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Original Article

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 photooxidative 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 utilization 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 have 10-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 a 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 with plants lacking *ntrc* alone. These data uncover a new role for NTRC in the control of photosynthetic yield.

Key-words: high-light acclimation; oxidative stress; photosynthesis; redox signalling; thioredoxin; thylakoid.

INTRODUCTION

In plants, not only are reactive oxygen species (ROS) 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, as molecular oxygen acts as a sink for PET-derived electrons (Takahashi & Murata 2008; Li *et al*. 2009). Adaptation to high light intensities is essential for plant survival and involves changes of gene expression and enzymatic activities in order to optimize photosynthesis and to avoid toxic ROS levels (Li *et al*. 2009; Foyer *et al*. 2012; Tikkanen *et al*. 2012; Spetea *et al*. 2014; Dietz 2015).

Photosystem II (PSII) is intrinsically sensitive to strong irradiance, as 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* synthesized copy in a costly repair process (Nixon *et al*. 2010; Tyystjarvi 2013; Jarvi *et al*. 2015). One of the protection mechanisms of plants against excess light involves thermal dissipation of the light energy captured by light-harvesting complex II (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) (Szabo *et al*. 2005; Jahns *et al*. 2009; Jahns & Holzwarth 2012; Ruban *et al*. 2012). VDE is located in the thylakoid lumen and is regulated by pH, the availability of ascorbate and, possibly, by its redox state (Pfundel & Dilley 1993; Bratt *et al*. 1995; Hall *et al*. 2010; Simionato *et al*. 2015). The effectiveness of qE in preventing production of singlet

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oxygen and photoinhibition has been confirmed in studies of an *Arabidopsis* mutant lacking PsbS (*npq4*) and in transgenic *Arabidopsis* overexpressing 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, as 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 *et al*. 2009; Jahns & Holzwarth 2012) and, hence, to down-regulate the protection of plants against excess light in order to allow for a more efficient utilization 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 (Munekage *et al*. 2002; DalCorso *et al*. 2008; Suorsa *et al*. 2012). Furthermore, plants devoid of the thylakoid H^+/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 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 (Serrato *et al*. 2004; Perez-Ruiz *et al*. 2006). NTRC constitutes a functional homodimer, where the NTR domain of one subunit reduces the active site of the thioredoxin domain in the other subunit (Perez-Ruiz & Cejudo 2009; Bernal-Bayard *et al*. 2012). NTRC is a very efficient reductant for the thiol-dependent peroxidase 2-Cys peroxiredoxin (2-Cys Prx) (Moon *et al*. 2006; Perez-Ruiz *et al*. 2006; Alkhalfioui *et al*. 2007; 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 (Serrato *et al*. 2004; Perez-Ruiz *et al*. 2006; Lepisto *et al*. 2009), while plants overexpressing NTRC are larger than wild-type (wt) plants (Toivola *et al*. 2013). This phenotype of the *ntrc* mutant is particularly pronounced under short-day photoperiods (Perez-Ruiz *et al*. 2006; Lepisto *et al*. 2009; Lepisto *et al*. 2013). 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 with wt plants (Serrato *et al*. 2004; Perez-Ruiz *et al*. 2006).

The possible implication of chloroplast redox enzymes, for example 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 (Serrato *et al*. 2004; Perez-Ruiz *et al*. 2006) 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 *ntrc–psbs* mutant was obtained by manually crossing the *psbs* mutant with the *ntrc* 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, 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).

The redox state of photosystem I (PSI) P700 was monitored by following the changes in absorbance of 30 min dark-adapted plants at 830 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 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 and Krieger-Liszkay (2012). Pieces of 15–20 mg of leaf per sample were infiltrated with a solution containing 50 mM 4-POBN, 4% ethanol, 2.5 μM Fe-ethylenediaminetetraacetic acid (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. Electron paramagnetic resonance (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; and number of scans, 2. Signals were normalized 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′-GAGGATCCGTTGATGCACTTAAA-3′ (BamH1) and reverse 5′-AACTGCAGCCTGACCTTCCTGAT-3′ (PstI), and ZE, forward 5′-AAGGATCCGCGGCGACG-3′ (BamH1) and reverse 5′-GCAAGCTTAGCTGTCTGAAGTAA-3′ (HindIII).

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 by 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 sodium dodecyl sulphate–polyacrylamide gel electrophoresis (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 visualized with enhanced chemiluminescence (ECL) using the Immobilon™ Western Chemiluminescent horseradish peroxidase (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 wt plants were diluted to a chlorophyll concentration of 5 μg mL^{-1}. 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 NH_4Cl (20 mM) were added at the indicated time points. For maximal fluorescence quenching, thylakoids were illuminated with red light at 500 μmol quanta m^{-2} s^{-1} intensity in the presence of 100 μM methyl viologen.

Measurement of ΔpH *in vivo* using the electrochromic shift 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 m^{-2} 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 normalization.

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 high-performance liquid chromatography (HPLC) as described by Hornero-Mendez *et al.* (2000). All operations were carried out under dimmed light to prevent isomerization and photodegradation 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 wt 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 wt plants even at higher light intensities (Fig. 1b). The *ntrc* rosettes weighed 12 ± 2.7 and 8.6 ± 1.9% of wt rosettes at 600

