The chloroplast NADPH thioredoxin reductase C, NTRC, controls non-photochemical quenching of light energy and photosynthetic electron transport in Arabidopsis

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Abstract

High irradiances may lead to photo-oxidative stress in plants and non-photochemical quenching (NPQ) contributes to protection against excess excitation. One of the NPQ mechanisms, qE, involves thermal dissipation of the light energy captured. Importantly, plants need to tune down qE under light-limiting conditions for efficient utilisation of the available quanta. Considering the possible redox control of responses to excess light implying enzymes, such as thioredoxins, we have studied the role of the NADPH thioredoxin reductase C (NTRC). Whereas Arabidopsis thaliana plants lacking NTRC tolerate high light intensities, these plants display drastically elevated qE, have larger trans-thylakoid ΔpH and ten-fold higher zeaxanthin levels under low and medium light intensities, leading to extremely low linear electron transport rates. To test the impact of the high qE on plant growth, we generated an ntrc-psbs double knockout mutant, which is devoid of qE. This double mutant grows faster than the ntrc mutant and has higher chlorophyll content. The photosystem II activity is partially restored in the ntrc-psbs mutant and linear electron transport rates under low and medium light intensities are twice as high as compared to plants lacking ntrc alone. These data uncover a new role for NTRC in the control of photosynthetic yield.

Keyword index

High light acclimation, oxidative stress, photosynthesis, redox signalling, thioredoxin, thylakoid.
INTRODUCTION

In plants, reactive oxygen species (ROS) are not only produced through normal aerobic metabolism, but additional ROS are also generated via the photosynthetic electron transport (PET) and oxygen evolution, which take place in the chloroplast (Foyer & Noctor, 2009). At elevated light intensities, when the PET rates exceed photosynthetic capacity, the excess light energy absorbed yields increased production of ROS, including superoxide anion radicals, hydrogen peroxide, hydroxyl radicals and singlet oxygen (Foyer & Noctor, 2009; Li et al., 2009). Adverse environmental conditions, such as extreme temperatures, high salinity and drought have in common that photosynthesis is slowed down and this leads to enhanced formation of ROS even under moderate light intensities, since molecular oxygen acts as sink for PET-derived electrons (Li et al., 2009; Takahashi & Murata, 2008). Adaptation to high light intensities is essential for plant survival and involves changes of gene expression and enzymatic activities in order to optimise photosynthesis and to avoid toxic ROS levels (Dietz, 2015; Foyer et al., 2012; Li et al., 2009; Spetea et al., 2014; Tikkanen et al., 2012).

Photosystem II (PSII) is intrinsically sensitive to strong irradiance, since excess excitation increases the production of singlet oxygen in the reaction centre, which impairs the adjacent proteins, in particular the D1 protein (Aro et al., 1993; Krieger-Liszkay et al., 2008; Tyystjarvi, 2013). Damaged inactive D1 protein becomes proteolytically degraded and replaced with a de novo synthesised copy in a costly repair process (Jarvi et al., 2015; Nixon et al., 2010; Tyystjarvi, 2013). One of the protection mechanisms of plants against excess light involves thermal dissipation of the light energy captured by LHCII, the light-harvesting antenna of PSII (Szabo et al., 2005). The fate of the light energy absorbed can be inferred from measurements of chlorophyll a fluorescence (Baker, 2008) and the loss of chlorophyll fluorescence that is not due to photochemistry is referred to as non-photochemical quenching (NPQ). The major component of NPQ in plants, qE, requires acidification of the thylakoid
lumen, the presence of the PSII PsbS protein (Li et al., 2000) and the conversion of the xanthophyll violaxanthin to zeaxanthin, via antheraxanthin, catalysed by violaxanthin de-epoxidase (VDE) (Jahns & Holzwarth, 2012; Jahns et al., 2009; Ruban et al., 2012; Szabo et al., 2005). VDE is located in the thylakoid lumen and is regulated by pH, the availability of ascorbate and, possibly, by its redox state (Bratt et al., 1995; Hall et al., 2010; Pfundel & Dilley, 1993; Simionato et al., 2015). The effectiveness of qE in preventing production of singlet oxygen and photoinhibition has been confirmed in studies of an Arabidopsis mutant lacking PsbS (npq4) and in transgenic Arabidopsis over-expressing PsbS (oePsbS), which induces very high levels of NPQ (Li et al., 2002; Roach & Krieger-Liszkay, 2012).

Plants in the field may experience a number of sudden changes in light intensity throughout the day. Therefore, rapid reversibility of NPQ should be advantageous, since efficient use of the incident light to promote photosynthesis under light-limiting conditions is as important as protection of the photosynthetic apparatus against excess excitation. Indeed, the time required for reversal of heat dissipation after each shift from high to low light is predicted to cause large losses in carbon assimilation by crop canopies (Zhu et al., 2004). The activity of zeaxanthin epoxidase (ZE), which converts zeaxanthin back to violaxanthin, is necessary to revert qE (Jahns & Holzwarth, 2012; Jahns et al., 2009) and hence, to down-regulate the protection of plants against excess light in order to allow a more efficient utilisation when light energy is scarce. ZE is thought to be constitutively active and the possible regulation of this enzyme is not well understood (Jahns et al., 2009). Numerous findings indicate that induction and reversal of the qE-component of NPQ may be more complex than previously thought. Heat stress reduces the ΔpH component of the trans-thylakoid proton motive force and affects qE (Zhang et al., 2009). Plants lacking the PGR5 or PGRL1 proteins, which function in the switch between linear electron flow (LEF) and cyclic electron flow (CEF), are unable to induce qE (DalCorso et al., 2008; Munekage et al., 2002; Suorsa et al., 2012).
Furthermore, plants devoid of the thylakoid \( \text{H}^+/\text{K}^+ \) antiporter KEA3 display retardation of recovery from NPQ after transfer from high to low light, suggesting a role for KEA3 in this process through direct control of the trans-thylakoid proton gradient (Armbruster et al., 2014). In addition, mutants affected in the thylakoid two-pore \( \text{K}^+ \) channel TPK3 have been shown to be deficient in NPQ (Carraretto et al., 2013). Probably, more factors regulating these processes remain to be identified.

Redox regulation is a means of regulating enzyme activity that implies reversible post-translational changes in the redox state of functional groups in the amino acid side chains (Buchanan & Balmer, 2005). Oxidation of reactive cysteine residues to sulphenic acids or disulphides may be reversed through the action of thioredoxins and glutaredoxins (Meyer et al., 2009). Chloroplasts contain a rich variety of thioredoxins, which receive reducing equivalents from the PET via ferredoxin-thioredoxin reductase (Schurmann & Buchanan, 2008) and in this manner catalyse light-dependent reduction of target enzymes. In addition, there is a chloroplast NADPH-dependent thioredoxin reductase, NTRC, which contains a thioredoxin domain fused to the C-terminus (Perez-Ruiz et al., 2006; Serrato et al., 2004). NTRC constitutes a functional homodimer, where the NTR domain of one subunit reduces the active site of the thioredoxin domain in the other subunit (Bernal-Bayard et al., 2012; Perez-Ruiz & Cejudo, 2009). NTRC is a very efficient reductant for the thiol-dependent peroxidase 2-Cys peroxiredoxin (2-Cys Prx) (Alkhalifioui et al., 2007; Moon et al., 2006; Perez-Ruiz et al., 2006; Puerto-Galan et al., 2015). Hence, a role in chloroplast oxidative stress tolerance has been proposed for this enzyme. The *Arabidopsis thaliana ntrc* knockout mutant is pale-green and displays retarded growth (Lepisto et al., 2009; Perez-Ruiz et al., 2006; Serrato et al., 2004), while plants overexpressing NTRC are larger than wild type plants (Toivola et al., 2013). This phenotype of the *ntrc* mutant is particularly pronounced under short-day photoperiods (Lepisto et al., 2009; Lepisto et al., 2013; Perez-Ruiz et al.,
The ntrc mutant was reported to be hypersensitive to several kinds of abiotic stress, such as high salinity and drought (Serrato et al., 2004), prolonged darkness (Perez-Ruiz et al., 2006) and heat (Chae et al., 2013). Under these conditions the already low growth rate of the ntrc mutant is further reduced in comparison to wild type plants (Perez-Ruiz et al., 2006; Serrato et al., 2004).

