

RESEARCH PAPER

Functional analysis of the pathways for 2-Cys peroxiredoxin reduction in *Arabidopsis thaliana* chloroplasts

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Abstract

Photosynthesis is a process that inevitably produces reactive oxygen species, such as hydrogen peroxide, which is reduced by chloroplast-localized detoxification mechanisms one of which involves 2-Cys peroxiredoxins (2-Cys Prxs). *Arabidopsis* chloroplasts contain two very similar 2-Cys Prxs (denoted A and B). These enzymes are reduced by two pathways: NADPH thioredoxin reductase C (NTRC), which uses NADPH as source of reducing power; and plastidial thioredoxins (Trxs) coupled to photosynthetically reduced ferredoxin of which Trx *x* is the most efficient reductant *in vitro*. With the aim of establishing the functional relationship between NTRC, Trx *x*, and 2-Cys Prxs *in vivo*, an *Arabidopsis* Trx *x* knock-out mutant has been identified and a double mutant (denoted $\Delta 2cp$) with <5% of 2-Cys Prx content has been generated. The phenotypes of the three mutants, *ntrc*, *trxx*, and $\Delta 2cp$, were compared under standard growth conditions and in response to continuous light or prolonged darkness and oxidative stress. Though all mutants showed altered redox homeostasis, no difference was observed in response to oxidative stress treatment. Moreover, the redox status of the 2-Cys Prx was imbalanced in the *ntrc* mutant but not in the *trxx* mutant. These results show that NTRC is the most relevant pathway for chloroplast 2-Cys Prx reduction *in vivo*, but the antioxidant function of this system is not essential. The deficiency of NTRC caused a more severe phenotype than the deficiency of Trx *x* or 2-Cys Prxs as determined by growth, pigment content, CO₂ fixation, and F_v/F_m , indicating additional functions of NTRC.

Key words: Chloroplast, oxidative stress, peroxiredoxin, thioredoxin.

Introduction

Oxygenic photosynthesis converts light energy into organic material, being a process of primary importance for the production of biomass and oxygen in the biosphere (Nelson and Ben-Shem, 2004; Rascher and Nedbal, 2006). Because photosynthesis involves the transport of electrons in the presence of oxygen, it is a process that inevitably produces reactive oxygen species (ROS), which act as oxidants and may cause cell damage (Apel and Hirt, 2004; Mittler *et al.*, 2004). In addition to this toxic effect, ROS have an

important signalling function (Van Breusegem *et al.*, 2008). This is the case for hydrogen peroxide (H₂O₂), which is well established as a signalling molecule in eukaryotes (Wood *et al.*, 2003; Mullineaux *et al.*, 2006), and singlet oxygen, which acts as a signal to activate gene expression in response to environmental stress in plants (Op den Camp *et al.*, 2003; Wagner *et al.*, 2004; Kim *et al.*, 2008).

In photosynthetic plant cells chloroplasts are a major source of ROS production (Pitzschke *et al.*, 2006), which

increases under adverse environmental conditions (Apel and Hirt, 2004; Ledford *et al.*, 2007). Therefore, the different antioxidant mechanisms, non-enzymatic and enzymatic, that operate in the chloroplast are important not only to avoid toxic ROS effects but also to modulate their signaling function. Non-enzymatic antioxidants may be classified into those which are hydrophilic, including glutathione and ascorbate (AsA), which reach millimolar concentrations in the chloroplast, and those which are lipophilic. Lipophilic antioxidants, such as β -carotene, zeaxanthine, and α -tocopherol, have an important scavenging role and protect thylakoid membranes and photosynthetic structures (Havaux *et al.*, 2005; Kruk *et al.*, 2005; Peñuelas and Munne-Bosch, 2005).

Enzymatic antioxidant systems include superoxide dismutases (SODs), ascorbate peroxidases (Apxs), catalases, glutathione peroxidases (Gpxs), and peroxiredoxins (Prxs) (Foyer and Noctor, 2009). In plants, these enzymes are encoded by gene families and, for most of them, excluding catalases, some of the isoforms are targeted to chloroplasts. Among the different SODs, Fe-SOD and CuZn-SOD are present in chloroplasts and their overexpression produces enhanced tolerance to different environmental stresses (Lee *et al.*, 2007). Apxs are haem-containing peroxidases, of which the chloroplast contains two isoforms localized in the stroma (sApx) and associated with the thylakoid (tApx), respectively (Shigeoka *et al.*, 2002). These peroxidases play an important protective role in the chloroplast because they participate in the AsA-dependent water–water cycle able to reduce H_2O_2 using AsA as reductant (Asada, 2006). Interestingly, *Arabidopsis* mutants devoid of both sApx and tApx showed phenotypic differences from wild-type plants only under severe oxidative stress imposed by treatment with methyl viologen (Kangasjärvi *et al.*, 2008). Gpxs are encoded by a gene family composed of eight genes in *Arabidopsis* with different cellular localization (Rodríguez-Milla *et al.*, 2003). Though these enzymes were initially described as glutathione dependent, their reduction by thioredoxins (Trxs) has recently been reported (Navrot *et al.*, 2006). An *Arabidopsis* mutant deficient in one of the isoforms, AtGpx3, is sensitive to oxidative stress (Miao *et al.*, 2006), thus supporting the antioxidant function of these enzymes.

The most recently described peroxidases in plants are Prxs, thiol-based enzymes encoded by a gene family of 10 members in *Arabidopsis* (Dietz, 2003). According to their reaction mechanism and biochemical properties, Prxs are classified into four types: typical 2-Cys Prx, atypical Prx Q and type II Prx, and 1-Cys Prx. The *Arabidopsis* chloroplast contains four Prxs: Prx Q, Prx IIE, and two very similar 2-Cys Prxs, namely 2-Cys Prx A and 2-Cys Prx B (Bréhélin *et al.*, 2003; Horling *et al.*, 2003; Dietz *et al.*, 2006). The highly conserved reaction mechanism involves a cysteine residue, termed peroxidatic, which attacks the peroxide and becomes transiently oxidized to sulphenic acid. This intermediate is subsequently attacked by a second cysteine residue, termed resolving, yielding water or the corresponding alcohol, and the two cysteine residues become oxidized,

forming a disulphide bridge (Dietz, 2003; König *et al.*, 2003; Hall *et al.*, 2009) that needs to be reduced for a new catalytic cycle. In chloroplasts, two pathways have been proposed to reduce typical 2-Cys Prxs. One is based on plastidial Trxs, such as Trx *x*, which is the most efficient reductant (Collin *et al.*, 2003), and CDSP32, which has been shown to interact with 2-Cys Prx *in vitro* and *in vivo* (Broin *et al.*, 2002; Rey *et al.*, 2002). The other pathway is based on a peculiar type of NADPH thioredoxin reductase (NTR), termed NTRC, with a joint Trx domain at the C-terminus (Serrato *et al.*, 2002, 2004). NTRC conjugates NTR and Trx activities to reduce 2-Cys Prxs with high efficiency (Pérez-Ruiz *et al.*, 2006; Pérez-Ruiz and Cejudo, 2009). Whereas the Trx-dependent pathway obtains reducing power from ferredoxin (Fd) reduced by the photosynthetic electron chain and mediated by ferredoxin-dependent thioredoxin reductase (FTR), NTRC uses NADPH as the source of reducing power. This difference is important because NADPH can be produced in darkness from glucose 6-phosphate via the oxidative pentose phosphate pathway, thus supplying reducing power under conditions when the levels of reduced Fd are lower (Spínola *et al.*, 2008). Previously, analysis based on *in vitro* assays with purified recombinant proteins showed that NTRC is more efficient than Trx *x* or CDSP32 as a reductant of 2-Cys Prx (Pérez-Ruiz *et al.*, 2006). The present study undertakes an *in vivo* assessment of these redox pathways. Following the identification of *Arabidopsis* mutants deficient in 2-Cys Prxs and Trx *x*, a comparative analysis with the previously reported NTRC-deficient mutant was performed with the aim of identifying the contribution of these Prxs and the two major pathways, NTRC dependent and Trx *x* dependent, to the overall mechanism of chloroplast detoxification of peroxides and maintenance of chloroplast redox homeostasis.

