RESEARCH PAPER

The contribution of NADPH thioredoxin reductase C (NTRC) and sulfiredoxin to 2-Cys peroxiredoxin overoxidation in Arabidopsis thaliana chloroplasts

Leonor Puerto-Galán, Juan M. Pérez-Ruiz, Manuel Guinea* and Francisco Javier Cejudo†

Instituto de Bioquímica Vegetal y Fotosíntesis, Universidad de Sevilla-CSIC, Avda Américo Vespucio, 49, 41092-Sevilla, Spain

* Present address: Umea Plant Science Centre, Department of Plant Physiology, Umea University, S-901 87 Umea, Sweden
† To whom correspondence should be addressed. E-mail: fjcejudo@us.es

Received 26 September 2014; Revised 27 November 2014; Accepted 27 November 2014

Abstract

Hydrogen peroxide is a harmful by-product of photosynthesis, which also has important signalling activity. Therefore, the level of hydrogen peroxide needs to be tightly controlled. Chloroplasts harbour different antioxidant systems including enzymes such as the 2-Cys peroxiredoxins (2-Cys Prxs). Under oxidizing conditions, 2-Cys Prxs are susceptible to inactivation by overoxidation of their peroxidatic cysteine, which is enzymatically reverted by sulfiredoxin (Srx). In chloroplasts, the redox status of 2-Cys Prxs is highly dependent on NADPH-thioredoxin reductase C (NTRC) and Srx; however, the relationship of these activities in determining the level of 2-Cys Prx overoxidation is unknown. Here we have addressed this question by a combination of genetic and biochemical approaches. An Arabidopsis thaliana double knockout mutant lacking NTRC and Srx shows a phenotype similar to the ntrc mutant, while the srx mutant resembles wild-type plants. The deficiency of NTRC causes reduced overoxidation of 2-Cys Prxs, whereas the deficiency of Srx has the opposite effect. Moreover, in vitro analyses show that the disulfide bond linking the resolving and peroxidatic cysteines protects the latter from overoxidation, thus explaining the dominant role of NTRC on the level of 2-Cys Prx overoxidation in vivo. The overoxidation of chloroplast 2-Cys Prxs shows no circadian oscillation, in agreement with the fact that neither the NTRC nor the SRX genes show circadian regulation of expression. Additionally, the low level of 2-Cys Prx overoxidation in the ntrc mutant is light dependent, suggesting that the redox status of 2-Cys Prxs in chloroplasts depends on light rather than the circadian clock.

Key words: Arabidopsis thaliana, chloroplast, 2-Cys peroxiredoxin, overoxidation, redox regulation, sulfiredoxin, thioredoxin reductase.

Introduction

Aerobic metabolism inevitably produces reactive oxygen species (ROS) including singlet oxygen, superoxide anions, or hydrogen peroxide. These species are highly oxidizing and may have a toxic effect, though they also exert important signalling activity. Hydrogen peroxide affects the expression of a large set of genes in plants (Vandenabeele et al., 2003; Vanderauwera et al., 2005), and is involved in different signal transduction pathways (Pitzschke et al., 2006; Laloi et al., 2007). In order to balance the toxic and signalling activities of hydrogen peroxide, its intracellular concentration needs to be tightly regulated. In this regard the activity of 2-Cys peroxiredoxins (2-Cys Prxs) has been proposed to exert a relevant function. 2-Cys Prxs are thiol-based peroxidases classified as typical and atypical, depending on whether they have dimeric...
or monomeric structures, respectively (Rhee and Woo, 2011). Both typical and atypical 2-Cys Prxs have a common reaction mechanism, which depends on two cysteine residues, termed peroxidatic and resolving. The thiolate form of the peroxidatic cysteine reacts with the peroxide and becomes transiently oxidized as sulfenic acid (\(-\text{SOH}\)), which then reacts with the resolving cysteine to render both cysteines oxidized as a disulfide bridge (Rhee et al., 2012). For a new catalytic cycle, this disulfide has to be reduced in a reaction catalysed by a protein disulphide reductase, which is most frequently thioredoxin (Trx), though glutaredoxins (Grx) and cyclophilins might also be involved (Dietz, 2011). However, in the presence of hydrogen peroxide, the sulfenic intermediate may become overoxidized to sulfonic (\(-\text{SO}_2\text{H}\)) or even sulfonic (\(-\text{SO}_3\text{H}\)) acid leading to the inactivation of the enzyme (Yang et al., 2002). 2-Cys Prx overoxidation not only causes the inactivation of the peroxidase activity, but also induces the oligomerization and chaperone activity of the enzyme (Jang et al., 2004). The sulfenic form of 2-Cys Prxs can be converted back into the sulfenic form in an ATP-dependent reaction catalysed by sulfiredoxin (Srx) (Biteau et al., 2003; Woo et al., 2003).