The possible implication of chloroplast redox enzymes, e.g. thioredoxins, as signalling molecules in high light acclimation and protection against excess excitation was postulated several years ago (Li et al., 2009). Recently, the relevance of thioredoxins in adaptation of plants to fluctuating light conditions has been considered (Nikkanen & Rintamaki, 2014). This motivated us to investigate a potential connection between light acclimation processes and NTRC, previously implied in peroxide detoxification and abiotic stress tolerance.

**MATERIALS AND METHODS**

**Plant material and growth conditions**

*Arabidopsis thaliana* plants were grown on soil in a growth chamber FITOCLIMA 700 EDTU from Aralab (Rio de Mouro, Portugal) under a photoperiod of 10 h light/14 h darkness and at temperatures of 22°C/20°C during the day/night cycle. The light intensities applied were 50 (low light), 120 (normal light), 600 (moderately high light), or 1000 μmol quanta·m⁻²·s⁻¹ (high light). Temperature and relative humidity (60%) were strictly controlled at all light intensities. Chlorophyll content was measured using the equations in (Lichtenthaler, 1987) after extraction from leaf discs with 100% methanol overnight.

The Arabidopsis mutants used were the ntrc knockout mutant (Perez-Ruiz et al., 2006; Serrato et al., 2004) and the trxx and Δ2cp mutants (Pulido et al., 2010). The psbs mutant was
selected from the line SALK_095156, which contains a T-DNA insertion in the second exon of the gene At1g44575 encoding the PSII PsbS subunit. The ntrec-psbs mutant was obtained by manually crossing the psbs mutant with the ntrec mutant. PCR analyses of these insertion lines were performed using the oligonucleotides in Supporting Information Tables S1 and S2.

Measurements of chlorophyll fluorescence and of the photosystem I P700 redox state

Room temperature chlorophyll a fluorescence was measured using a pulse-amplitude modulation fluorometer (DUAL-PAM-100, Walz, Effeltrich, Germany). The maximum quantum yield of PSII was assayed after incubation of plants in the dark for 30 min by calculating the ratio of the variable fluorescence, $F_v$, to maximal fluorescence, $F_m$, $(F_v/F_m)$. Induction-recovery curves were performed using red (635 nm) actinic light at the intensities specified for each experiment during 8 min. Saturating pulses of red light at 10 000 μmol quanta·m$^{-2}$·s$^{-1}$ intensity and 0.6 s duration were applied every 60 s and recovery in darkness was recorded for up to 12 min. The parameters Y(II), Y(NPQ) and Y(NO), corresponding to the respective quantum yields of PSII photochemistry, non-photochemical quenching (NPQ) and non-regulated basal quenching (NO), were calculated by the DUAL-PAM-100 software according to the equations in (Kramer et al., 2004b). Measurements of relative linear electron transport rates were based on chlorophyll fluorescence of pre-illuminated plants applying stepwise increasing actinic light intensities up to 2000 μmol quanta·m$^{-2}$·s$^{-1}$. For measurement of post-illumination fluorescence induction, dark-adapted leaves were subjected to one saturating pulse and illuminated with actinic light at 126 μmol quanta·m$^{-2}$·s$^{-1}$ intensity, whereafter fluorescence was recorded for 5 min in darkness.

Chlorophyll fluorescence imaging of whole rosettes was performed using an IMAGING-PAM M-Series instrument (Walz, Effeltrich, Germany).
The redox state of photosystem I P700 was monitored by following changes in absorbance of 30 min dark-adapted plants at 830 nm versus 875 nm using the DUAL-PAM-100. To probe the maximum extent of P700 oxidation, leaves were illuminated with far red light superimposed on the saturating pulse of red light.

**Electron paramagnetic resonance (EPR) for measurement of peroxide-derived hydroxyl radicals in vivo**

Spin trapping assays with N-(4-pyridylmethylene)-tert-butylamine N,N’-dioxide (4-POBN) were carried out using leaves from plants grown at 120 µmol quanta·m⁻²·s⁻¹ essentially as in (Michelet & Krieger-Liszkay, 2012). Pieces of 15 - 20 mg of leaf per sample was infiltrated with a solution containing 50 mM 4-POBN, 4% ethanol, 2.5 µM Fe-EDTA, and 20 mM phosphate buffer (pH 7.0). Infiltrated leaves were incubated in 250 µl of the same solution for 1 h under the light conditions indicated. EPR spectra of the 4-POBN/α-hydroxyethyl adducts were recorded at room temperature in a standard quartz flat cell using an e-scan X-band spectrometer (Bruker, Rheinstetten, Germany). The following parameters were used: microwave frequency, 9.73 GHz; modulation frequency, 80 kHz; modulation amplitude, 1 G; microwave power, 6.3 mW; number of scans, 2. Signals were normalised to leaf weight.

**Cloning, expression and purification**

For the expression of proteins, the coding sequences for VDE (At1g08550) and ZE (At5g67030), without chloroplast- and luminal transit peptides, were amplified by PCR using gene-specific oligonucleotides, which included restriction sites for cloning into the pQE-30 vector (Qiagen Sciences, Germantown, MD, USA). The sequences of these oligonucleotides were as follows: VDE, forward 5’-GAGGATCCGTTGATGCACTTTAAA-3’ (BamH1) and reverse 5’-AAGGATCCGACCTCAGCTTTCTGAT-3’ (PstI); ZE, forward 5’-AAGGATCCGCGGACG-3’ (BamH1) and reverse 5’-
Proteins were expressed in *Escherichia coli* XL1-Blue cells from Stratagene (Agilent Technologies, Santa Clara, CA, USA). The recombinant proteins were purified by nickel-affinity chromatography using the Ni-NTA Agarose resin (Qiagen). Purified recombinant rice NTRC was prepared as in (Serrato *et al.*, 2004), but using nickel-affinity chromatography.

**Electrophoresis and protein gel blot analysis**

Total leaf proteins or thylakoid proteins were extracted as described in (Lepisto *et al.*, 2009), the chlorophyll concentrations were measured in 80% acetone as in (Lichtenthaler, 1987), and the protein concentrations were determined according to (Markwell *et al.*, 1978). Proteins were resolved using SDS-PAGE gels at 12% or 10% acrylamide concentration. Antibodies against purified recombinant VDE were raised in rabbit and used at 1:2500 dilution. Antibodies against PsbS, ZE and the ATP synthase γ subunit (AtpC) were from Agrisera (Vännäs, Sweden) and used at dilutions of 1:2000, 1:3000 and 1:5000, respectively. The antibody against NTRC (Serrato *et al.*, 2004) was used at dilution 1:1000. Signals were visualised with enhanced chemiluminescence (ECL) using the Immobilon™ Western Chemiluminescent HRP Substrate reagent from Millipore (Billerica, MA, USA). Alkylation of cysteine thiols was performed using MM(PEG)24 from Thermo Fisher Scientific Inc. (Rockford, IL, USA).