Materials and methods

Growth conditions and plant material

Arabidopsis thaliana wild-type (ecotype Columbia) and mutant plants were grown in soil supplemented with Hoagland medium in growth chambers under long-day conditions (16 h light/8 h darkness) at 22 °C during the day and 20 °C during the night. The light intensity was set at 140 $\mu E m^{-2} s^{-1}$. The 2-Cys Prx A–2-Cys Prx B double mutant, denoted $\Delta 2cp$, was obtained by manual crossing of the single mutants *2cpA*, line SALK_065264, and *2cpB*, line SALK_017213, previously characterized (Kirchsteiger *et al.*, 2009). Seeds resulting from this cross were checked for heterozygosity of T-DNA insertions in the *2cpA* and *2cpB* genes. The plants were then selfed and double homozygous plants were detected in the progeny by PCR analysis of genomic DNA using oligonucleotides a (5'-GAGAAGTTGAACACCGA-3') and b (5'-GGGGACAAAGTGAGAATC-3') for the *2cpA* gene, and a' (5'-CCACCTGAACCAAGAAAG-3') and b' (5'-CCTGCAAGACAACATCAC-3') for the *2cpB* gene in conjunction with the oligonucleotide T (5'-TGGTTCACGTAGTGGGCCATCG-3') located in the T-DNA. A T-DNA homozygous line SALK_128914 (Alonso *et al.*, 2003) in the single gene encoding Trx *x* of *Arabidopsis* (At1g50320 locus) was selected by PCR analysis of genomic DNA with oligonucleotides c (5'-GCCATGGACTC-TATCGTCTC-3') and d (5'-CCTTCCCTTCTGCTCCCT-3') in

conjunction with the T oligonucleotide. The NTRC knock-out mutant was described previously (Serrato *et al.*, 2004).

Treatment with methyl viologen

Leaf discs, 6 mm in diameter, were excised from equivalent leaves of wild-type and mutant plants that had been grown for 21–25 d. Discs were immediately incubated in sterile distilled water supplemented with methyl viologen up to 10 μM concentration and incubated during 48 h under continuous light (30 $\mu\text{E m}^{-2} \text{s}^{-1}$).

Quantitative PCR (qPCR) and western blot analysis

Total RNA (1 μg) extracted from ground leaf tissue using Trizol™ was retro-transcribed by means of a QuantiTect™ RT-kit (Qiagen). Real-time PCR was performed in a total reaction volume of 20 μl containing primers (4 μM), cDNA (40 ng), and 10 μl of iQ™ SYBR Green Supermix (Bio-Rad). The results obtained from three independent biological samples (three analytical replicates each) are represented as $2^{-\Delta\Delta\text{CT}}$ (threshold cycle) as described by Livak and Schmittgen (2001). *Ubiquitin 10* was used as housekeeping gene. Gene-specific primers used are described in Supplementary Table S1 available at *JXB* online. Fluorescence of PCR products was determined continuously by the iQ5 cycler (Bio-Rad).

Western blot analysis was performed as previously described (Kirchsteiger *et al.*, 2009) using as probes anti-NTRC, anti-NTRB, and anti-2-Cys Prx antibodies, previously described. Anti-Trx *x* polyclonal antibodies were produced after immunization of rabbits with purified recombinant Trx *x* at the Servicio de Protección Animal (Sevilla University, Spain). The anti-Prx Q antibody was purchased (Agrisera, Vännäs, Sweden), and anti-Prx IIE was kindly provided by Dr N. Rouhier (University of Nancy, France). For optimized resolution, SDS-PAGE was performed with 12% or 15% acrylamide/bisacrylamide gels and loading of 15 μg of total protein extract, or 7.5 μg for 2-Cys Prx, respectively, under reducing conditions.

Determination of the photosynthetic rate and PSII photochemical efficiency

Photosynthetic gas exchange was measured using a portable infrared gas analyser (model LI-6400, LI-COR Biosciences, Inc., Lincoln, NE, USA), which allows environmental conditions inside the chamber to be precisely controlled. Air temperature in the chamber was set at 25 °C, and the relative humidity was maintained at 50%. The CO_2 assimilation rate was determined at 1000 $\mu\text{E m}^{-2} \text{s}^{-1}$ with the upper leaf of wild-type and mutant plants grown for 3 weeks as described above. Plants treated with continuous light were grown in a growth chamber at 22 °C at a constant light intensity of 140 $\mu\text{E m}^{-2} \text{s}^{-1}$. For the treatment of prolonged darkness, plants grown under a 16 h/8 h photoperiod were subjected to darkness during 72 h before the determination of CO_2 assimilation rate, which was performed at 1000 $\mu\text{E m}^{-2} \text{s}^{-1}$.

Parameters of chlorophyll fluorescence emission were measured at 22 °C with the chlorophyll fluorometer PAM 2000 (Walz, Effeltrich, Germany). The maximal quantum yield of PSII (F_v/F_m) was calculated from the parameters using the following equation: $F_v/F_m = (F_m - F_o)/F_m$, where F_o is the initial minimal fluorescence emitted from leaves dark-adapted for 15 min and F_m is the maximal fluorescence elicited by saturating actinic light.

Determination of photosynthetic pigments, carbonyl groups, H_2O_2 , AsA, and glutathione

The content of total chlorophyll and carotenoids was determined according to the method of Lichtenthaler and Wellburn (1983), as previously reported (Pérez-Ruiz *et al.*, 2006). Carbonyl groups were assayed using the dinitrophenyl hydrazine method, according to Romero-Puertas *et al.* (2002). H_2O_2 was analysed by using PeroXOquant Quantitative Peroxide Assay Kits (Pierce). AsA and

dehydroascorbate (DHA) were analysed in microplates using the α - α' bipyridyl method according to Gillespie and Ainsworth (2007). Total AsA was measured after incubation of the samples with dithiothreitol (DTT) and the oxidized form (DHA) was determined as the difference between total and reduced AsA. To quantify the glutathione contents, leaf tissues frozen with liquid nitrogen were homogenized in cold extraction buffer containing 0.1 N HCl and 1 mM EDTA. Homogenates were centrifuged at 15 000 g for 15 min at 4 °C. Thiols were reduced at 4 °C for 15 min by mixing 400 μl of extracted samples with 600 μl of 200 mM CHES (pH 9.2) and 100 μl of 250 mM NaBH_4 . For derivatization, a 330 μl aliquot was added to 20 μl of 15 mM monobromobimane and kept in the dark at room temperature for 15 min. The reaction was stopped by adding 250 μl of 0.25% (v/v) methanesulphonic acid. Derivatized thiols were separated and quantified by reverse-phase HPLC as described in Domínguez-Solís *et al.* (2001).