In eukaryotic organisms, the interaction of the peroxidatic and resolving cysteines is limited by two motifs, GGLG and YF, at the C-terminal region of the 2-Cys Prxs; this structural constraint makes the sulfenic intermediate more susceptible to overoxidation (Wood et al., 2003). It has been proposed that the inactivation of 2-Cys Prxs by overoxidation results in a gain of function of the eukaryotic enzymes promoting a further increase of hydrogen peroxide concentration, which may thus be used for signalling purposes: the so-called floodgate hypothesis (Wood et al., 2003). In contrast, 2-Cys Prxs of prokaryotic organisms, with the exception of some cyanobacterial strains (Pascual et al., 2010), are less sensitive to inactivation by overoxidation so that hydrogen peroxide is efficiently detoxified and has less relevance as a second messenger in these organisms. More recently, the circadian oscillation of 2-Cys Prx overoxidation has been shown, which persists without transcription in mammalian and algal cells (O’Neill and Reddy, 2011; O’Neill et al., 2011). The extension of these analyses to other organisms led to the proposal that 2-Cys Prx overoxidation constitutes a conserved marker of circadian rhythms, thus linking the redox status of the cell with the circadian clock (Edgar et al., 2012).

In plants, Prxs are encoded by a gene family formed by 9–10 genes (Dietz, 2003). Remarkably, four of these Prxs, typical 2-Cys Prx A and 2-Cys Prx B, and atypical Prx Q and Prx IIE, are localized in the chloroplast in Arabidopsis thaliana (Dietz, 2003), typical 2-Cys Prxs being among the most abundant proteins of this organelle (Dietz et al., 2006). As in other organisms, the catalytic cycle of chloroplast 2-Cys Prxs requires the reduction of the disulfide bridge linking the peroxidatic and resolving cysteines. The Trx-like CDS32 (Broin et al., 2002), Trx x (Collin et al., 2003) and an NADPH thioredoxin reductase (NTR) with a joint Trx domain at the C-terminus, termed NTRC (Serrato et al., 2004), have been proposed as putative reductants of 2-Cys Prxs in chloroplasts. NTRC, which is exclusive to oxygenic photosynthetic organisms, is localized in plastids (Serrato et al., 2004; Moon et al., 2006; Kirchsteiger et al., 2012) and assays in vitro revealed that it is a very efficient reductant of 2-Cys Prxs (Moon et al., 2006; Pérez-Ruiz et al., 2006; Alkalifiou et al., 2007; Pérez-Ruiz and Cejudo, 2009); this has been further supported by analyses in vivo (Kirchsteiger et al., 2009; Muthuramalingam et al., 2009; Pulido et al., 2010).

As mentioned above, 2-Cys Prxs have emerged as key players in the connection of cell redox homeostasis and circadian rhythms (Edgar et al., 2012). However, the biochemical mechanism that allows the oscillation of 2-Cys Prx overoxidation is unknown (Stangherlin and Reddy, 2013). It is assumed that overoxidation is favoured by oscillating oxidizing conditions, while the decrease of the level of overoxidation might be due to Srx activity. However, there are organisms lacking Srx that still show circadian oscillation of 2-Cys Prx overoxidation (Stangherlin and Reddy, 2013). 2-Cys Prxs from chloroplasts undergo overoxidation (Kirchsteiger et al., 2009); in addition, these organelles harbour both NTRC, an efficient system for 2-Cys Prx reduction (Kirchsteiger et al., 2009), and Srx (Liu et al., 2006; Rey et al., 2007; Iglesias-Baena et al., 2010), the enzymes that may play a central role determining the redox status of 2-Cys Prxs (Puerto-Galán et al., 2013). Thus, plant chloroplasts constitute an excellent system for elucidating the biochemical mechanisms controlling any oscillation of 2-Cys Prx overoxidation and its relationship with the redox status of this organelle.