**Determination of ATP hydrolysis and ΔpH in vitro using 9-aminoacridine fluorescence**

Thylakoids isolated from wild type plants were diluted to a chlorophyll concentration of 5 µg/ml. The thylakoid suspension was incubated in the presence of 2.5 µM 9-aminoacridine and with the additions of 100 µM NADPH and 5 µM NTRC, 100 µM NADPH alone or 5 mM DTT for 5 min. The fluorescence at 420 – 550 nm was recorded using the DUAL-PAM-100 fluorometer. ATP (50 µM) and NH₄Cl (20 mM) were added at the indicated time points.
For maximal fluorescence quenching thylakoids were illuminated with red light at 500 μmol quanta m² s⁻¹ intensity in the presence of 100 μM methyl viologen.

**Measurement of ΔpH in vivo using the electrochromic shift (ECS) assay**

Spectroscopic measurements of electrochromic shift (ECS) based on absorbance changes at 520 nm were performed essentially as in (Takizawa et al., 2007) using a JTS-10 Joliot type spectrometer (BioLogic, Claix, France). ECS signals and dark relaxation were measured after 15 min illumination of plants with actinic light at intensities between 12 and 940 μmol quanta m² s⁻¹. The slowly-reversible component ECS_{inv} is proportional to the ΔpH. For each leaf the maximum signal was obtained using a saturating laser flash. The flash-induced signal was used for normalisation.

**Analysis of xanthophylls**

Pigments were extracted by grinding 0.5 g of leaves in liquid nitrogen and resuspending the leaf powder in 10 ml 90% acetone. Quantitative analysis of the carotenoids involved in the xanthophyll cycle (violaxanthin, antheraxanthin and zeaxanthin) was performed using HPLC as described in (Hornero-Mendez et al., 2000). All operations were carried out under dimmed light to prevent isomerisation and photo-degradation of pigments.

**RESULTS**

**The Arabidopsis ntrc mutant tolerates high light intensities**

In order to address the possible involvement of NTRC in long-term high light acclimation we examined the growth of Arabidopsis ntrc and wild type plants at different light intensities. Elevated light intensities did not further compromise the growth of the ntrc plants (Fig. 1a).
The rosettes of the mutant plants maintained nearly their proportions to those of wild type plants even at higher light intensities (Fig. 1b). The ntnc rosettes weighed 12±2.7 % and 8.6±1.9 % of wild type rosettes at 600 and 1000 μmol quanta m⁻² s⁻¹, respectively, as compared to 10±3.8 % at 120 μmol quanta m⁻² s⁻¹. Regarding the amount of total chlorophyll per leaf area, the mutant plants had about 60% of the values of wild type plants at all light intensities examined (Fig. 1c).

To test short-term high light tolerance, plants grown at a light intensity of 120 μmol quanta m⁻² s⁻¹ were transferred to 800 μmol quanta m⁻² s⁻¹ and the integrity of PSII was assayed as variable fluorescence normalised to maximal fluorescence (Fv/Fm). Before the transfer to high light the ntnc plants had an Fv/Fm ratio of about 0.78, which is slightly lower than the typical 0.83 of wild type plants (Fig. 1d). After three hours of illumination at 800 μmol quanta m⁻² s⁻¹ these values had decreased to 0.74 and 0.76, respectively. The results indicate that the ntnc mutant does not suffer increased damage to PSII (Fig. 1d). Assays were also performed using detached leaves in the presence and absence of lincomycin, which inhibits chloroplast protein synthesis and, therefore, impedes PSII repair. These experiments confirmed that there is hardly any difference in sensitivity to photoinhibition between mutant and wild type plants (Supporting Information Fig. S1).

Under high light conditions the increase in formation of superoxide anion radicals leads to enhanced production hydrogen peroxide and hydroxyl radicals (Foyer & Noctor, 2009; Li et al., 2009). Taking into account that NTRC is an important source of electrons for one of the most abundant chloroplast peroxidases, the 2-Cys Prx (Alkhalfioui et al., 2007; Moon et al., 2006; Perez-Ruiz et al., 2006), we examined the content of superoxide, peroxides and hydroxyl radicals in plants at their growth light intensity (120 μmol quanta m⁻² s⁻¹) and after exposure to high light (500 μmol quanta m⁻² s⁻¹) or darkness by an indirect spin trapping
assay (Heyno et al., 2009). To this end, leaves were immersed in 4-POBN/ethanol in the presence of Fe-EDTA, as catalyst for the Haber-Weiss reaction, while illuminated and electron paramagnetic resonance (EPR) spectra of the 4-POBN/α-hydroxyethyl adducts were recorded (Supporting Information Fig. S1). Notably, the ntrc plants had 40% higher content of peroxide-derived hydroxyl radicals than the corresponding wild type controls at growth light intensity, but not under strong illumination (Fig. 1e). These results on ROS content in the ntrc mutant are in agreement with a previous report (Lepisto et al., 2013). This would speak against a function of NTRC in peroxide detoxification at high irradiances.

The absence of NTRC causes drastically enhanced NPQ at lower light intensities

To analyse the function of NTRC in plant adaptation to changes of light intensity, we measured the ability of the ntrc mutant to adapt to a sudden increase in light intensity. Chlorophyll fluorescence was monitored during illumination with actinic light followed by darkness (Fig. 2a). The intensity of the actinic light in this experiment was about twice the growth light of these plants. The saturating light pulse-induced peaks of maximal fluorescence during illumination ($F_{m'}$) were smaller than the maximal fluorescence in the dark-adapted state ($F_m$) for both wild type and ntrc mutant plants (Fig. 2a). This shows that the mutant is capable of responding adequately to an increase in light intensity by inducing NPQ. During the subsequent dark period, fluorescence maxima were recovered in both wild type and mutant plants with similar kinetics (Fig. 2a).

At actinic light intensities lower than the growth light intensity NPQ is normally not observed, except shortly after the onset of light due to transient acidification of the thylakoid lumen (Kalituho et al., 2007). This was confirmed in the fluorescence curves recorded for the wild type plants (Fig. 2b). In contrast, the ntrc mutant displayed extensive NPQ lasting during the entire illumination period, as revealed by the dramatic reduction of the $F_{m'}$ values
This response is abnormal and distinguishes the plants lacking NTRC from wild type plants. In order to compare quantitatively the photosynthetic performance of these plants during induction and recovery, the respective quantum yields of NPQ, PSII photochemistry and non-regulated, or basal, energy dissipation (NO) were analysed according to Kramer et al. (2004b). At 75 μmol quanta m$^{-2}$ s$^{-1}$ actinic light the fraction of total energy dissipated through NPQ in the ntrc mutant plants was as high as 0.5 under steady state photosynthesis, whereas the corresponding value for wild type plants was close to 0.05 (Fig. 2c). Accordingly, the effective quantum yield of PSII in the light was more than four times lower in the mutant as compared to wild type plants, while NO did not differ (Fig. 2c). Chlorophyll fluorescence imaging showed that the high-NPQ phenotype is particularly pronounced in the young expanding leaves of the ntrc plants and that there is variegation within single leaves (Fig. 2d). Since some aspects of the ntrc mutant phenotype depend on the day length during growth (Lepisto et al., 2009; Lepisto et al., 2013; Perez-Ruiz et al., 2006), and these experiments were performed using plants grown under short-day conditions (10 h light and 14 h darkness), we also measured chlorophyll fluorescence of plants grown under long-day (16 h light/8 h dark) photoperiod. The ntrc mutant plants grown under long photoperiods also displayed exceptionally high and persisting NPQ at low light intensities (Supporting Information Fig. S2).