Determination of SOD and Apx activities

SOD was assayed by native isoelectric focusing (IEF) in 5% acrylamide vertical slab gels (pH 3–7). The anode and cathode solutions were 20 mM acetic acid and 25 mM NaOH, respectively, and the proteins were focused sequentially at 150 V for 30 min, 200 V for 2 h, and 400 V for 2 h, at 4 °C. Individual isoenzymes were stained by a photochemical method using NBT and identified by their sensitivity to H_2O_2 and KCN (Sandalio and del Río, 1988). Isoenzyme activity was quantified by densitometry in the Quantity One (Bio-Rad), recording the area under the peaks, and the total SOD activity was obtained by addition of the areas of all the isoforms. Results are expressed as a percentage of each isoform from the mutants in comparison with the wild type, arbitrarily considered 100%. For determination of soluble Apx activity, leaf tissues were ground in liquid nitrogen and homogenized in potassium phosphate buffer pH 7.6, supplemented with 20% (w/v) sorbitol, 1 mM EDTA, 5 mM AsA, and 2% (w/v) polyvinylpyrrolidone. Extracts were then centrifuged at 12 000 g for 30 min and activity was measured in the supernatant as described by Maruta *et al.* (2010) by the oxidation of AsA at 290 nm.

Results

Comparative analysis of Arabidopsis mutants deficient in chloroplast 2-Cys Prxs, Trx *x*, and NTRC

Previous results based on *in vitro* assays established that chloroplast 2-Cys Prxs are reduced by different Trxs, of which the *x*-type was the most efficient electron donor (Collin *et al.*, 2003). However, NTRC, a bimodular protein conjugating both NTR and Trx activity, showed even higher catalytic efficiency than Trx *x* as reductant of 2-Cys Prx (Pérez-Ruiz *et al.*, 2006). The *Arabidopsis* genome contains two genes encoding very similar isoforms of typical 2-Cys Prx, namely A and B. Single T-DNA insertion mutants of *Arabidopsis* deficient in either 2-Cys Prx A or 2-Cys Prx B have been described and do not show a phenotype significantly different from that of the wild-type plants, when grown under standard conditions (Kirchsteiger *et al.*, 2009). To analyse further the function of chloroplast 2-Cys Prxs, a double mutant deficient in both 2-Cys Prx A and 2-Cys Prx B was obtained by manual crossing of the corresponding single mutants and selection of lines homozygous for the T-DNA insertions in both genes, as determined by PCR analysis of genomic DNA

(Supplementary Fig. S1 at *JXB* online). The double mutant, termed $\Delta 2cp$, did not contain detectable amounts of 2-Cys Prx B transcripts and had only minimal amounts of 2-Cys Prx A transcripts, as determined by qPCR analysis (Fig. 1A). Accordingly, the content of 2-Cys Prx protein was severely reduced in the double mutant, being almost undetectable in western blot analysis of leaf protein extracts (Fig. 1B). Though not visible in Fig. 1B, when western blots were overloaded a faint band could be detected, which corresponded to 2-Cys Prx A as deduced from the electrophoretic mobility of 2-Cys Prx A and 2-Cys Prx B in the respective single mutants (Kirchsteiger *et al.*, 2009). Therefore, it was concluded that the $\Delta 2cp$ mutant was a severe knock-down containing <5% of the wild-type content of 2-Cys Prx A and no 2-Cys Prx B.

The second objective of this study was to establish the physiological relevance of NTRC and Trx *x*, previously identified as the most efficient reductants of 2-Cys Prx *in vitro* (Collin *et al.*, 2003; Pérez-Ruiz *et al.*, 2006). To analyse the function of Trx *x* and make a comparison with the NTRC-dependent pathway, a T-DNA insertion mutant, SALK_128914, in the single gene encoding Trx *x* of *Arabidopsis* (At1g50320 locus) was isolated (Supplementary Fig. S2 at *JXB* online). The homozygous line for the T-DNA, the *trxx* mutant, contained no detectable Trx *x* mRNA (Fig. 2A) and, accordingly, no Trx *x* protein in leaf

extracts (Fig. 2B). Based on these results it is concluded that the *trxx* mutant is a knock-out for Trx *x*.

When grown under standard long-day conditions, the NTRC knock-out mutant showed the previously reported characteristic phenotype (Serrato *et al.*, 2004; Pérez-Ruiz *et al.*, 2006) of retarded growth, lower fresh weight, and pale green leaves with a lower content of photosynthetic pigments than wild-type plants (Fig. 3A–C). In comparison, the 2-Cys Prx-deficient mutant, $\Delta 2cp$, showed a slightly lower fresh weight and photosynthetic pigment content, total chlorophyll, and carotenoids, though the phenotype of these plants resembled more that of wild type than that of NTRC-deficient plants (Fig. 3A–C). No difference from the wild type in the chlorophyll *a*:chlorophyll *b* ratio was detected in these mutants (not shown). Similarly, the Trx *x* knock-out mutant exhibited a phenotype more similar to the wild type than to NTRC-deficient plants (Fig. 3A). However, the size of the rosette leaves was larger in the Trx *x* knock-out than in the wild-type plants (Fig. 3A). This result was confirmed by an increase of fresh weight of the rosette leaves (Fig. 3B) although there was no significant difference in the photosynthetic pigment content (Fig. 3C).

2-Cys Prxs, Trx x, and NTRC are not essential components of the mechanism of response to oxidative stress

To test the antioxidant and metabolic significance of 2-Cys Prxs, Trx *x*, and NTRC, several markers of oxidative stress and photosynthetic activity were analysed in plants grown under standard conditions and in response to different treatments. All three mutants showed altered redox homeostasis as revealed by the increase in the levels of protein carbonylation, which was duplicated in the *ntrc* and *trxx*

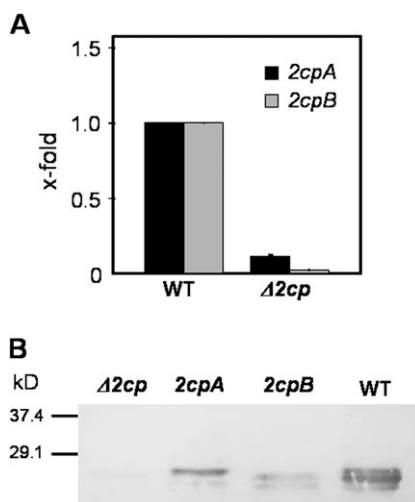


Fig. 1. Characterization of the *Arabidopsis* $\Delta 2cp$ double mutant. (A) qPCR analysis of *2cpA* and *2cpB* transcripts in wild-type and $\Delta 2cp$ double mutant plants. The amount of transcripts in the $\Delta 2cp$ double mutant was represented as arbitrary units relative to the respective levels in the wild-type plant, which was set to 1.0. (B) Western blot analysis of the content of 2-Cys Prx in the $\Delta 2cp$ double mutant as compared with the respective single mutants *2cpA* and *2cpB*. Protein samples (7.5 μ g of protein) extracted from leaves of the wild type, *2cpA* and *2cpB* single mutants, and the $\Delta 2cp$ double mutant were subjected to SDS-PAGE (15% acrylamide) under reducing conditions, electrotransferred onto nitrocellulose sheets, and probed with anti-2-Cys Prx antibody. Molecular mass markers, in kD, are indicated on the left.