In this work we have analysed the contribution of NTRC and Srx to the redox status of chloroplast 2-Cys Prxs by a combination of genetic and biochemical approaches. Moreover, we have analysed whether the NTRC and SRX genes are involved in determining any oscillation of 2-Cys Prx overoxidation in plant chloroplasts. To that end, we have generated an ntrc-srx double mutant of Arabidopsis, the phenotype of which, as compared with the srx and ntrc single mutants, shows that the deficiency of NTRC exerts a dominant effect over the deficiency of Srx on plant performance. This notion was further supported by studies in vitro showing that the reduction of the disulfide bridge linking the peroxidatic and resolving cysteines is necessary for the overoxidation of plastidial 2-Cys Prxs. In addition, an NTRC-independent, light-dependent component contributing to the redox status of chloroplast 2-Cys Prxs was uncovered. Overall, our data suggest that 2-Cys Prx overoxidation in plant chloroplasts responds to light rather than to circadian oscillations.

Materials and methods

Growth conditions and plant material

Arabidopsis thaliana wild-type (ecotype Columbia) and mutant plants were routinely grown in soil in growth chambers under long-day (16-h light/8-h dark) or short-day (8-h light/16-h dark) conditions at 22°C during the light and 20°C during the dark and a light intensity of 140 μE m⁻² s⁻¹. The ntrc mutant, SALK_012208, has previously been reported (Serrato et al., 2004). A homozygous line, SALK_05324 (Alonso et al., 2003), with a T-DNA insertion at the single gene encoding Srx (AT1G31170) from Arabidopsis, here termed the srx mutant, was selected by PCR analysis with the oligonucleotides described in Supplementary Table S1. These ntrc and
srx single mutants were used to obtain the ntrc-srx double mutant by manual crossing. Seeds resulting from this cross were checked for heterozygosity of the T-DNA insertions in the NTRC and Srx genes. Plants were then self-crossed and double homozygous lines were identified in the progeny by PCR analysis of genomic DNA using oligonucleotides described in Supplementary Table S1. Seeds were surface sterilized using chlorine gas for 16 h, plated on germination media, Murashige and Skoog medium (Duchefa), pH 5.8 containing 0.6% Gelrite (Duchefa) and 0.5% (w/v) sucrose and stratified at 4°C for 2–3 days. For circadian experiments, seedlings were grown for 10 days under long-day conditions and harvested at 4 h-intervals during a period of 24 h (16-h light/8-h dark), which was followed by a second 24-h period under continuous light or continuous darkness. Samples were immediately frozen in liquid nitrogen and kept at −80°C until required.

RNA extraction and RT-qPCR analysis
Total RNA was extracted using Trizol reagent (Invitrogen). cDNA synthesis was performed with 5 μg of total RNA using the Maxima first-strand cDNA synthesis kit (Fermentas) according to manufacturer’s instructions. Real-time quantitative PCR (RT-qPCR) was performed using an IQ5 real-time PCR detection system (Bio-Rad). A standard thermal profile (95°C, 3 min; 40 cycles at 95°C for 10 s, and 60°C for 30 s) was used for all reactions. After the PCR, a melting curve analysis (55–94°C at 0.5°C/30s.) was performed to confirm the specificity of the amplicon and to exclude primer-dimers or non-specific amplification. Oligonucleotides used for RT-qPCR analyses are described in Supplementary Table S2. Expression levels were normalized using four reference genes: ACTIN and 18S rRNA (Woodson et al., 2013), UBIQUITIN (Ventriglia et al., 2008), and the Ser/Thr protein phosphatase 2A subunit A3 (PP2A-A3) (Czechowski et al., 2005). Oligonucleotides for Srx gene analyses were designed in exons 4 and 5 of splice variants AT1G31170.1, AT1G31170.2, and AT1G31170.3; the splice variant AT1G31170.4 was not amplified.

Protein extraction and western blot analysis
For protein extraction, plant tissues were ground with a mortar and pestle in liquid nitrogen. Extraction buffer [100 mM Tris-HCl pH 7.9; 1% (v/v) glycerol; 1 mM EDTA; 10 mM MgCl₂; 1 mM PMSF; and 1% (v/v) protease inhibitor cocktail for plant cell and tissue extracts (Sigma-Aldrich)] was immediately added, and the sample given a swirl on a vortex, and then centrifuged at 13 000g at 4°C for 20 min. Total protein content was quantified using the Bradford reagent (Bio-Rad) and proteins were subjected to SDS-PAGE, under reducing or non-reducing conditions, as indicated in the figures legends. Western blots were performed as previously described (Kirchsteiger et al., 2009). The anti-2-Cys Prx antibody was diluted in the same blocking buffer overnight at 4°C. The abundance of 2-Cys Prx in Arabidopsis chloroplasts allowed the redox status of these proteins to be studied in leaf extracts and, thus, isolation of chloroplasts was not necessary.