**Photosystem I in the ntrc mutant suffers donor side limitations in the light, but not lack of acceptors**

Once established that the lack of NTRC strongly influences the levels of NPQ and PSII activity, we proceeded to investigate the photosystem I (PSI) activity. It should be noted that various biosynthetic processes, such as starch synthesis, are deficient in the ntrc mutant (Michalska et al., 2009; Toivola et al., 2013) and, therefore, a shortage of electron acceptors for PSI might be expected. Furthermore, poor consumption of ATP associated with deficient
biosynthesis might lead to activation of NPQ. To test the possibility that the role of NTRC in starch synthesis could be related to the high NPQ in the ntrc mutant, we focused on ADP glucose pyrophosphorylase (AGPase), a key enzyme in this process, which was previously reported to be reduced and activated by NTRC (Michalska et al., 2009). An Arabidopsis mutant lacking the regulatory small subunit of the AGPase, aps1, is devoid of leaf starch and grows poorly, except under continuous light conditions (Ventriglia et al., 2008). However, aps1 shows a pattern of NPQ identical to wild type plants (Supporting Information Fig. S3), indicating that the role of NTRC in starch synthesis is not relevant for the increased energy dissipation.

Measurements of the PSI activity in wild type and ntrc plants based on P700 absorbance changes showed that the redox state of P700 during illumination is affected in plants lacking NTRC (Supporting Information Fig. S4). Analysis of the respective quantum yields of PSI activity, Y(I), donor side limitations, Y(ND), and acceptor side limitations, Y(NA), revealed that the ntrc mutant has low effective PSI quantum yield under growth light intensity (Fig. 3). This turned out to be the result of limitations on the donor side of PSI in the electron transport chain during illumination (Fig. 3). In contrast, wild type and ntrc plants were indistinguishable with respect to acceptor side limitations (Fig. 3). Thus, the absence of NTRC leads to a deficiency affecting specifically the supply of electrons to PSI in the light. However, the demand for electrons from PSI appears not to be altered.

**The influence of NTRC on NPQ is unrelated to its function as reductant for the 2-Cys peroxiredoxin**

NTRC is known to be an important electron donor to the chloroplast 2-Cys Prx (Perez-Ruiz et al., 2006; Pulido et al., 2010) and, since ROS are involved in the regulation of various responses to excess light (Li et al., 2009), we addressed the relevance of this function for the
control of NPQ. A severe knock-down mutant with minimal levels of 2-Cys Prx, the Δ2cp mutant (Pulido et al., 2010), was examined for its capacity to induce NPQ as a function of light intensity and compared to ntrc and wild type plants. In addition, mutant plants devoid of the chloroplast thioredoxin Trx x (Pulido et al., 2010) were included in these experiments, since Trx x has been proposed as an alternative electron donor for 2-Cys Prx (Collin et al., 2003). Plants were grown at four different light intensities and fluorescence induction-recovery curves were recorded using three actinic light intensities for each set of plants. The yields of NPQ for the Δ2cp and trxx mutants were similar to those of wild type plants (Fig. 4 and Supporting Information Fig. S5), except the Δ2cp plants grown at high light intensities, which had less NPQ under lower actinic light. In contrast, the yields of NPQ for the ntrc mutant were higher under all conditions tested (Fig. 4 and Supporting Information Fig. S5). This indicates that the effect that NTRC exerts on NPQ is not related to reduction of 2-Cys Prx.

To compare the photosynthetic performance of these plants, the relative rates of linear PET were analysed under gradually increasing intensities of actinic light up to 2000 μmol quanta m⁻² s⁻¹ (Fig. 5). Wild type plants adapted to higher light intensities had higher linear PET rates, but the electron transport for the ntrc mutant remained extremely slow at all intensities examined. The electron transport rates of plants deficient in Trx x and 2-Cys Prx were comparable to those of the wild type (Fig. 5). The yields of NPQ in these experiments were always higher for ntrc plants, particularly at lower light intensities (Supporting Information Fig. S6).

The absence of NTRC leads to alterations of the xanthophyll cycle

In order to establish the reason why plants lacking NTRC have higher levels of NPQ, different factors contributing to NPQ were analysed. The presence of the xanthophyll
zeaxanthin is a prerequisite for formation of the qE component of NPQ, while violaxanthin is inactive in this process (Jahns & Holzwarth, 2012; Jahns et al., 2009). Therefore, we determined the levels of the xanthophyll cycle pigments violaxanthin, antheraxanthin and zeaxanthin in wild type and ntrc mutant plants. Samples were collected at the end of the night and following the onset of light. As expected, wild type leaves displayed only a minor increase in the content of antheraxanthin and zeaxanthin 20 min after dawn, which reverted after 40 min of illumination (Fig. 6a). Then, as the light was turned off and after an additional 90 min of darkness, the original violaxanthin levels were nearly completely recovered in wild type leaves. In contrast, the ntrc mutant showed ten-fold higher levels of zeaxanthin throughout the 40 min of illumination (Fig. 6a). This implies that the high NPQ of the ntrc plants at normal light intensities is a direct consequence of excess zeaxanthin and antheraxanthin.

Since the relative content of xanthophyll cycle enzymes could affect the composition of xanthophylls, the levels of both VDE and ZE were analysed. However, VDE, the enzyme responsible for the conversion of violaxanthin to zeaxanthin, and ZE, which catalyses the conversion of zeaxanthin back to violaxanthin, were equally abundant in ntrc mutant and wild type plants (Fig. 6b). Hence, there is no evidence for differences in the content of xanthophyll cycle enzymes that could contribute to explaining the observed changes in xanthophyll composition.

The qE component of NPQ depends on ΔpH and, hence, is sensitive to nigericin (see (Ruban et al., 2012)). Treatment with nigericin in vivo reduced markedly the NPQ in ntrc plants (Fig. 7a). To test the short-term effect of thiol reduction in vivo in the ntrc mutant, detached leaves were treated with DTT, a known inhibitor of VDE (Yamamoto & Kamite, 1972), before fluorescence was recorded. Indeed, the yield of NPQ in the ntrc mutant leaves treated with DTT decreased prominently (Fig. 7b). The titration showed that incubation with 2 mM DTT...
could reduce the yield of NPQ to wild type levels (Fig. 7c). Concomitantly, the effective quantum yield of PSII in the light increased significantly in the mutant leaves upon incubation with DTT (Fig. 7d). Taken together, the effects of nigericin and DTT and the elevated zeaxanthin levels indicate that the energy-dependent quenching qE is permanently activated under low-light conditions in ntrc plants.

**The redox state of the xanthophyll cycle enzymes VDE and ZE in vivo**

It is conceivable that NTRC could modulate the activities of VDE and/or ZE through reduction of regulatory disulphides. VDE is known to be inhibited by disulphide reduction *in vitro* (Hall *et al*., 2010; Simionato *et al*., 2015; Yamamoto & Kamite, 1972), though the physiological meaning of this remains unknown. The *Arabidopsis* VDE contains twelve cysteines in the sequence of the mature protein and, yet, a single substitution of cysteine-72 to tyrosine is sufficient to abolish activity (Niyogi *et al*., 1998). ZE contains nine cysteine residues, five of which are conserved in orthologues from monocotyledons and four are also conserved in green algae (Supporting Information Fig. S7). Thiol-based redox regulation of ZE has not been previously reported, though ZE was found to be inhibited *in vivo* by photo-oxidative stress under high light conditions (Reinhold *et al*., 2008).

Thus, to test whether VDE is a redox-regulated enzyme, the redox state of VDE *in vivo* in the ntrc mutant and wild type plants was examined (Fig. 8a) taking advantage of the fact that reduced VDE form migrates slower on SDS-PAGE (Supporting Information Fig. S8). While the most oxidised form of VDE is somewhat more abundant in the ntrc mutant in the dark, there is no significant difference between the mutant and wild type VDE redox state in the light (Fig. 8a). This would rule out a possible role for NTRC in reductive inactivation of VDE in the light.
Purified recombinant ZE was found to be prone to formation of intermolecular disulphides under non-reducing conditions and purified NTRC is able to catalyse the reduction of these disulphides in vitro (Supporting Information Fig. S9). In contrast, analysis of the redox state of ZE in vivo using alkylation with methyl-polyethylene glycol₂₄-maleimide, MM(PEG), showed no changes in the wild type and the ntrc mutant (Fig. 8b). This indicates that ZE is not a target for redox regulation under normal light conditions.