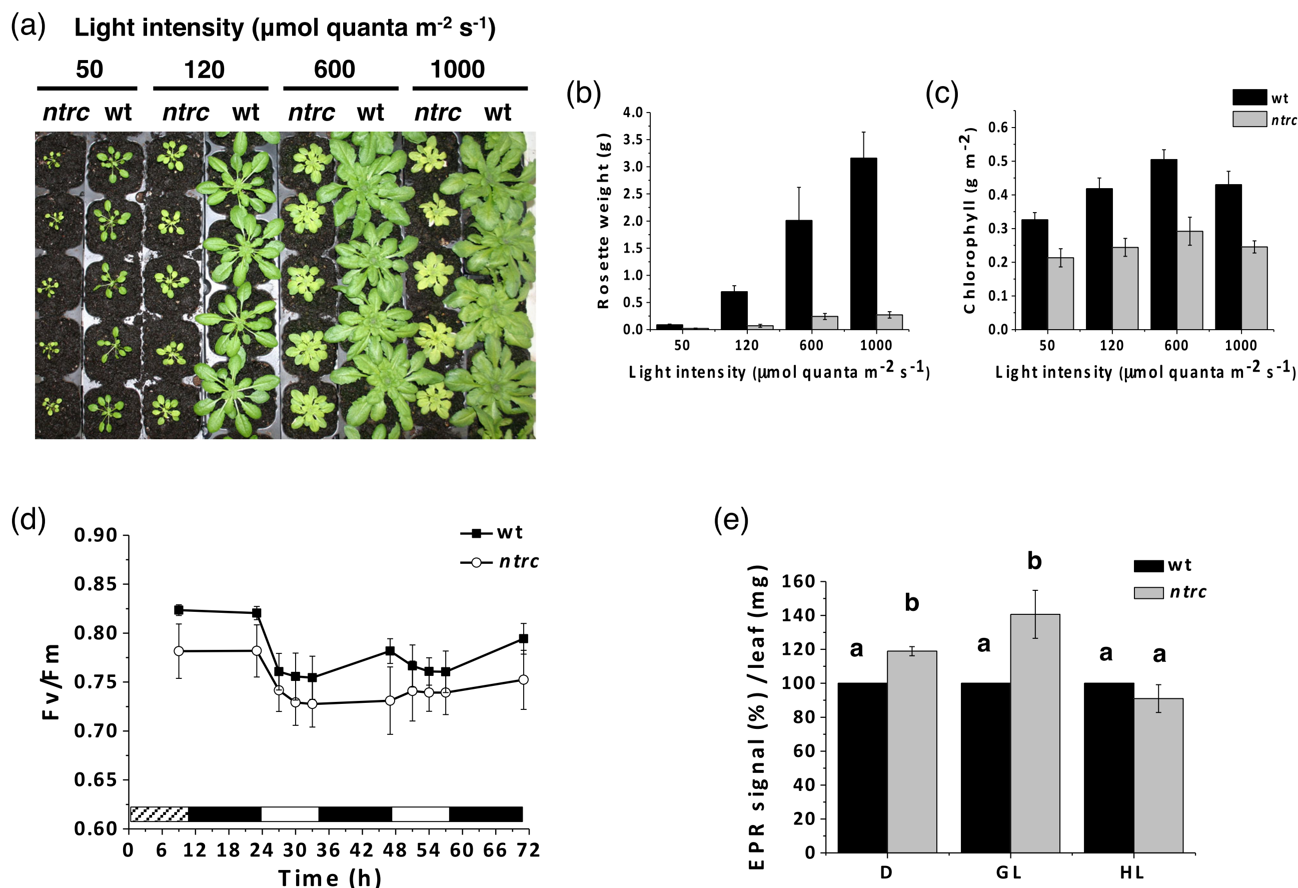

Figure 1. Tolerance of *Arabidopsis* wild-type (wt) and *ntrc* knockout mutant plants to different irradiances. Plants were grown under a photoperiod of 10 h light/14 h darkness at 50, 120, 600 or 1000 μmol quanta m^{-2} s^{-1} intensity. (a) wt and *ntrc* mutant plants grown for 5 weeks at the respective light intensities. (b) Fresh weight of rosettes from 5-week-old plants. Each data point is the mean of 10–20 plants, and standard deviations (SDs) are presented as error bars. (c) Content of total chlorophylls *a* and *b* determined in leaf discs from leaves of 5-week-old plants. Each data point is the mean of discs from 10 plants ± SDs. (d) Five-week-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 (three leaves from each), and SDs are presented as error bars. (e) Relative content of peroxide-derived hydroxyl radicals based on electron paramagnetic resonance (EPR) signals from wt and *ntrc* mutant leaves in the dark, D; growth light, GL (120 μmol quanta m^{-2} s^{-1}); and high light, HL (500 μmol quanta m^{-2} s^{-1}). The presence of hydroxyl radicals was shown by indirect spin trapping with *N*-(4-pyridylmethylene)-*tert*-butylamine *N*,*N*′-dioxide (4-POBN)/ethanol in the presence of Fe-ethylenediaminetetraacetic acid (EDTA). The signal from the *ntrc* mutant is expressed as a percentage 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 ($P < 0.05$).

and 1000 μmol quanta $m^{-2} s^{-1}$, respectively, as compared with 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 wt 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 normalized to maximal fluorescence (F_v/F_m). Before the transfer to high light, the *ntrc* plants had an F_v/F_m ratio of about 0.78, which is slightly lower than the typical 0.83 of wt plants (Fig. 1d). After 3 h 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 *ntrc* 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 wt plants (Supporting Information Fig. S1).

Under high-light conditions, the increase in formation of superoxide anion radicals leads to enhanced production of 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 (Moon *et al*. 2006; Perez-Ruiz *et al*. 2006; Alkhalfioui *et al*. 2007), 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 a catalyst for the Haber–Weiss reaction, while illuminated and 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 wt 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 NADPH thioredoxin reductase C causes drastically enhanced non-photochemical quenching 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 wt 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 wt 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 wt 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 (Fig. 2b). This response is abnormal and distinguishes the plants lacking NTRC from wt 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) was 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 wt 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 with wt 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). Because some aspects of the *ntrc* mutant phenotype depend on the day length during growth (Perez-Ruiz *et al*. 2006; Lepisto *et al*. 2009; Lepisto *et al*. 2013) 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 it is established that the lack of NTRC strongly influences the levels of NPQ and PSII activity, we proceeded to investigate the 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 adenosine diphosphate (ADP) glucose pyrophosphorylase (AGPase), a key enzyme in this process, which was previously

