Col-0 and ntrc plants under SD and LD conditions.
Supplementary Fig. S4. Starch accumulation in chloroplasts of A. thaliana plants grown under SD conditions.
Supplementary Fig. S5. Correlation between the initial rate of starch degradation at night and the starch content at the end of the photoperiod in A. thaliana Col-0 and ntrc plants.
Supplementary Fig. S6. Interaction of the TRX domain of NTRC with 2-CysPrx B and the small subunit of AGPase in a yeast two-hybrid assay.

Supplementary Table S1. Rosette dry weights in A. thaliana Col-0 and ntrc plants grown under various L/D rhythms for equal hours of light.

Supplementary Table S2. H$_2$O$_2$-derived hydroxyl radical production by A. thaliana thylakoids measured by spin-trapping EPR spectroscopy.

Supplementary Table S3. Primers and description of the yeast two-hybrid assay.

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