Another potential target for redox control through NTRC is the γ-subunit of the thylakoid ATP synthase, which is known to mediate activation of ATP synthesis (see (Hisabori et al., 2013)). A possible deficiency in the reduction of the γ-subunit would lead to a build-up of the trans-thylakoid proton gradient and, thus, enhanced NPQ. In the dark this subunit is present in the oxidised form both in the wild type and the ntrc mutant (Fig. 8c). After only 1 min of illumination the γ-subunit is fully reduced in the wild type, while a minor amount remains oxidised in the ntrc mutant plants in the light (Fig. 8c).

**The trans-thylakoid ΔpH is larger in plants lacking NTRC under low light intensities**

Given that low pH of the thylakoid lumen is a key factor for formation of qE and an active ATP synthase is a prerequisite for consumption of protons, it is relevant to know whether NTRC is capable of activating this enzyme. Activity of the ATP synthase was assayed in vitro by measuring the loss of 9-aminoacridine fluorescence in thylakoid suspensions after addition of ATP. ATP hydrolysis leads to build-up of a proton gradient that can be measured since the protonated form of the fluorophore is sequestered in the thylakoid lumen, which leads to quenching (Schuldiner et al., 1972). NH₄Cl functions as an uncoupler and promotes recovery of fluorescence. The addition of NTRC together with NADPH stimulated ATP hydrolysis and induction of ΔpH, whereas NADPH alone was hardly able to activate ATP hydrolysis (Fig. 9a). Addition of DTT was used as positive control. In order to assess
quantitatively these measurements, quenching of 9-aminoacridine fluorescence was compared to that observed in illuminated thylakoids using methyl viologen as electron acceptor, which yields a large ΔpH and high levels of fluorescence quenching (Fig. 9b).

The trans-thylakoid proton motive force, Δψ and ΔpH, can be measured in vivo using the electrochromic shift (ECS) technique (Takizawa et al., 2007). Thus, ΔpH in wild type and mutant plants was compared at different light intensities using ECS (Fig. 9c). At intensities below 200 μmol quanta m⁻² s⁻¹ the ΔpH was consistently larger in ntrc mutant plants, indicating that a low luminal pH promotes high qE in these plants.

Cyclic electron flow (CEF) around PSI could lead to acidification of the thylakoid lumen and concomitant induction of qE (Livingston et al., 2010). Reduction of the plastoquinone (PQ) pool dependent on the NADPH dehydrogenase (NDH) may form part of a CEF pathway and can be measured as transient post-illumination chlorophyll fluorescence (Shikanai et al., 1998). Therefore, we compared the induction of post-illumination fluorescence in wild type and ntrc plants. Whereas in wild type plants the rise of chlorophyll fluorescence after turning off the actinic light was only transitory, in the ntrc mutant this fluorescence remained high (Fig. 9d). This shows that the contribution of NDH activity to reduction of the PQ pool is higher in the ntrc mutant.

**Knocking out the psbs gene improves growth of the ntrc mutant**

The PsbS protein is a subunit of PSII required for induction of the qE (Li et al., 2000), and transgenic plants overexpressing PsbS have higher NPQ (Li et al., 2002; Roach & Krieger-Liszkay, 2012). Therefore, the content of PsbS was examined in the ntrc mutant plants and compared to the wild type. The levels of the PsbS protein were similar in mutant and wild type plants (Fig. 10a).
Since the PsbS protein is necessary for qE, a double mutant lacking both NTRC and PsbS presumably would be devoid of qE and, thus, would provide useful insight into the nature of the NPQ of the ntrc mutant. Furthermore, such a double mutant would permit establishing the specific impact of qE on the ntrc mutant phenotype. The Arabidopsis ntrc-psbs double mutant was obtained by manual crossing of the corresponding single mutants (Supporting Information Fig. S10), and the absence of the two proteins NTRC and PsbS was confirmed (Fig. 10b). Chlorophyll fluorescence imaging showed that the ntrc-psbs mutant has low NPQ similar to the psbs single mutant (Fig. 10c). In addition, the effective PSII quantum yield was higher in ntrc-psbs than in the ntrc mutant (Fig. 10c). When grown at moderate light intensity (120 µmol quanta m\(^{-2}\) s\(^{-1}\)) for ten weeks under a short-day photoperiod, the double mutant plants were larger than ntrc plants (Fig. 10d and e). The rosette weight and chlorophyll content of ntrc-psbs mutant plants were twice as high as compared to those of the single ntrc mutant (Fig. 10f and g).

Measurements of chlorophyll fluorescence showed that the effective PSII quantum yield was higher in plants lacking both NTRC and PsbS than in plants lacking NTRC alone (Fig. 11a). Indeed, qE was missing in both the psbs mutant and in the ntrc-psbs double mutant (Fig.11b). The linear electron transport rates at growth light and low light intensities were significantly higher in the ntrc-psbs double mutant than in the ntrc mutant (Fig. 11c). However, the double mutant is hypersensitive to high irradiances similarly to the psbs single mutant (Fig. 11c). The PSI activity was partially restored in the ntrc-psbs plants (Fig. 11d), due to the relief of the donor side limitations observed in the ntrc plants (Fig. 11e). A slight increase in acceptor side limitations (Fig. 11f) counteracts the complete rescue of PSI activity in the double mutant. In summary, abolishing qE in plants lacking NTRC stimulates the linear PET and improves photosynthesis under low to medium light intensities.
DISCUSSION

Short- and long-term acclimation of plants to high light intensities is crucial for their survival during excess excitation to avoid toxic ROS levels and to protect the photosystems I and II (Dietz, 2015; Spetea et al., 2014; Tikkanen et al., 2012). Nevertheless, the success of a plant also depends on the capacity to restrict the induction of acclimation mechanisms, such as qE, to situations of need in order to avoid wasting the energy absorbed. Given the possible redox control and implication of thioredoxins in protection mechanisms against excess light (Li et al., 2009) we have investigated the role of the chloroplast NADPH-dependent thioredoxin reductase NTRC.

In this study we found that plants lacking NTRC tolerate light intensities up to 1000 µmol quanta m\(^{-2}\) s\(^{-1}\). This is in agreement with a previous study by (Toivola et al., 2013), where growth of the ntrc mutant at 600 µmol quanta m\(^{-2}\) s\(^{-1}\) was found not to be further compromised, and suggests that NTRC is not involved primarily in molecular mechanisms controlling long-term acclimation to high light intensities. A complete knockout mutant for the chloroplast 2-Cys Prx was recently reported to be hypersensitive to photoinhibition upon a transfer from low to high irradiances (Awad et al., 2015). Since the 2-Cys Prx is a substrate for NTRC (Alkhalfioui et al., 2007; Moon et al., 2006; Perez-Ruiz et al., 2006; Puerto-Galan et al., 2015), more photoinhibition in plants lacking NTRC might be expected. However, ntrc plants grown to adult stage at 120 µmol quanta m\(^{-2}\) s\(^{-1}\) and transferred to 800 µmol quanta m\(^{-2}\) s\(^{-1}\) light intensity did not show exacerbated photoinhibition. This indicates that NTRC is not required for short-term acclimation to high light and suggests that the 2-Cys Prx benefits from electron donors other than NTRC at high light intensities.