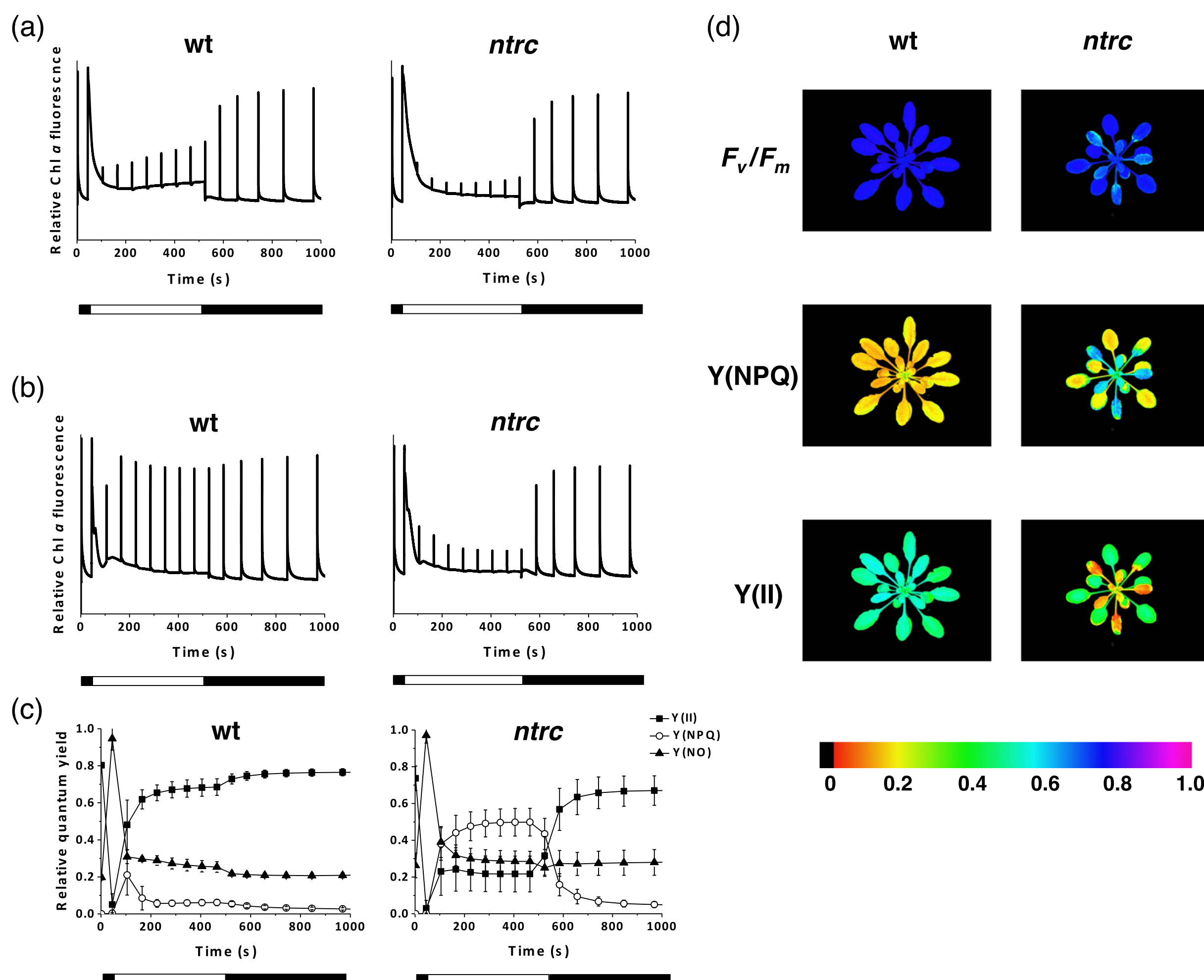

Figure 2. Chlorophyll fluorescence in wild-type (wt) and *ntrc* mutant plants. Fluorescence was measured with a pulse-amplitude modulation fluorometer using attached leaves of 5-week-old plants grown at 120 μmol quanta m^{-2} s^{-1} light intensity. Following 30 min dark incubation and determination 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. (a) 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); photosystem II (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 four plants and standard deviations (SDs) are indicated by error bars. (d) False-colour images representing F_v/F_m in wt 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.

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 wt 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 wt 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 a 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, wt 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 NADPH thioredoxin reductase C on non-photochemical quenching is unrelated to its function as a reductant for the 2-Cys peroxiredoxin

NADPH thioredoxin reductase C is known to be an important electron donor to the chloroplast 2-Cys Prx (Perez-Ruiz *et al.* 2006; Pulido *et al.* 2010), and because ROS are involved in

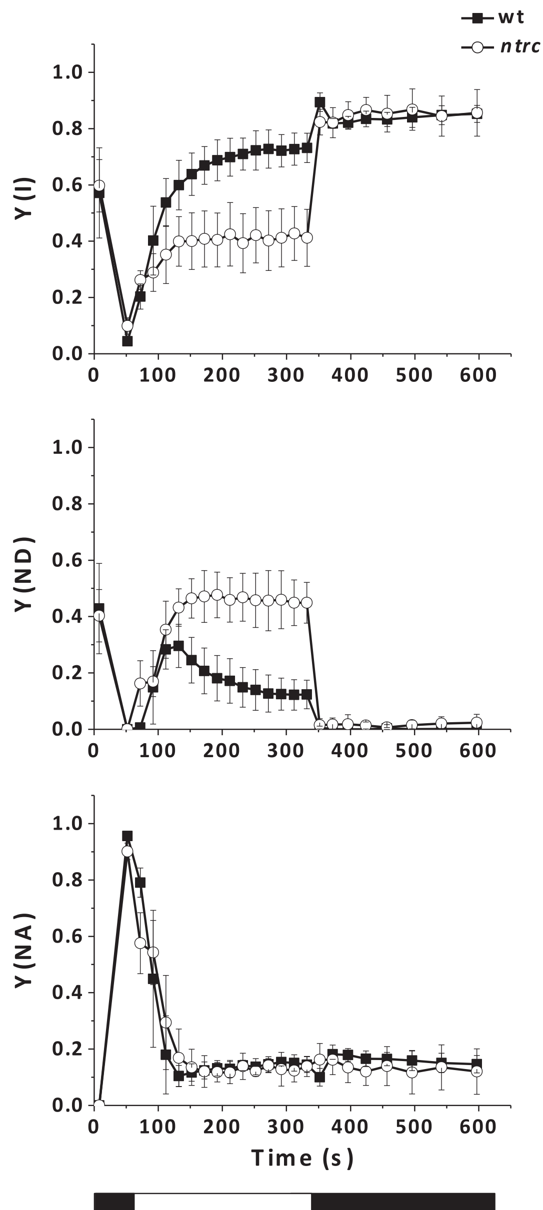

Figure 3. Activity of photosystem I (PSI) in wild-type (wt) and *ntrc* mutant plants. The redox state of the photosystem I (PSI) reaction centre P700 was monitored through the changes in absorbance at 830 versus 875 nm. Five-week-old plants grown at 120 μmol quanta m^{-2} s^{-1} light intensity were 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 m^{-2} 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 six plants, and standard deviations (SDs) are shown as error bars.

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 knockdown mutant with minimal levels of 2-Cys Prx, the Δ*2cp* mutant (Pulido *et al.* 2010), was examined for its capacity to induce NPQ as a function of light intensity and compared with *ntrc* and wt plants. In addition, mutant plants devoid of the chloroplast thioredoxin Trx *x* (Pulido *et al.* 2010) were included in these experiments, as 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 Δ*2cp* and *trxx* mutants were similar to those of wt plants (Fig. 4 and Supporting Information Fig. S5), except the Δ*2cp* 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). wt 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 with those of the wt (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 NADPH thioredoxin reductase C 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 *et al.* 2009; Jahns & Holzwarth 2012). Therefore, we determined the levels of the xanthophyll cycle pigments violaxanthin, antheraxanthin and zeaxanthin in wt and *ntrc* mutant plants. Samples were collected at the end of the night and following the onset of light. As expected, wt 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 wt leaves. In contrast, the *ntrc* mutant showed 10-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.