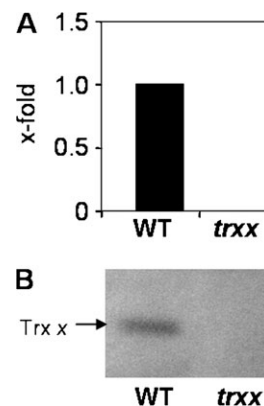


Fig. 2. Characterization of the *Arabidopsis* T-DNA insertion mutant in the Trx *x* gene. (A) qPCR analysis of Trx *x* transcripts in wild-type and *trxx* mutant plants. The amount of transcripts in the mutant was represented as arbitrary units relative to the respective level in the wild-type plant, which was set to 1.0. (B) Western blot analysis of the Trx *x* protein content. Protein samples (15 μ g of protein) extracted from leaves of the wild type and *trxx* mutant were subjected to SDS-PAGE (15% acrylamide) under reducing conditions, electrotransferred onto nitrocellulose sheets, and probed with anti-Trx *x* antibody.

mutants and reached up to an almost 5-fold increase in the 2-Cys Prx knock-down mutant (Table 1). The increase in protein carbonylation might be produced by the accumulation of H_2O_2 due to the deficiency of 2-Cys Prx in the $\Delta 2cp$ mutant, or the pathways of 2-Cys Prx reduction in *ntrc* and *trxx* mutants. The $\Delta 2cp$ mutant and, to a lesser extent, the *ntrc* mutant showed significant increases in H_2O_2 in leaves (Table 1). However, the content of H_2O_2 was not significantly affected by the deficiency of Trx *x* (Table 1). These results indicate the involvement of these enzymes in the maintenance of the redox homeostasis of the chloroplast. However, unlike the deficiency of NTRC, which has a clear effect on plant growth, the deficiency of 2-Cys Prxs A and B and Trx *x* had only a slight effect, at least under standard growth conditions.

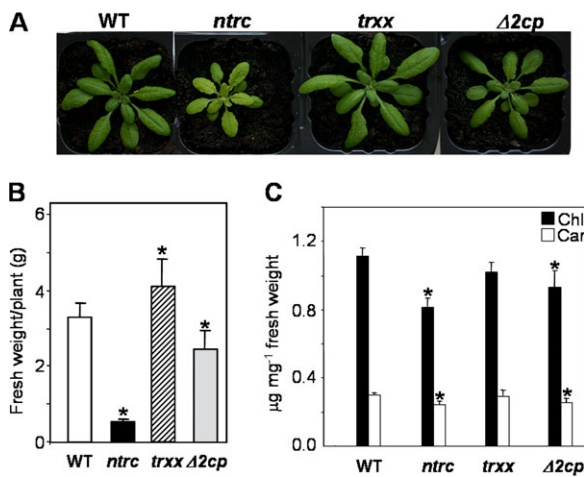


Fig. 3. Growth and contents of photosynthetic pigments in *Arabidopsis* wild-type and mutant lines grown under standard long-day conditions. (A) Wild-type and mutant plants, as indicated, after 24 d of growth under long-day conditions. (B) Fresh weight of the rosette leaves of wild-type and mutant plants after 33 d of growth. Mean values plus standard errors are represented. (C) Content of photosynthetic pigments (total chlorophyll and carotenoids) in leaves from *Arabidopsis* wild-type and mutant plants after 33 d of growth. Pigments were extracted from four independent plant samples and the mean values \pm SE are indicated. Asterisks mark significant differences from the wild type ($P \leq 0.025$, *t*-Student).

Table 1. Content of carbonyl groups and hydrogen peroxide in leaves from *Arabidopsis* wild-type and mutant lines

Line	Carbonyl groups (nmol DNPH mg^{-1} protein)	H_2O_2 (nmol g^{-1} fresh weight)
Wild type	68.7 \pm 13.1 c	769.5 \pm 24.6 c
<i>ntrc</i>	157.6 \pm 17.3 b	991.1 \pm 7.6 b
<i>trxx</i>	162.4 \pm 43.1 b	767.7 \pm 11.4 c
$\Delta 2cp$	321.8 \pm 62.4 a	1116.1 \pm 37.9 a

Values are means \pm SE of at least two replicates of three independent samples. Values with different letters are significantly different ($P \leq 0.05$) as determined by Tukey's multiple range test.

To analyse further the involvement of these enzymes in the response to oxidative stress, leaf discs from the three mutants were incubated in the presence of the photooxidative agent methyl viologen under continuous light. Treatment with increasing concentrations of methyl viologen provoked a decrease in the total chlorophyll content (Fig. 4A) and dramatically affected the carotenoid content (Fig. 4B). However, the loss of photosynthetic pigments was similar in discs from the wild type and the mutants, hence indicating that 2-Cys Prxs, Trx *x*, and NTRC are not essential components of the protection mechanism against oxidative stress.

NTRC and Trx *x* use NADPH and reduced Fd, respectively, as the source of reducing power for 2-Cys Prx reduction. Whereas during the day reduced Fd and NADPH are products of the photosynthetic electron transport chain, during the night, when the level of reduced Fd is low, NADPH can be produced from sugars by the oxidative pentose phosphate pathway. Thus, the NADPH-dependent NTRC pathway becomes more important in the dark, as suggested by the hypersensitivity of the *ntrc* mutant to prolonged darkness (Pérez-Ruiz *et al.*, 2006). To analyse further the function of both pathways, the effect of

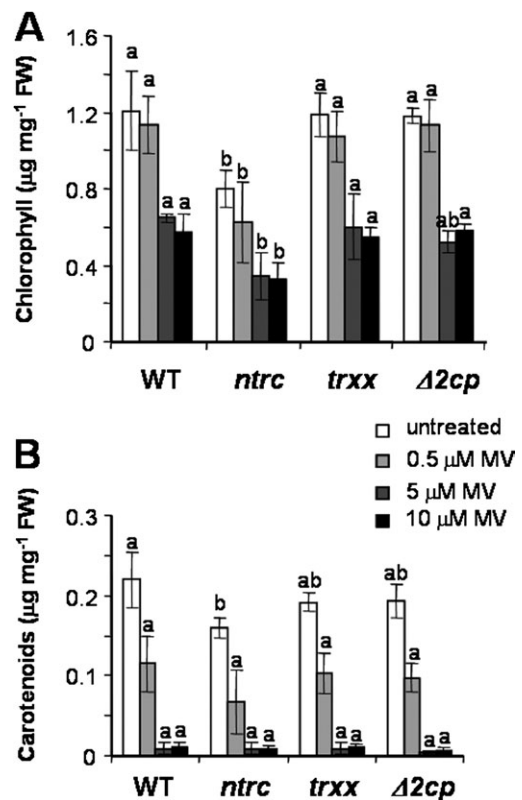


Fig. 4. Effect of oxidative stress treatments on leaf discs. Leaf discs from wild-type and mutant plants were incubated in the presence of increasing concentrations of methyl viologen, as indicated, for 48 h under continuous light ($30 \mu E m^{-2} s^{-1}$), and the contents of total chlorophyll (A) or carotenoids (B) were determined. The assay was performed three times, and the values are presented as the mean \pm SE. Letters indicate significance of difference ($P \leq 0.05$) as determined by Tukey's multiple range test comparing the four *Arabidopsis* lines for each methyl viologen treatment.