While exploring the light responses of the ntrc mutant a new distinctive feature was uncovered. At light intensities equal to the irradiance applied during growth, or even lower
light intensities, these plants present drastically elevated levels of NPQ, which are retained throughout the illumination period. This characteristic is unique to the plants lacking NTRC and is not found in wild type plants or the Δ2cp and trxx mutants. To understand the influence of NTRC on NPQ, the factors contributing to different elements of dissipation of the light energy should be considered. NPQ comprises at least three components: qT, qI and qE. The qT component is the quenching resulting from state transitions, which is particularly important in algae, and qI is related to photoinhibition (Baker, 2008; Szabo et al., 2005). The major component in plant leaves is qE, which is also referred to as ΔpH-dependent quenching (Szabo et al., 2005) or energy-dependent quenching (Baker, 2008; Kramer et al., 2004a). Both a trans-thylakoid pH gradient and the xanthophyll zeaxanthin must be present in order to produce qE. The trans-thylakoid pH gradient alone was previously proven not to be sufficient to maintain NPQ in the absence of zeaxanthin (Gilmore et al., 1994) and constitutively high concentrations of zeaxanthin in a ZE knockout mutant, npq2-1, were not sufficient to produce NPQ in the absence of ΔpH (Niyogi et al., 1998). The qE component of NPQ also depends on the presence of the PSII subunit PsbS and the content of this protein determines the extent of maximal NPQ (Li et al., 2002). In this study, we found that the elevated NPQ at low light intensities in plants lacking NTRC has fast dark-light induction and light-dark relaxation kinetics, is inhibited by nigericin and correlates with high levels of zeaxanthin. Furthermore, DTT inhibited NPQ in vivo in ntrc plants with a concomitant recovery of effective PSII quantum yield, which also demonstrated the integrity of the photosynthetic machinery in plants lacking NTRC. All these data indicate that qE is affected specifically in ntrc plants. This was eventually demonstrated using double mutant plants devoid of both NTRC and PsbS, which have very low NPQ and lack the characteristic initial peak of qE observed in wild type plants upon a dark-light transition.
The two enzymes that participate in the xanthophyll cycle, VDE and ZE, were present in equal amounts in the ntrc mutant and in wild type plants. Hence, the large differences regarding xanthophyll composition during illumination must be due to alterations of the activities of either or both enzymes in the mutant. VDE, which is located in the thylakoid lumen, is activated by the decrease in luminal pH that occurs upon a transition from darkness to light (Bratt et al., 1995; Jahns et al., 2009; Pfundel & Dilley, 1993). Recently, the thylakoid lumen has attracted interest as a compartment where a number of regulatory proteins reside, many of which are plausible targets for redox regulation (Buchanan & Luan, 2005; Hall et al., 2010; Jarvi et al., 2013). Notably, VDE is a thioredoxin target in vitro and inhibited by disulphide reduction (Hall et al., 2010; Simionato et al., 2015). A putative interaction between NTRC, located in the chloroplast stroma, and VDE might occur through a transmembrane pathway for disulphide-dithiol exchange, such as the DsbD-like system that involves HCF164 and CcdA (Karamoko et al., 2013; Motohashi & Hisabori, 2006; Motohashi & Hisabori, 2010). Thus, we tested whether NTRC is responsible for possible reduction and inactivation of VDE. However, we could not observe differences in the redox state of VDE in the light between wt and ntrc plants that would explain the high zeaxanthin levels in the latter.

Regarding ZE, there are previous reports indicating that this enzyme is inactivated by oxidation. Treatment of plants with cadmium ions results in inhibition of its activity (Latowski et al., 2005), and ZE is also inhibited in vivo by photo-oxidative stress under high light conditions (Reinhold et al., 2008). We found that ZE is prone to thiol oxidation, which causes the protein to form multimeric aggregates of high molecular mass. NTRC is able to reduce these ZE aggregates in vitro, suggesting a function in the activation of zeaxanthin epoxidation converting this xanthophyll back to violaxanthin, which would lead to cessation of the NPQ. Nevertheless, under normal light conditions in vivo the redox state of ZE does
not change in either wt or ntrc plants and could therefore not contribute to explaining the high zeaxanthin levels in the ntrc mutant.

Inhibition of the high NPQ observed in ntrc plants in vivo by nigericin draws the attention to the significance of ΔpH in this process. The electrochromic shift experiments demonstrated that the trans-thylakoid ΔpH is larger under low and medium light intensities in plants lacking NTRC. This is most likely the cause for the elevated qE and is sufficient to explain the high zeaxanthin levels found in the ntrc mutant under moderate light intensities as a consequence of activation of VDE.

NTRC has been reported to reduce and activate AGPase, a key enzyme in starch synthesis (Michalska et al., 2009). Moreover, NTRC functions in the synthesis of chlorophyll (Perez-Ruiz et al., 2014; Richter et al., 2013) and in the shikimate pathway (Lepisto et al., 2009). Thus, it seemed plausible that deficiencies in biosynthetic pathways would lead to lack of electron acceptors, poor utilisation of ATP and, consequently, increased acidification of the thylakoid lumen during illumination. This in turn would result in the high and stable levels of NPQ observed. However, a mutant lacking the regulatory small subunit of the AGPase, aps1 (Ventriglia et al., 2008) that is devoid of leaf starch, shows a pattern of NPQ identical to wild type plants. Moreover, our determinations of PSI activity based on P700 absorbance show that the ntrc mutant does not suffer more limitations at the acceptor side of PSI than the wild type. In contrast, whereas the PSI donor side limitations are gradually relieved after a dark-light transition in the wild type, these limitations persist in the plants lacking NTRC during the entire period of illumination. These results speak against the idea that limited activation of chloroplast biosynthetic processes would contribute to the high-qE phenotype of ntrc plants.
Wild type plants transferred from darkness to weak or moderate light display a brief initial peak of NPQ that relaxes within less than 2 min, as protons accumulating in the thylakoid lumen are consumed through ATP synthesis. This phenomenon has been suggested to reflect the light-induced activation of the ATP synthase (Kalituho et al., 2007). An attractive hypothesis would be that NADPH, the substrate for NTRC, might regulate ATP synthesis through NTRC-mediated reduction of the ATP synthase γ-subunit, which is well known to be redox-regulated (Hisabori et al., 2013). Indeed, plants with a defective chloroplast ATP synthase have high constitutive NPQ at low light intensities (Dal Bosco et al., 2004). The ATPase was activated by NTRC and NADPH in vitro, indicating a direct effect of NTRC on the activation of the ATP synthase. Light-induced reduction of the ATP synthase γ-subunit in vivo proved to be somewhat less efficient in the ntrc mutant than in wt plants. However, it is uncertain whether this alone would account for the increase in ΔpH observed in the ntrc mutant and it is obvious that there are other enzymes more efficient than NTRC in catalysing this reaction in vivo.

The distribution of photosynthetic electron flow between LEF and CEF is crucial for balancing the synthesis of ATP and NADPH and for acclimation of plants to fluctuating light conditions (Allahverdiyeva et al., 2015; Kramer et al., 2004a, Foyer et al., 2012). Interestingly, measurements of constitutive high qE under moderate light conditions have been applied previously to screen for high CEF (hcef) mutants, which have higher rates of proton translocation and larger proton motive force (Livingston et al., 2010). The thioredoxin Trx m4 has recently been shown in vivo to inhibit CEF dependent on the NDH (Courteille et al., 2013). However, mutants lacking Trx m4 did not show enhanced NPQ or lower effective PSII quantum yield in the light (Courteille et al., 2013). The ntrc mutant displays elevated and stable post-illumination chlorophyll fluorescence indicative of an enhanced NDH activity and possible high CEF. Nevertheless, the limitation of electron donation to PSI in the ntrc
mutant in comparison to wild type plants indicates that there is not more CEF in the mutant. Hence, this mechanism cannot contribute to the larger ΔpH found in the ntrc plants. The precise mechanism of action of NTRC in down-regulation of qE remains to be determined and might involve one or several means to limit influx and to enhance efflux of protons from the thylakoid lumen, such as inhibition of NDH and the PGR5/PGRL1 pathways or activation of the KEA3 antiporter. Another possibility is that reduction of the ATP synthase γ-subunit might not be sufficient to fully activate this enzyme and that the simultaneous reduction of other subunits, perhaps catalysed by NTRC, would be required, since the α-, β-, δ- and ε-subunits are also potential thioredoxin targets (Lindahl & Kieselbach, 2009).