Because 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 wt

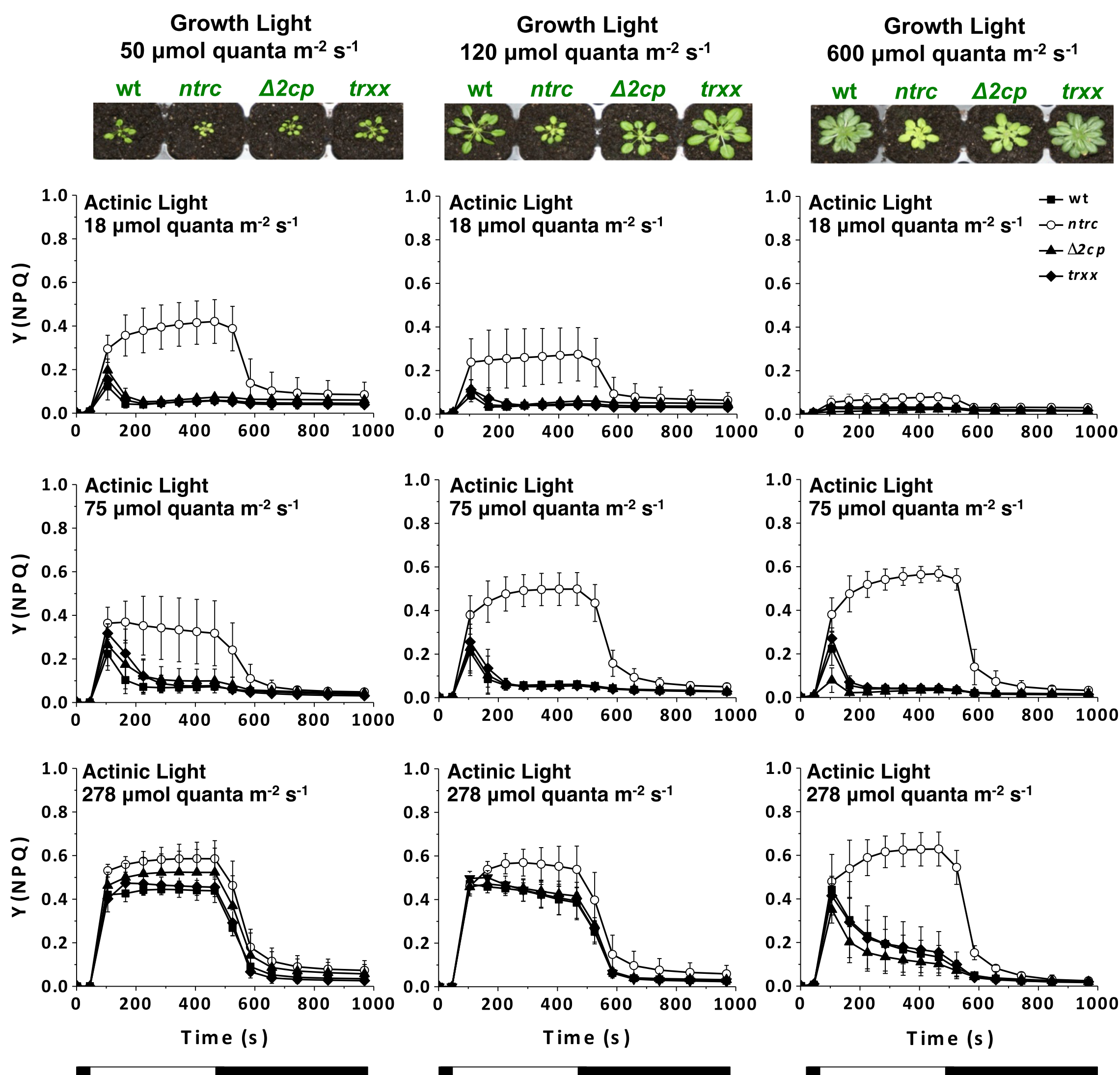

Figure 4. Non-photochemical quenching (NPQ) in wild-type (wt) and *ntrc*, *Δ2cp* and *trxx* mutant plants grown under different irradiances. Chlorophyll fluorescence was measured using attached leaves of 5-week-old plants grown at 50, 120 and 600 μmol quanta m^{-2} s^{-1}. Plants were kept in the dark 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 four plants, and standard deviations (SDs) are indicated by error bars.

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 (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 wt 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 violaxanthin de-epoxidase and zeaxanthin epoxidase *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* (Yamamoto & Kamite 1972; Hall *et al.* 2010; Simionato

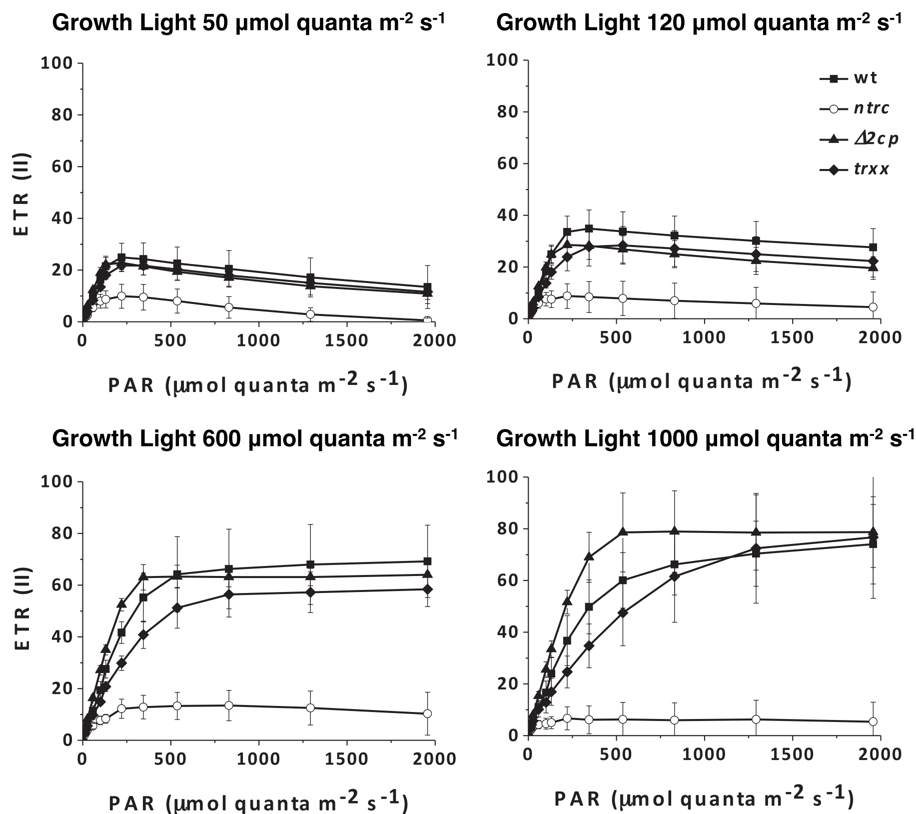

Figure 5. Linear photosynthetic electron transport in wild-type (wt) and *ntrc*, *Δ2cp* and *trxx* mutant plants grown under different irradiances. Chlorophyll fluorescence was measured with a pulse-amplitude modulation fluorometer using attached leaves of plants grown at 50, 120, 600 and 1000 μmol quanta m^{-2} s^{-1} for 5 weeks. Relative electron transport rates of photosystem II (PSII), ETR(II), were determined during stepwise increasing photosynthetically active radiation (PAR). Each data point is the mean of the ETR(II) from eight plants, and standard deviations (SDs) are presented as error bars.

et al. 2015), although the physiological meaning of this remains unknown. The *Arabidopsis* VDE contains 12 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, although ZE was found to be inhibited *in vivo* by photooxidative 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 wt plants was examined (Fig. 8a), taking advantage of the fact that reduced VDE forms migrates slower on SDS-PAGE (Supporting Information Fig. S8). While the most oxidized form of VDE is somewhat more abundant in the *ntrc* mutant in the dark, there is no significant difference between the mutant and wt VDE redox states 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 the 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 glycol$_{24}$-maleimide, MM(PEG), showed no changes in the wt 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 the activation of ATP synthesis (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 oxidized form in both the wt and the *ntrc* mutant (Fig. 8c). After only 1 min of illumination, the γ subunit is fully reduced in the wt, while a minor amount remains oxidized in the *ntrc* mutant plants in the light (Fig. 8c).