treatments affecting the source of reducing power, prolonged darkness and continuous light, on photosynthetic parameters of wild-type and mutant plants was determined. Under control conditions or in continuous light the photochemical efficiency (F_v/F_m) did not show any significant difference between the three mutants and the wild-type plants, whereas prolonged darkness significantly decreased F_v/F_m only in the *ntrc* mutant (Fig. 5A). CO_2 fixation of the *ntrc* mutant was inhibited under control conditions, as previously reported by Pérez-Ruiz *et al.* (2006), but not in the $\Delta 2cp$ or *trxx* mutants (Fig. 5B). The rate of CO_2 fixation was affected by continuous light in the *ntrc* and *trxx* mutants and, to a lower extent, in the $\Delta 2cp$ mutant (Fig. 5B). The treatment with prolonged darkness, which reduced the CO_2 fixation activity of the wild-type plants, completely abolished this activity in the NTRC-deficient plants, whereas the *trxx* mutant did not differ significantly from the wild type (Fig. 5B). Therefore, although the continuous light treatment had a significant effect on the CO_2 fixation activity of the *trxx* mutant, the relative changes in F_v/F_m and CO_2 fixation parameters showed that photosynthetic activities are more affected by the deficiency of NTRC than Trx *x* or 2-Cys Prx.

NTRC and Trx *x* have different effects on the redox status of 2-Cys Prxs

Kirchsteiger *et al.* (2009) reported recently that the redox status of plastidial 2-Cys Prx is imbalanced in NTRC

knock-out plants. Thus, the availability of the *trxx* mutant allowed testing of which of the two pathways, Trx *x* or NTRC, enables the most efficient reduction *in vivo*. For this purpose, the redox status of 2-Cys Prx of leaves harvested during the day from both the *ntrc* and *trxx* mutants was analysed. The ratio of reduced (monomeric) to oxidized (dimeric) forms of 2-Cys Prxs in the *trxx* mutant was indistinguishable from that of the wild-type plants. In clear contrast, reduced 2-Cys Prx was almost undetectable in the *ntrc* mutant (Fig. 6). These results indicate that the redox status of 2-Cys Prx is imbalanced in the absence of NTRC, whereas it is not affected by the absence of Trx *x*, thus suggesting that NTRC is the prevailing pathway for 2-Cys Prx reduction *in vivo*.

Analysis of compensatory effects between different antioxidant systems

The last set of experiments addressed the possibility that the deficiency of NTRC, Trx *x*, or 2-Cys Prxs could alter the expression of alternative antioxidant mechanisms. The amount of *NtrC* transcripts, undetectable in the NTRC knock-out mutant, was not significantly affected in the $\Delta 2cp$ mutant and only slightly increased in the *trxx* mutant (Fig. 7A). Similarly, the amount of Trx *x* transcripts was only slightly affected in the *ntrc* or $\Delta 2cp$ mutants, as compared with wild-type plants (Fig. 7B). The most significant variations were observed for the levels of 2-Cys Prx transcripts: the *ntrc* mutant contained a lower amount of 2-Cys Prx transcripts, significantly reduced for 2-Cys Prx

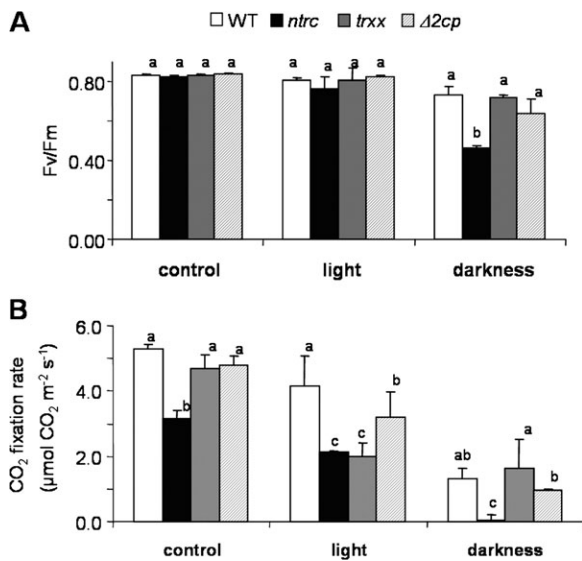


Fig. 5. PSII photochemical efficiency and CO_2 fixation in *Arabidopsis* wild-type and mutant lines. Plants were either grown under standard long-day conditions (control), with continuous light (light), or grown as in control conditions and then subjected to 3 d of continuous darkness (darkness). Then six leaves from different plants of wild-type and mutant lines were used for determination of the initial PSII photochemical efficiency (F_v/F_m) (A) and CO_2 fixation rate, which was determined as described in the Materials and methods at a light intensity of $1000 \mu\text{E m}^{-2} \text{ s}^{-1}$ (B). Values are represented as the means \pm SE. Letters indicate significance of difference ($P \leq 0.05$) as determined by Tukey's multiple range test.

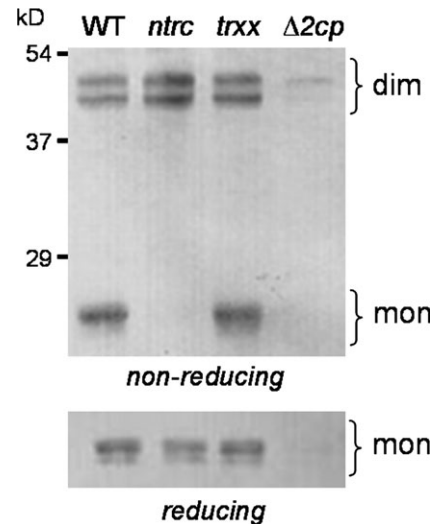


Fig. 6. Redox status of 2-Cys Prx in *Arabidopsis* wild-type and mutant lines. Protein extracts from leaves of the wild type, and *trxx*, *ntrc*, and $\Delta 2cp$ mutants were prepared during the day and subjected to SDS-PAGE (15% acrylamide) under non-reducing (15 μg of protein) and reducing (7.5 μg of protein) conditions, as indicated, electrotransferred onto nitrocellulose sheets, and probed with the anti-2-Cys Prx antibody. Positions of molecular mass markers, in kD, are marked on the left. dim, dimeric partially or fully oxidized form; mon, monomeric reduced forms of 2-Cys Prx.

B, whereas the *trxx* mutant showed wild-type levels of 2-Cys Prx B but an ~2-fold increase in 2-Cys Prx A transcripts (Fig. 7C). In addition, no significant variation of the content of the respective proteins, NTRC, 2-Cys Prxs, or Trx *x*, was observed, except for the total content of 2-Cys Prx in the *ntrc* mutant, which was lower (Fig. 7D). Furthermore, the amounts of the other plastidial Prxs, Prx IIE and Prx Q, were not altered in any of the three mutants analysed (Fig. 7D). Therefore, despite minor variations of the transcript levels in each of the mutants, no compensatory mechanisms based on the induction of NTRC, Trx *x*, or chloroplast-localized Prxs were observed.