In summary, our results show that plants lacking NTRC accumulate more protons in the thylakoid lumen at low and moderate light intensities, which results in rapid synthesis of zeaxanthin and high levels of dissipation of the light energy absorbed by LHCII. This, in turn, leads to low effective quantum yield of PSII and, hence, to shortage of electron donors for PSI. A further implication of this enhanced dissipation and slow electron transport is that less energy is available for biosynthesis. The ntrc mutant was previously reported to have lower rates of carbon dioxide fixation, particularly at lower light intensities (Perez-Ruiz et al., 2006). Hence, the low starch content in ntrc plants (Lepisto et al., 2013; Michalska et al., 2009; Toivola et al., 2013) may also reflect the modest production of photosynthates. Indeed, this view affords an explanation to several aspects of the ntrc mutant phenotype. Plants that are continuously starved for light energy should be undersized and grow slowly (Lepisto et al., 2009; Perez-Ruiz et al., 2006; Serrato et al., 2004). Furthermore, longer days should promote growth of such plants by adding to the quantity of total energy absorbed and this is also true for plants lacking NTRC (Lepisto et al., 2009; Lepisto et al., 2013; Perez-Ruiz et al., 2006). Here we show that more electrons can be forced through the LEF pathway in
plants lacking both NTRC and PsbS than in plants lacking NTRC alone. This rescues part of the ntrc phenotype with respect to growth and pigmentation.

In conclusion, NTRC is required for down-regulation of qE-mediated energy dissipation and stimulation of LEF, which is essential for plant growth, particularly during early leaf development. NTRC is not involved in long- or short-term high light tolerance, nor is it implied in peroxide detoxification at high light intensities. On the contrary, plants lacking NTRC are over-protected against light even at low and moderate irradiances leading to a state of permanent starvation for light energy.

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Figure 1. Tolerance of Arabidopsis wild type and ntrc knockout mutant plants to different irradiances. Plants were grown under a photoperiod of 10 h light/14 h darkness at 50, 120, 600, or 1000 μmol quanta m⁻² s⁻¹ intensity. (a) Wild type (wt) and ntrc mutant plants grown for 5 weeks at the respective light intensities. (b) Fresh weight of rosettes from 5-weeks old plants. Each data point is the mean of 10 to 20 plants and SDs are presented as error bars. (c) Content of total chlorophylls a and b determined in leaf discs from leaves of 5-weeks old plants. Each data point is the mean of discs from 10 plants ±SDs. (d) Five-weeks old plants grown at 120 μmol quanta m⁻² s⁻¹ light intensity were transferred to an intensity of 800 μmol quanta m⁻² s⁻¹. Time 0 corresponds to dawn of the last day in normal light. Each data point is the mean of the $F_v/F_m$ values measured from 12 plants (3 leaves from each) and SDs are presented as error bars. (E) Relative content of peroxide-derived hydroxyl radicals based on EPR signals from wild type and ntrc mutant leaves in the dark, D, growth light, GL (120 μmol quanta m⁻² s⁻¹), and in high light, HL (500 μmol quanta m⁻² s⁻¹). The presence of hydroxyl radicals was shown by indirect spin trapping with 4-POBN/ethanol in the presence of Fe-EDTA. The signal from the ntrc mutant is expressed as % of the corresponding wt control sample ± SE. Darkness, n = 5; growth light, n = 8; high light, n = 4. Significantly different values according to Student’s t-test are marked with different letters (p < 0.05).
Figure 2. Chlorophyll fluorescence in wild type and ntrc mutant plants. Fluorescence was measured with a pulse-amplitude modulation fluorometer using attached leaves of 5-weeks old plants grown at 120 μmol quanta m⁻² s⁻¹ light intensity. Following 30 min dark incubation and determination of $F_0$ and $F_m$, the actinic light was turned on and saturating pulses were applied every 60 s. After 8 min illumination measurements were continued for another 10 min in the dark. White and black bars below graphs indicate periods of illumination with actinic light and darkness, respectively. (b) Induction and recovery curves with 278 μmol quanta m⁻² s⁻¹ intensity actinic light. (b) Induction and recovery curves applying 75 μmol quanta m⁻² s⁻¹ intensity actinic light. (c) Quantum yields of NPQ, Y(NPQ), PSII photochemistry, Y(II), and non-regulated energy dissipation, Y(NO), based on experiments such as in (b). Y(NPQ), open circles; Y(II), closed squares; Y(NO), closed triangles. Each data point is the mean of the values from 4 plants and SDs are indicated by error bars. (d) False-colour images representing $F_v/F_m$ in wild type and ntrc mutant plants and the respective Y(NPQ) and Y(II) after 3 min of 80 μmol quanta m⁻² s⁻¹ intensity illumination. Images of ntrc plants have been enlarged to facilitate viewing.
Figure 3. Activity of PSI in wild type and ntrc mutant plants. The redox state of the PSI reaction centre P700 was monitored through the changes in absorbance at 830 nm versus 875 nm. Five-weeks old plants grown at 120 μmol quanta m² s⁻¹ light intensity were kept in the dark for 30 min prior to the measurements. Following the initial determination of maximal oxidation of P700 the actinic light at an intensity of 126 μmol quanta m² s⁻¹ was turned on and saturating pulses were applied every 20 s. After 5 min the actinic light was switched off and measurements were continued for another 5 min. The quantum yields of PSI photochemistry, Y(I), donor side limitations Y(ND) and acceptor side limitations Y(NA) are based on saturating pulse analyses. White and black bars below graphs indicate periods of illumination with actinic light and darkness, respectively. Each data point is the mean of the values from 6 plants and SDs are shown as error bars.
Figure 4. NPQ in wild type and ntrc, Δ2cp and trxx mutant plants grown under different irradiances. Chlorophyll fluorescence was measured using attached leaves of five-weeks old plants grown at 50, 120 and 600 μmol quanta m$^{-2}$ s$^{-1}$. Plants were kept in the dark for 30 min prior to measurements. Three different actinic light intensities of 18, 75 and 278 μmol quanta m$^{-2}$ s$^{-1}$ were used and saturating pulses were applied every 60 s. Quantum yields of NPQ, Y(NPQ), were calculated from saturating pulse analyses. White and black bars below graphs indicate periods of illumination with actinic light and darkness, respectively. Each data point is the mean of the Y(NPQ) from 4 plants and SDs are indicated by error bars.
Figure 5. Linear photosynthetic electron transport in wild type and ntrc, Δ2cp and trxx mutant plants grown under different irradiances. Chlorophyll fluorescence was measured with a pulse-amplitude modulation fluorometer using attached leaves of plants grown at 50, 120, 600 and 1000 μmol quanta m⁻² s⁻¹ for 5 weeks. Relative electron transport rates of PSII, ETR(II), were determined during stepwise increasing photosynthetically active radiation (PAR). Each data point is the mean of the ETR(II) from 8 plants and SDs are presented as error bars.
Figure 6. Levels of xanthophyll cycle pigments and VDE and ZE proteins in wild type and ntrc mutant plants. (a) Leaves of 5-weeks old plants grown at 120 μmol quanta m$^{-2}$ s$^{-1}$ light intensity were harvested just before the onset of light in the morning (0 min) and after 20 and 40 min of illumination with light at 120 μmol quanta m$^{-2}$ s$^{-1}$ intensity. Thereafter, plants were kept in the dark for another 90 min and samples were taken again (130 min). Pigments were extracted from the leaves and the levels of violaxanthin (Vx), antheraxanthin (Ax) and zeaxanthin (Zx) were determined. The amounts of xanthophylls are expressed as μg per g fresh weight of leaves. Values are the mean ± SD of the xanthophyll levels determined in leaves from 3 plants. (b) Proteins extracted from the leaves were analysed by protein gel immunoblot using antibodies against VDE and ZE. Numbers above each lane indicate the quantity of chlorophyll in μg.
Figure 7. Effects of nigericin and DTT in vivo on the NPQ and PSII effective quantum yield of wild type and ntrc mutant plants. Leaves from 5-weeks old plants grown at 120 μmol quanta m$^{-2}$ s$^{-1}$ light intensity were floated on 100 μm nigericin (a) or DTT solutions (b) in the dark for three hours. Chlorophyll fluorescence was measured during 4 min illumination with actinic light at 75 μmol quanta m$^{-2}$ s$^{-1}$ intensity followed by 4 min darkness and the yields of NPQ were determined. (c) Yields of NPQ in DTT-treated leaves after 3 min illumination. (d) PSII effective quantum yields in DTT-treated leaves after 3 min illumination. Each data point is the mean of the Y(NPQ) or Y(II) from 6 leaves and SDs are presented as error bars. Significantly different values according to Tukey (ANOVA) in (d) are marked with different letters ($p < 0.01$).
Figure 8. The redox state in vivo of the xanthophyll cycle enzymes, VDE and ZE, and of the ATP synthase γ subunit. Total leaf proteins from wild type and ntrc mutant plants in the dark or after 1 hour in the light at 120 μmol quanta m⁻² s⁻¹ intensity were extracted in the presence of 10% trichloroacetic acid to preserve the thiol redox state. (a) Proteins were solubilised for SDS-PAGE in the presence of 40 mM N-ethyl maleimide to avoid oxidation of thiols and electrophoresed in the presence or absence of DTT. VDE was detected by immunoblot analysis. D, dark; L, light; M, molecular mass protein standard. (b) Protein thiols were alkylated with 10 mM MM(PEG) during solubilisation for SDS-PAGE and electrophoresed in the presence of DTT. ZE was detected by immunoblot. The left lanes of each gel contain control samples without alkylation. D, dark; L, light. (c) Samples were taken from leaves in the dark and after 1, 5 and 10 min of illumination. Protein thiols were alkylated with MM(PEG) prior to electrophoresis and the γ subunit of the ATP synthase was detected by immunoblot.
Figure 9. The effect of NTRC on the trans-thylakoid \( \Delta pH \) in vitro and in vivo