The trans-thylakoid ΔpH is larger in plants lacking NADPH thioredoxin reductase C 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

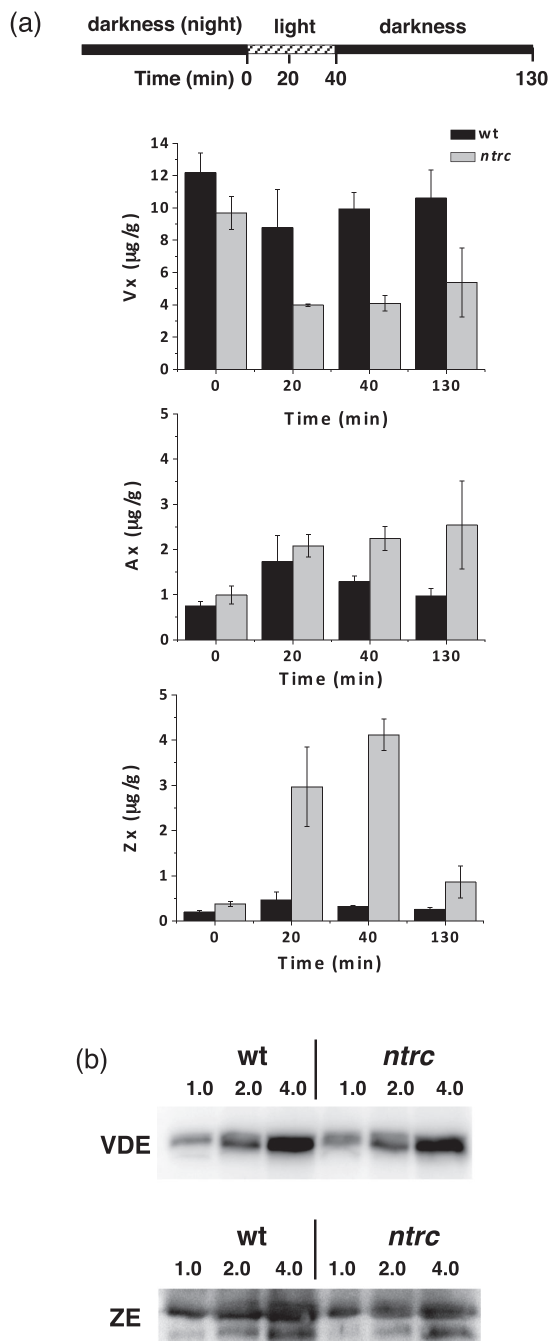

Figure 6. Levels of xanthophyll cycle pigments and violaxanthin de-epoxidase (VDE) and zeaxanthin epoxidase (ZE) proteins in wild-type (wt) and *ntrc* mutant plants. (a) Leaves of 5-week-old plants grown at 120 μmol quanta m^{-2} s^{-1} light intensity were harvested just before the onset of light in 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 micrograms per gram fresh weight of leaves. Values are the mean ± standard deviation (SD) of the xanthophyll levels determined in leaves from three 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 micrograms.

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 the build-up of a proton gradient that can be measured because the protonated form of the fluorophore is sequestered in the thylakoid lumen, which leads to quenching (Schuldiner *et al.* 1972). NH_4Cl 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). The addition of DTT was used as a positive control. In order to assess quantitatively these measurements, quenching of 9-aminoacridine fluorescence was compared with that observed in illuminated thylakoids using methyl viologen as an electron acceptor, which yields a large ΔpH and high levels of fluorescence quenching (Fig. 9b).

The trans-thylakoid proton motive force, $\Delta\psi$ and ΔpH, can be measured *in vivo* using the ECS technique (Takizawa *et al.* 2007). Thus, ΔpH in wt 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 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 wt and *ntrc* plants. Whereas in wt 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 the 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 with the wt. The levels of the PsbS protein were similar in mutant and wt plants (Fig. 10a).

Because 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 of 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

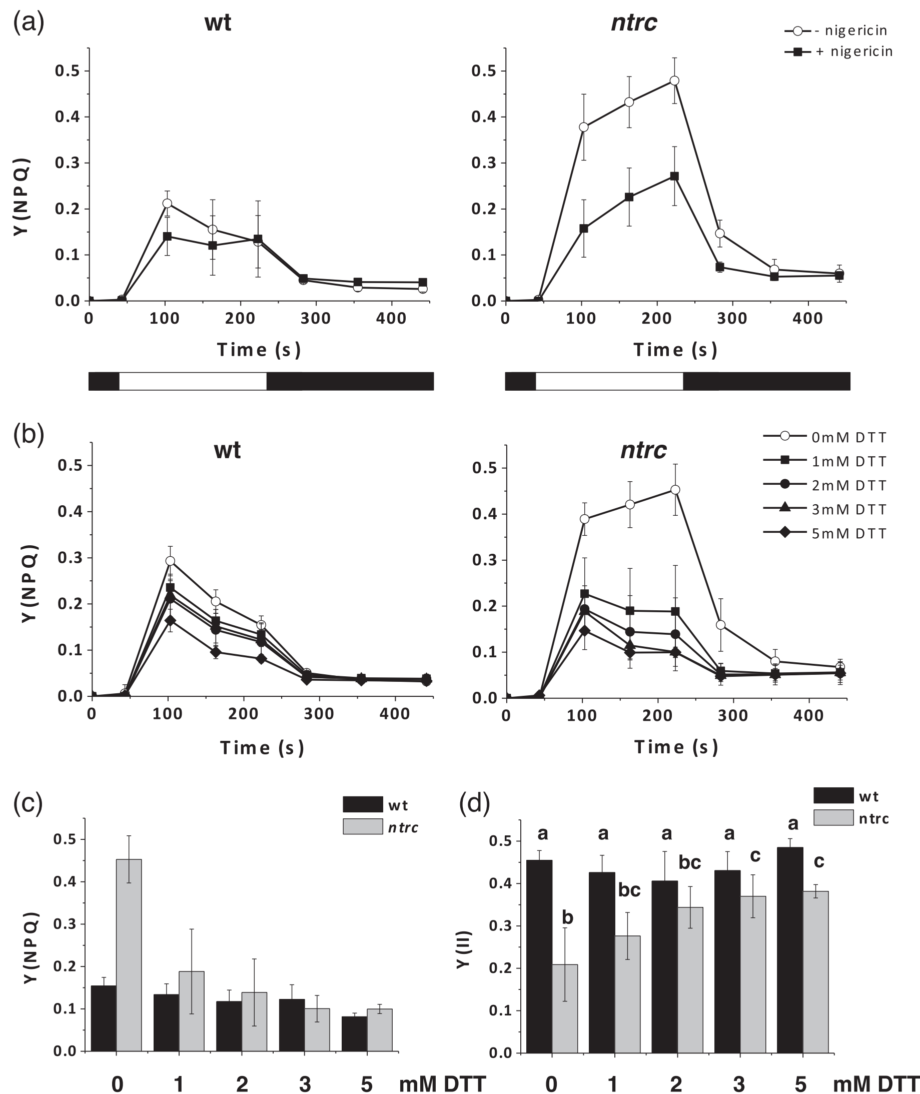

Figure 7. Effects of nigericin and dithiothreitol (DTT) *in vivo* on the non-photochemical quenching (NPQ) and photosystem II (PSII) effective quantum yield of wild-type (wt) and *ntrc* mutant plants. Leaves from 5-week-old plants grown at 120 μmol quanta m^{-2} s^{-1} light intensity were floated on 100 μm nigericin (a) or DTT solutions (b) in the dark for 3 h. 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 six leaves, and standard deviations (SDs) are presented as error bars. Significantly different values according to Tukey (ANOVA) in (d) are marked with different letters ($P < 0.01$).