In addition, the possibility was addressed that compensatory mechanisms could involve other antioxidants such as Apx or SOD activities. Total soluble Apx activity in leaves from the three mutants was lower than in wild-type plants (Table 2). As Apx is encoded by a gene family in *Arabidopsis*, the expression of the different Apx genes was analysed by qPCR with gene-specific primers. Both the *ntrc* and $\Delta 2cp$ mutants contained reduced amounts of transcripts of all the Apx genes analysed (Fig. 8), which was more pronounced than the observed reduction of Apx activity. The *trxx* mutant showed the lowest level of Apx activity (Table 2) despite having a higher content of Apx transcripts than the *ntrc* and

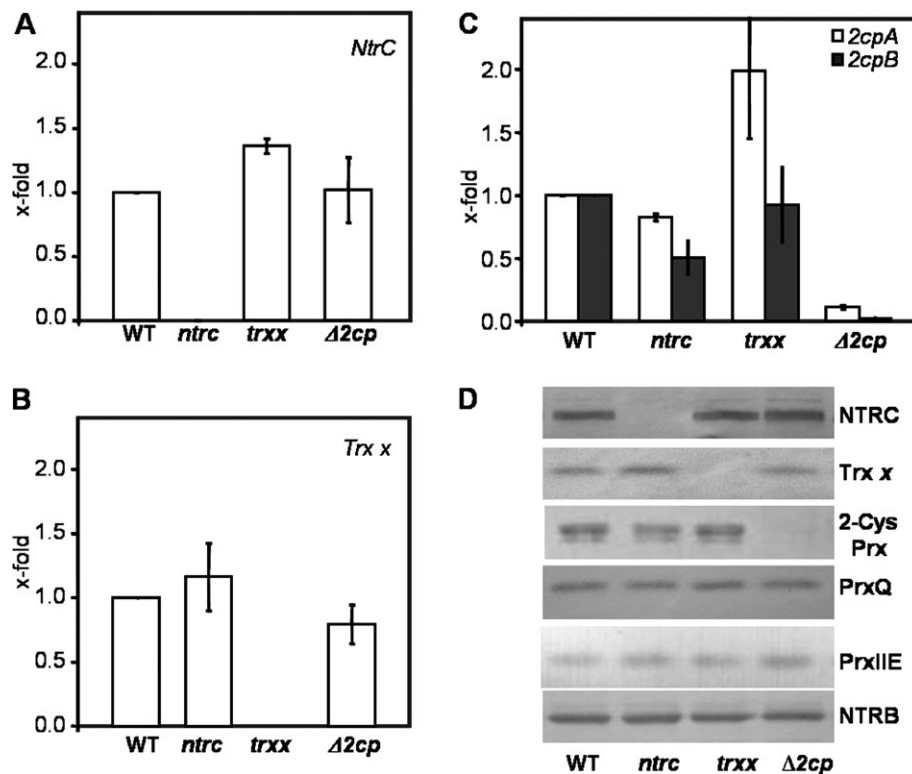


Fig. 7. Analysis of *NtrC*, *Trx x*, and 2-Cys *Prx* expression in *Arabidopsis* wild-type and mutant lines. (A–C) qPCR analysis of *NtrC*, *Trx x*, and *2cpA* and *2cpB* transcripts in leaves from wild-type and mutant lines, as indicated. The transcript amounts are represented as arbitrary units relative to the respective levels in the wild-type plants set to 1.0. Mean values of three independent determinations and the standard errors are represented. (D) Western blot analysis of protein contents of NTRC, *Trx x*, and the four chloroplast-localized Prxs in *Arabidopsis* wild-type and mutant lines, as indicated. Protein samples (15 μ g of protein, except for 2-Cys Prx where 7.5 μ g were loaded) extracted from leaves of wild-type and mutant lines were subjected to SDS–PAGE (12–15% acrylamide) under reducing conditions, electrotransferred onto nitrocellulose sheets, and probed with the corresponding antibody shown on the right. The anti-NTRB antibody was used to check equal loading.

Table 2. Apx activity, glutathione, and AsA pools from *Arabidopsis* wild type and mutant lines

	Wild type	<i>ntrc</i>	<i>trxx</i>	$\Delta 2cp$
Apx activity (μ mol AsA $\text{min}^{-1} \text{mg}^{-1}$ protein)	1.7 \pm 0.03 a	1.5 \pm 0.1 ab	1.0 \pm 0.05 c	1.33 \pm 0.04 b
AsA+DHA (μ mol AsA g^{-1} FW)	1.60 \pm 0.12 c	2.78 \pm 0.73 a	2.70 \pm 0.72 a	2.44 \pm 0.13 b
Reduced AsA/(AsA+DHA)	0.56 \pm 0.027 a	0.61 \pm 0.040 b	0.69 \pm 0.022 c	0.52 \pm 0.033 a
Total GSH (nmol g^{-1} FW)	213 \pm 9.8 a	252 \pm 15.9 a	210 \pm 16.1 a	252 \pm 22.6 a
Reduced GSH/total GSH	0.83 \pm 0.07 a	0.79 \pm 0.05 ab	0.75 \pm 0.006 ab	0.68 \pm 0.007 b

Values are expressed as means \pm SE of four different extracts from two independent experiments. Values with different letters are significantly different ($P \leq 0.05$) as determined by Tukey's multiple range test.

trxx mutants (Fig. 8). The analysis of SOD activity was performed by native IEF, which enabled separation of the six different isoforms. On the basis of their inhibition by KCN and H₂O₂ (not shown), three of the isoforms were identified as Fe-SODs, one as Mn-SOD, and two as CuZn-SODs (Fig. 9). Total SOD activity was considerably reduced in the *trxx* mutant, which showed reduced levels of all isoforms (Fig. 9). SOD activity of the *ntrc* mutant was similar to that of the wild type, because the reduced level of Fe-SOD and Mn-SOD was compensated for by the increase in CuZn-SOD, in contrast to the $\Delta 2cp$ mutant, which showed reduced CuZn-SOD but increased Fe-SOD and Mn-SOD (Fig. 9).

Finally, the content and redox state of AsA and glutathione was also analysed. For unknown reasons, wild-type plants showed a high degree of oxidation of the AsA pool (Table 2), which might be due to the time (hour of the day) of harvesting and the age of the plants, both factors showing an influence on the AsA redox state. Nevertheless, results obtained for wild-type and mutant plants were comparable because the extracts were obtained from plants grown under the same conditions and harvested at the same time. The total amount of AsA was significantly increased in the three mutants, but the proportion of reduced AsA was increased only in the *ntrc* and *trxx* mutants and not in the $\Delta 2cp$ mutant (Table 2). The redox state of glutathione was altered in all of the three mutants; however, the most significant change was observed in the 2-Cys Prx-deficient plants (Table 2).