(a) Quenching of 9-aminoacridine fluorescence as indicator of \( \Delta pH \) formed during ATP hydrolysis. Isolated thylakoids were incubated for 5 min either with 100 \( \mu M \) NADPH and 5 \( \mu M \) NTRC, NADPH alone or 5 mM DTT. Grey and black arrows indicate the additions of 50 \( \mu M \) ATP and 20 mM \( NH_4Cl \), respectively. (b) Quantitative comparison of 9-aminoacridine fluorescence quenching from measurements such as those in (a). Values were normalised to those obtained from illuminated thylakoids using 100 \( \mu M \) methyl viologen as electron acceptor, which was taken as 100% quenching. Each data point is the mean from 4 to 5 measurements \( \pm \)SD. (c) Wild type and \( ntrc \) plants were grown for five weeks at 120 \( \mu mol \) quanta m\(^{-2}\) s\(^{-1}\) light intensity. Electrochromic shift (ECS) signals and dark relaxation were measured at 520 nm after 15 min illumination with actinic light at intensities between 12 and 940 \( \mu mol \) quanta m\(^{-2}\) s\(^{-1}\). The component ECS\(_{inv}\) is proportional to the \( \Delta pH \). The lower panel is a close-up of the range at lower light intensities. Each data point is the mean of 4 leaves \( \pm \)SD (d) Chlorophyll fluorescence induction of wild type and \( ntrc \) plants was measured in the dark after illumination for 5 min with 126 \( \mu mol \) quanta m\(^{-2}\) s\(^{-1}\) intensity actinic light and typical traces are shown.
Figure 10. Phenotypic analysis of the ntrc-psbs double knockout mutant. (a) Proteins extracted from wt and ntrc leaves were analysed by protein gel immunoblot using antibodies against PsbS. Numbers above each lane indicate the quantity of chlorophyll in µg. (b) Leaf proteins of double and single mutants were examined by protein gel immunoblot using antibodies against PsbS and NTRC. Samples corresponding to 2 µg chlorophyll were loaded in each lane. (c) Plants were grown for 5 weeks at 120 µmol quanta m\(^{-2}\) s\(^{-1}\) light intensity under a photoperiod of 8 h light/16 h darkness. False-colour images representing \(F_v/F_m\) in wild type (wt) and mutant plants and the respective Y(NPQ) and Y(II) after 1 and 3 min of 80 µmol quanta m\(^{-2}\) s\(^{-1}\) intensity actinic light. Images of ntrc plants have been enlarged to facilitate viewing. (d) Plants grown for 10 weeks at 120 µmol quanta m\(^{-2}\) s\(^{-1}\) light intensity under a photoperiod of 8 h light/16 h darkness. (e) The largest rosette leaves from representative 10-weeks old plants of wild type, ntrc and the ntrc-psbs mutant. (f) Fresh weight of rosettes from 10-weeks old plants. Each value is the mean of 8 to 12 plants and SDs are presented as error bars. (g) Content of total chlorophylls \(a\) and \(b\) determined in leaf discs from 10-weeks old plants. Each data point is the mean of discs from 12 plants and SDs are shown as error bars. Significantly different values according to Student’s t-test are indicated with different letters (p < 0.01).
Figure 11. Photosynthetic performance of the \textit{ntrc-psbs} double mutant. Chlorophyll fluorescence and P700 absorbance were measured with a pulse-amplitude modulation fluorometer using plants grown for five weeks at 120 \(\mu\)mol quanta m\(^{-2}\) s\(^{-1}\) light intensity. Prior to all measurements the plants were kept in the dark for 30 min. For chlorophyll fluorescence, 75 \(\mu\)mol quanta m\(^{-2}\) s\(^{-1}\) actinic light was turned on and saturating pulses were applied every 60 s. After 6 min the actinic light was switched off and measurements were continued for another 6 min. White and black bars indicate periods of illumination with actinic light and darkness, respectively. (a) Quantum yields of PSII photochemistry, \(Y(\text{II})\). (b) Quantum yields of NPQ, \(Y(\text{NPQ})\). (c) Relative linear electron transport rates ETR(II) as a function of actinic light intensity. Values are the means of 6 measurements \(\pm\) SD.

For measurements of P700 absorbance actinic light at an intensity of 126 \(\mu\)mol quanta m\(^{-2}\) s\(^{-1}\) was used and saturating pulses were applied every 20 s. The quantum yields of (d) PSI photochemistry, \(Y(\text{I})\); (e) donor side limitations, \(Y(\text{ND})\); (f) acceptor side limitations \(Y(\text{NA})\) after 3 min of illumination are displayed. Significantly different values according to Tukey ANOVA are marked with different letters (\(p < 0.05\)).
SUMMARY

Excess excitation and the means by which plants respond to defend themselves against light-induced damage have been extensively studied and the importance of qE is well documented. Nevertheless, the success of a plant also depends on the capacity to restrict the induction of acclimation mechanisms, such as qE, in order to avoid wasting the energy absorbed. Given the possible redox control and implication of thioredoxins in protection mechanisms against excess light, we have investigated the role of the chloroplast NADPH-dependent thioredoxin reductase NTRC. This enzyme has been previously implied in peroxide detoxification and abiotic stress tolerance.

Surprisingly, NTRC proved to be essential, not for induction of protection mechanisms under strong irradiance, but for maintenance of optimal photosynthetic efficiency under normal light conditions and down-regulation of qE through control of the trans-thylakoid pH gradient.