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 10 weeks under a short-day photoperiod, the double-mutant plants were larger than *ntrc* plants (Fig. 10d,e). The rosette weight and chlorophyll content of *ntrc–psbs* mutant plants were twice as high as compared with those of the single *ntrc* mutant (Fig. 10f,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 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 similar to the *psbs* single mutant (Fig. 11c). The PSI activity was partially restored in the *ntrc–psbs* plants (Fig. 11d), owing 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-term and long-term acclimation of plants to high light intensities is crucial for their survival during excess excitation

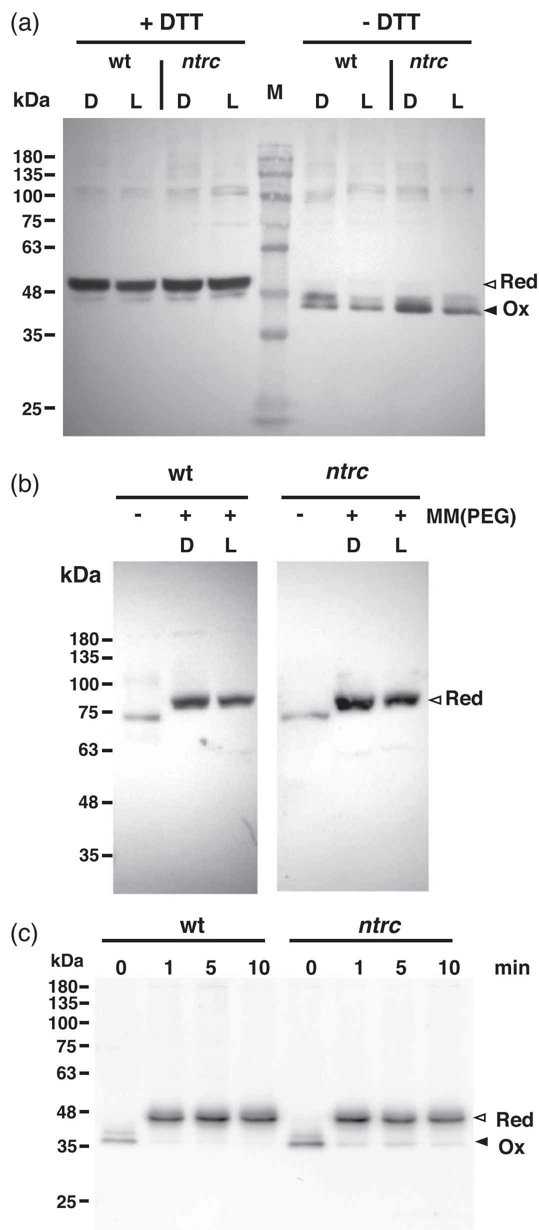

Figure 8. The redox state *in vivo* of the xanthophyll cycle enzymes, violaxanthin de-epoxidase (VDE) and zeaxanthin epoxidase (ZE), and of the ATP synthase γ subunit. Total leaf proteins from wild-type (wt) and *ntrc* mutant plants in the dark or after 1 h in the light at 120 μmol quanta m^{-2} s^{-1} intensity were extracted in the presence of 10% trichloroacetic acid to preserve the thiol redox state. (a) Proteins were solubilized for sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of 40 mM *N*-ethyl maleimide to avoid oxidation of thiols and electrophoresed in the presence or absence of dithiothreitol (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 solubilization 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.

to avoid toxic ROS levels and to protect the PSI and PSII (Tikkanen *et al.* 2012; Spetea *et al.* 2014; Dietz 2015). 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). Because the 2-Cys Prx is a substrate for NTRC (Moon *et al.* 2006; Perez-Ruiz *et al.* 2006; Alkhalfioui *et al.* 2007; 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 wt plants or the Δ*2cp* 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 (Szabo *et al.* 2005; Baker 2008). 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 (Kramer *et al.* 2004a; Baker 2008). 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