Discussion

The reducing power necessary for Prx-dependent peroxide reduction is provided either by NADPH, in a reaction

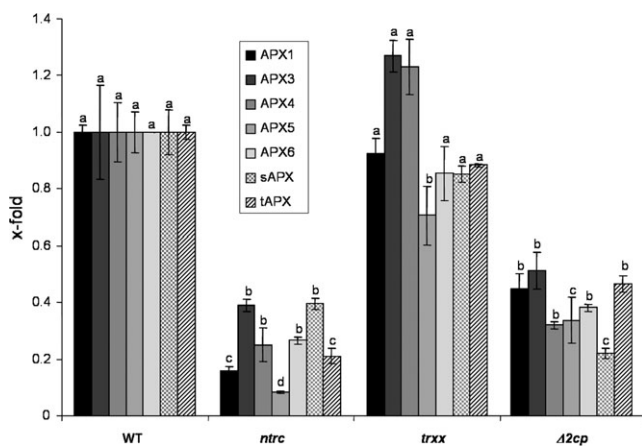


Fig. 8. Expression analysis of the Apx gene family in *Arabidopsis* wild-type and mutant lines. RNA samples isolated from leaves of the corresponding *Arabidopsis* wild-type and mutant lines were retro-transcribed and the amount of transcripts of the Apx genes, as indicated, was determined by qPCR. Values are represented as arbitrary units relative to the respective levels in the wild-type plants set to 1.0. Mean values of three independent determinations and the standard errors are represented. Letters indicate significance of difference ($P \leq 0.05$) as determined by Tukey's multiple range test comparing each gene in wild-type and mutant lines.

catalysed by NTRC (Moon *et al.*, 2006; Pérez-Ruiz *et al.*, 2006; Alkilfioui *et al.*, 2007), or by reduced Fd through the action of different plastidial Trxs, of which Trx *x* is the most efficient (Broin *et al.*, 2002; Collin *et al.*, 2003, 2004; Rey *et al.*, 2005). Previous *in vitro* activity assays suggested that NTRC is a more efficient catalyst than Trx *x* in terms of kinetic parameters (Pérez-Ruiz *et al.*, 2006). While the catalytic activity, the conformational dynamics, the reductive regeneration, and the function in the context of photosynthesis have been described (Baier and Dietz, 1999; König *et al.*, 2002; Collin *et al.*, 2004; Dietz *et al.*, 2006; Pérez-Ruiz *et al.*, 2006), the precise role of 2-Cys Prxs in chloroplast physiology still awaits clarification. Likewise, the physiological significance of the two pathways of reducing power transfer to 2-Cys Prx is not known. The aim of this work was to analyse this relationship by a comparative analysis of *Arabidopsis* mutants.

First, the functional relevance of chloroplast 2-Cys Prxs was addressed. Among the different peroxide detoxification systems of the chloroplast, Prxs are characterized by a low overall catalytic efficiency due to slow regeneration, which is compensated for by their high abundance and the capacity of these enzymes to reduce not only H₂O₂ but also organic peroxides (König *et al.*, 2002; Rouhier and Jacquot, 2002). These characteristics suggest an important role for Prxs in chloroplast peroxide detoxification. This notion is further supported by the fact that four of the 10 Prxs encoded by the *Arabidopsis* genome are targeted to the chloroplast (Bréhélin *et al.*, 2003; Horling *et al.*, 2003). *Arabidopsis* mutants deficient in individual chloroplast-localized Prxs (2-Cys Prx A, 2-Cys Prx B, Prx Q, and Prx

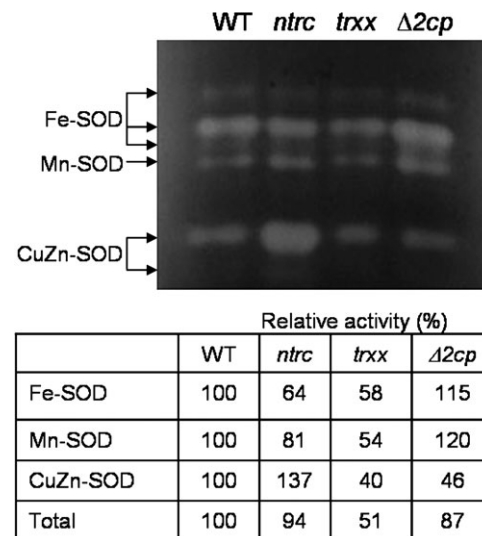


Fig. 9. In-gel assay of SOD activity in *Arabidopsis* wild-type and mutant lines. Protein extracts from leaves (80 μ g of protein) were subjected to IEF (pH 3–7) and stained with NBT. SOD isoforms are indicated on the left and each band was quantified and expressed as a percentage of each isoform from the mutants in comparison with the wild type, which was arbitrarily considered 100%. The assay was repeated at least three times, and a representative gel is shown.

IIE) show no significant difference in phenotype as compared with wild-type plants when grown under standard conditions (Lamkemeyer *et al.*, 2006; Petersson *et al.*, 2006; Romero-Puertas *et al.*, 2007; Kirchsteiger *et al.*, 2009), thus suggesting redundant functions for these and other peroxidases. The function of 2-Cys Prx was previously addressed by the construction of antisense plants, which showed a phenotype with alterations in developmental and photosynthetic parameters during early development of seedlings on agar plates (Baier and Dietz, 1999; Baier *et al.*, 2000). However, the altered phenotype disappeared during further plant development as the antisense plants accumulated 2-Cys Prx protein to wild-type amounts during leaf maturation. As a consequence, the effects of 2-Cys Prx deficiency on performance of mature photosynthesizing leaves still need clarification. Thus compared with the studies with leaky antisense constructs, the newly generated double mutant, $\Delta 2cp$, which contains no 2-Cys Prx B and only trace amounts of 2-Cys Prx A (Fig. 1), represents a novel genetic model to study the physiological significance of 2-Cys Prx since the genetic defect is stable. The deficiency of 2-Cys Prx provoked alterations in the redox homeostasis as shown by the accumulation of H_2O_2 in leaves and the higher level of protein carbonylation (Table 1), which is indicative of the involvement of these enzymes in the mechanism of peroxide detoxification of the chloroplast. However, despite these symptoms of altered redox homeostasis, only minor differences were detected in the fresh weight and pigment content of leaves of the $\Delta 2cp$ mutant plants (Fig. 3). Moreover, photosynthetic efficiency, as determined by F_v/F_m and the CO_2 assimilation rate, was either not altered under control conditions or slightly reduced in $\Delta 2cp$ mutant plants treated with continuous light or prolonged darkness (Fig. 5). Therefore, though 2-Cys Prxs are involved in the maintenance of the redox homeostasis of the chloroplast, the redox imbalance observed can be compensated for by alternative chloroplast antioxidant and repair systems without a significant loss of fitness. Apparently, these enzymes are not essential to protect the chloroplast against oxidative damage, at least under standard growth conditions. This notion was further supported by the similar effect of increasing concentrations of methyl viologen on leaf discs from wild-type and 2-Cys Prx-deficient plants (Fig. 4).

The major objective of this work was to establish which of the two pathways of 2-Cys Prx reduction, NTRC or Trx *x*, is more relevant *in vivo*. The analysis focused on the comparison of the previously reported *ntrc* mutant (Serrato *et al.*, 2004) with a mutant characterized in this work, *trxx*, which is a knock-out for Trx *x* (Fig. 2). The deficiency of each of these components provoked alterations in redox homeostasis as shown by the higher level of protein carbonylation, though to a lesser extent than in the 2-Cys Prx-deficient plants (Table 1). The deficiency of NTRC caused a dramatic effect on plant growth (Fig. 3), in concordance with the significant loss of photosynthetic efficiency of the *ntrc* mutant both under standard growth conditions and in response to continuous light or darkness

treatments (Fig. 5). In a converse manner, the absence of Trx *x* did not affect F_v/F_m and modified the CO_2 assimilation rate only during continuous light treatment (Fig. 5). The limited effect of Trx *x* deficiency on the photosynthetic parameters analysed here is in agreement with the lack of negative effect on growth or pigment content of the mutant plants (Fig. 3). Therefore, the comparison of the two mutants suggests a higher overall physiological relevance of NTRC.