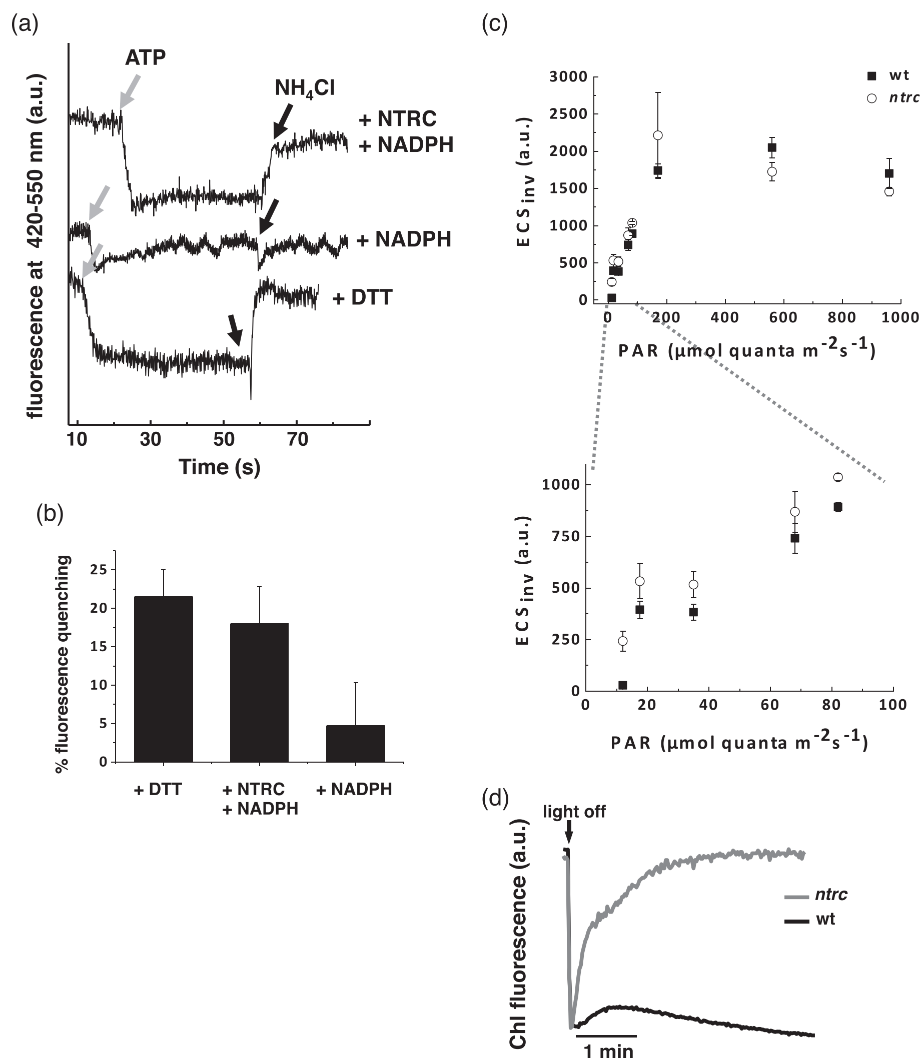

Figure 9. The effect of NADPH thioredoxin reductase C (NTRC) on the trans-thylakoid ΔpH *in vitro* and *in vivo*. (a) Quenching of 9-aminoacridine fluorescence as an indicator of ΔpH formed during ATP hydrolysis. Isolated thylakoids were incubated for 5 min with either 100 μM NADPH and 5 μM NTRC or NADPH alone or 5 mM dithiothreitol (DTT). Grey and black arrows indicate the additions of 50 μM ATP and 20 mM NH_4Cl, respectively. (b) Quantitative comparison of 9-aminoacridine fluorescence quenching from measurements such as those in (a). Values were normalized to those obtained from illuminated thylakoids using 100 μM methyl viologen as an electron acceptor, which was taken as 100% quenching. Each data point is the mean from four to five measurements ± SD. (c) Wild-type (wt) and *ntrc* plants were grown for 5 weeks at 120 μmol 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 μmol quanta $m^{-2}\,s^{-1}$. The component ECS_{inv} is proportional to the ΔpH. The lower panel is a close-up of the range at lower light intensities. Each data point is the mean of four leaves ± standard deviation (SD). (d) Chlorophyll fluorescence induction of wt and *ntrc* plants was measured in the dark after illumination for 5 min with 126 μmol quanta $m^{-2}\,s^{-1}$ intensity actinic light, and typical traces are shown.

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 wt 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 wt 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 (Pfundel & Dilley 1993; Bratt *et al*. 1995; Jahns *et al*. 2009). 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

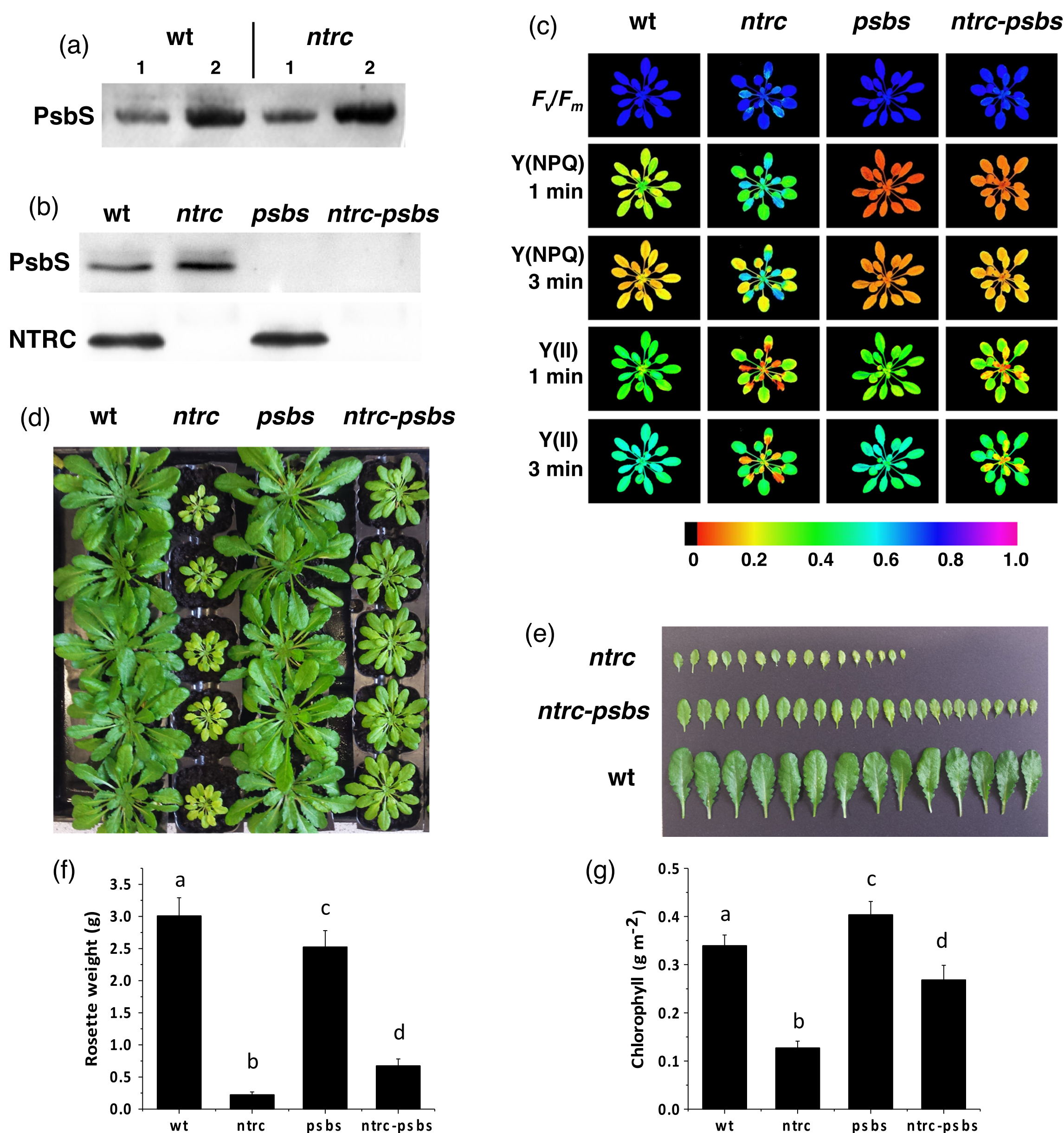

Figure 10. Phenotypic analysis of the *ntrc–psbs* double-knockout mutant. (a) Proteins extracted from wild-type (wt) and *ntrc* leaves were analysed by protein gel immunoblot using antibodies against PsbS. Numbers above each lane indicate the quantity of chlorophyll in micrograms. (b) Leaf proteins of double and single mutants were examined by protein gel immunoblot using antibodies against PsbS and NADPH thioredoxin reductase C (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 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-week-old plants of wt, *ntrc* and *ntrc–psbs* mutants. (f) Fresh weight of rosettes from 10-week-old plants. Each value is the mean of 8–12 plants, and standard deviations (SDs) are presented as error bars. (g) Content of total chlorophylls *a* and *b* determined in leaf discs from 10-week-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 ($P < 0.01$).

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 (Motohashi & Hisabori 2006, 2010; Karamoko *et al.* 2013). 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 photooxidative stress under high-light conditions (Reinhold *et al.* 2008). We

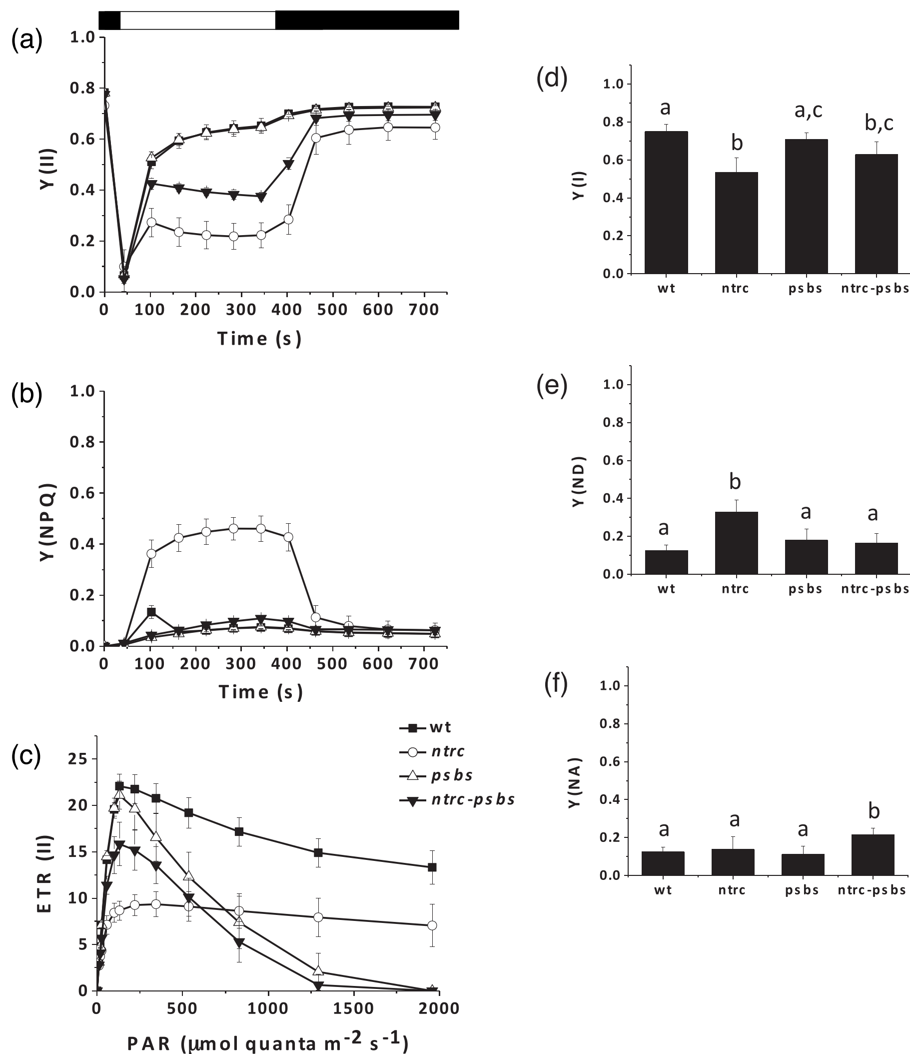

Figure 11. Photosynthetic performance of the *ntrc–psbs* double mutant. Chlorophyll fluorescence and P700 absorbance were measured with a pulse-amplitude modulation fluorometer using plants grown for 5 weeks at 120 μmol quanta $m^{-2} s^{-1}$ light intensity. Prior to all measurements, the plants were kept in the dark for 30 min. For chlorophyll fluorescence, 75 μmol 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 photosystem II (PSII) photochemistry, Y(II). (b) Quantum yields of non-photochemical quenching (NPQ), Y(NPQ). (c) Relative linear electron transport rates ETR(II) as a function of actinic light intensity. Values are the means of six measurements ± standard deviations (SD). For measurements of P700 absorbance, actinic light at an intensity of 126 μmol quanta $m^{-2} s^{-1}$ was used, and saturating pulses were applied every 20 s. The quantum yields of (d) photosystem I (PSI) photochemistry, Y(I); (e) donor-side limitations, Y(ND); and (f) acceptor-side limitations Y(NA) after 3 min of illumination are displayed. Significantly different values according to Tukey ANOVA are marked with different letters ($P < 0.05$).

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 ECS 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 the activation of VDE.

NADPH thioredoxin reductase C 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 (Richter *et al.* 2013; Perez-Ruiz *et al.* 2014) 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 utilization 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), which is devoid of leaf starch, shows a pattern of NPQ identical to wt 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 wt. In contrast, whereas the PSI donor-side limitations are gradually relieved after a dark–light transition in the wt, these limitations persist in 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 (Kramer *et al*. 2004a; Foyer *et al*. 2012; Allahverdiyeva *et al*. 2015). 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 *m*4 has recently been shown *in vivo* to inhibit CEF dependent on the NDH (Courteille *et al*. 2013). However, mutants lacking Trx *m*4 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 with wt 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, as 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 a 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 (Michalska *et al*. 2009; Lepisto *et al*. 2013; 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 (Serrato *et al*. 2004; Perez-Ruiz *et al*. 2006; Lepisto *et al*. 2009). 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 (Perez-Ruiz *et al*. 2006; Lepisto *et al*. 2009, 2013). 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-term or short-term high-light tolerance, nor is it implied in peroxide detoxification at high light intensities. On the contrary, plants lacking NTRC are overprotected against light even at low and moderate irradiances, leading to a state of permanent starvation for light energy.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Photoinhibition and relative reactive oxygen species content in leaves from wild -type and *ntrc* mutant plants.
Figure S2. Non-photochemical quenching (NPQ) in wild -type and *ntrc* mutant plants grown under long-day conditions.
Figure S3. Non-photochemical quenching (NPQ) in the aps1 mutant in comparison towith wild -type and ntrc mutant plants.
Figure S4. Redox state of the photosystem I P700 in wild -type and *ntrc* mutant plants.
Figure S5. Non-photochemical quenching (NPQ) in wild -type and *ntrc*, *Δ2cp* and *trx x* mutant plants grown at high light intensity.
Figure S6. Non-photochemical quenching (NPQ) as a function of light intensity in wild -type and *ntrc, Δ2cp* and *trx x* mutant plants grown at different light intensities.
Figure S7. Domain architectures and positions of cysteines in the *Arabidopsis thaliana* VDE and ZE.
Figure S8. Migration of reduced and oxidised VDE in SDS-PAGE.

Figure S9. NTRC reduces intermolecular disulphides of ZE in vitro.
Figure S10. Isolation of a *psbs* knockout mutant and *ntrc-psbs* double knockout mutants.
Table S1. DNA sequences of specific oligonucleotides used as primers for genotyping *ntrc, psbs* and *ntrc-psbs* knockout mutants.
Table S2. Primer pairs of specific oligonucleotides used for genotyping *ntrc, psbs* and *ntrc-psbs* knockout mutants, and their templates.