Concerning the specific roles of NTRC and Trx *x* in 2-Cys Prx reduction, the redox status of 2-Cys Prx in the respective mutants was analysed by gel electrophoresis under non-reducing conditions. The reduced 2-Cys Prx, which is detected as a monomeric form in denaturing gels, was hardly detectable in the *ntrc* mutant. In contrast, the level of reduced 2-Cys Prx in the *trxx* mutant was similar to that of the wild-type plants (Fig. 6), thus showing that the deficiency of NTRC, but not of Trx *x*, caused the imbalance in the redox status of this enzyme. In agreement with these results, the level of hydrogen peroxide, which was increased in the *ntrc* and $\Delta 2cp$ mutants, was unaltered in the *trxx* mutant (Table 1). Altogether, the results suggest that the NTRC pathway is more important than the FTR/Trx *x* pathway for 2-Cys Prx reduction *in vivo*, in agreement with the higher catalytic regeneration efficiency of NTRC observed *in vitro* with the purified recombinant enzymes (Pérez-Ruiz *et al.*, 2006). The recent finding, based on FRET (fluorescence resonance energy transfer) analysis, that NTRC interacts with 2-Cys Prx A but not with Trx *x* in protoplasts from *Arabidopsis* leaves (Muthuramalingam *et al.*, 2009), lends further support to this proposal.

Though the 2-Cys Prx-deficient mutant showed slightly retarded growth compared with the wild-type plant, its phenotype was less severe than the phenotype shown by the *ntrc* mutant (Fig. 3). Moreover, the *ntrc* mutant was more sensitive than the $\Delta 2cp$ mutant to continuous light and prolonged darkness in terms of F_v/F_m and CO_2 fixation (Fig. 5). The different phenotypes of the *ntrc* and $\Delta 2cp$ mutants suggest that beside the function of NTRC exerted through the efficient reduction of 2-Cys Prx, the enzyme may play additional roles not related to the 2-Cys Prx. One of the functions already established of NTRC in conjunction with 2-Cys Prx is the activation of protochlorophyllide synthesis, which takes place through the stimulation of an aerobic cyclase by the NTRC/2-Cys Prx system, as observed *in vitro* (Stenbaek *et al.*, 2008). Beyond the functions of NTRC in the context of 2-Cys Prx reduction, NTRC was recently implicated in the mechanism of redox activation of ADP-glucose pyrophosphorylase (AGPase), and thus in starch metabolism both in leaves and in roots (Michalska *et al.*, 2009). In addition, transcript and metabolite profiling revealed altered contents of amino acids and auxin in the *ntrc* mutant, a phenotype which was more severe under short-day conditions (Lepistö *et al.*, 2009). It is therefore likely that the activity of NTRC as a protein disulphide reductase (Pérez-Ruiz and Cejudo, 2009; Pérez-Ruiz *et al.*, 2009) adjusts the redox status of several target proteins involved in different metabolic and/or signalling pathways,

thus explaining the pleiotropic phenotype of the NTRC knock-out plant.

The deficiency of NTRC, but not of Trx *x* or 2-Cys Prx, provoked a lower rate of CO₂ fixation under control conditions (Fig. 5), which is additional evidence of the unique role of NTRC in different aspects of chloroplast function. As expected, a pre-treatment of plants with prolonged darkness, which affected the rate of CO₂ fixation in all mutants, showed a more severe effect in the *ntrc* mutant, in agreement with previous data showing hypersensitivity of this mutant to prolonged darkness (Pérez-Ruiz *et al.*, 2006). Surprisingly, continuous light, which might favour the FTR/Trx *x* pathway, provoked a similar decrease of the CO₂ fixation rate in the *ntrc* and the *trxx* mutants, suggesting that Trx *x* may become important under certain environmental conditions.

Despite the different symptoms of imbalanced redox homeostasis detected in the three mutants, *ntrc*, *trxx*, and $\Delta 2cp$, only the *ntrc* mutant showed a clear phenotypic variation. The results suggest that the antioxidant function of these proteins might be compensated for by alternative chloroplast antioxidant systems. Despite minor variations in the expression of the different chloroplast-localized Prxs, NTRC, and Trx *x*, no significant induction of these systems was detected in any of the mutants (Fig. 7). Moreover, although CuZn-SOD activity was increased in the *ntrc* mutant, total SOD and Apx activities did not increase in any of the three mutants (Table 2, Fig. 9). In the case of the *ntrc* and the $\Delta 2cp$ mutants, the decrease of Apx activity was expected since these plants showed a lower content of transcripts of the Apx gene family (Fig. 8). Thus, the possible compensatory effect exerted by additional detoxification systems seems not to require the induction of such systems. Similarly, *Arabidopsis* mutants deficient in stromal or thylakoid Apx (Maruta *et al.*, 2010) or the double mutant deficient in both chloroplast-localized Apxs (Kangasjärvi *et al.*, 2008) show no altered phenotype when grown under standard conditions, nor induction of the chloroplast-localized Prxs unless plants are subjected to treatments such as high light and, particularly, high light combined with high temperature (Kangasjärvi *et al.*, 2008).

Despite the lack of induction of alternative enzymatic antioxidants, all three mutants showed increased levels of the AsA pool, and the reduction state (ratio AsA/AsA+DHA) was increased in the *ntrc* and *trxx* mutants (Table 2). Moreover, although no significant differences were detected in the total content of glutathione, its redox balance was altered, showing a lower proportion of reduced glutathione, which was most significant in the $\Delta 2cp$ mutant (Table 2). Therefore, the redox buffer capacity of non-enzymatic antioxidants such as glutathione or AsA may be important to compensate for the deficiency of any of the enzymatic antioxidant systems analysed here.

In summary, the results reported here show that the deficiency of NTRC, Trx *x*, or 2-Cys Prx affects the redox homeostasis of the chloroplast. However, the moderate differences in phenotype caused by the deficiency of Trx *x* and 2-Cys Prx, in contrast to the dramatic effect caused by

the deficiency of NTRC, strongly supports the central role of NTRC in the redox regulation of the chloroplast, not only related to the response of the plant to oxidative stress.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. (A) Schematic representation of the structure of *2cpA* and *2cpB* genes from *Arabidopsis* showing the location of the T-DNA insertions, as previously reported (Kirchsteiger *et al.*, 2009), used for construction of the double mutant. The position of the oligonucleotides used for PCR analysis is marked with arrows. (B) PCR analysis of the presence of the T-DNA insertions in genomic DNA isolated from the wild type and homozygous double mutant (Hom) for both insertions. DNA size markers, in kbp, were loaded on the left. T denotes the primer specific for the T-DNA insertion, while a, a', b, and b' represent the gene-specific primers as shown in A.

Figure S2. (A) Schematic representation of the structure of the Trx *x* gene from *Arabidopsis* showing the position of the T-DNA insertion in line SALK_125897 and the oligonucleotides used for PCR analysis. (B) PCR analysis of genomic DNA isolated from the wild type and a homozygous line for the T-DNA insertion in the *trxx* gene. DNA size markers, in kbp, are shown on the left. T denotes the primer specific for the DNA insertion; c and d represent the gene-specific primers as shown in A.

Table S1. Gene-specific oligonucleotides used for qPCR analysis.

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