Expression and purification of recombinant 2-Cys Prxs A and B and analysis of overoxidation in vitro
Recombinant 2-Cys Prxs A and B from Arabidopsis were produced in Escherichia coli XL1-Blue with a His-tag at the N-terminus, as previously described (Kirchsteiger et al., 2009). Recombinant proteins were purified from crude extracts of E. coli cultures by chromatography in pre-packed Hi-Trap affinity columns (GE Healthcare). Recombinant proteins, 2-Cys Prxs A and B, at a concentration of 0.1 μg μl⁻¹ in 100 mM phosphate buffer (pH 7.4), 0.5 M NaCl, and 10% (v/v) glycerol, were incubated for 30 min with or without 20 mM DTT and then for another period of 30 min with increasing concentrations of H₂O₂ (0.1–10 mM). Protein samples (0.125 μg of protein) were subjected to SDS-PAGE, under reducing or non-reducing conditions, blotted on nitrocellulose membranes and probed with anti-SO₂₃ antibody.

Determination of chlorophylls and the Fv/Fm ratios
Leaf discs were weighed and frozen in liquid N₂. After extraction in 1 ml methanol for 16 h at 4°C, chlorophyll levels were measured spectrophotometrically, as described in Porra et al. (1989), and normalized to fresh weight. Room temperature chlorophyll 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, Fv, to maximal fluorescence, Fm (Fv/Fm).

Results
NTRC exerts a dominant effect over Srx on 2-Cys Prx overoxidation
Previous analyses have shown that the level of overoxidation of plastidial 2-Cys Prxs is highly dependent on NTRC (Kirchsteiger et al., 2009) and Srx (Iglesias-Baena et al., 2010), but the contribution of these activities to maintain the redox status of 2-Cys Prxs is not yet known. To address this question an Arabidopsis double knockout mutant deficient in NTRC and Srx was obtained by manual crossing of the respective single mutants. The double mutant, termed ntrc-srx, was effectively deficient in both transcripts as shown by RT-qPCR analysis (Fig. 1A). In agreement with previous results (Liu et al., 2006; Rey et al., 2007; Iglesias-Baena et al., 2010), the srx mutant showed a visual phenotype very similar to wild-type plants, whereas the ntrc mutant showed the characteristic growth retarded, pale-green leaf phenotype (Fig. 1B, C), as previously described (Serrato et al., 2004; Pérez-Ruiz et al., 2006; Lepistö et al., 2009). The phenotype of the ntrc-srx double mutant is very similar to the ntrc mutant and is also sensitive to photoperiod, short-day conditions resulting in a more severe effect (Fig. 1B) than long-day conditions (Fig. 1C). The contents of chlorophylls a and b, which were characteristically lower in leaves of ntrc mutant plants, were decreased at the same level in the ntrc-srx double mutant, in contrast with the srx mutant, which showed wild-type levels of these pigments (Fig. 2A). Finally, a treatment of continuous darkness showed a similar effect on photosystem II (PSII) efficiency, as determined by the Fv/Fm ratio, in the ntrc-srx double mutant and the ntrc mutant, whereas the srx mutant behaved like the wild-type plants (Fig. 2B). Therefore, the phenotypic characteristics analysed here revealed the high similarity of the ntrc-srx double mutant and the ntrc mutant, whereas the srx mutant was similar to the wild type. These results show that the deficiency of NTRC exerts a dominant effect on the deficiency of Srx on plant phenotype.
Analysis of the redox status of the chloroplast 2-Cys Prxs, based on non-reducing SDS-PAGE, further supported the hypothesis that the ntrc-srx double mutant phenocopies the ntrc single mutant. The deficiency of Srx caused the expected increase in the level of monomeric 2-Cys Prxs, reflecting an increase of the overoxidized form of these enzymes (Fig. 3A); this was confirmed by western blot analysis of samples subjected to SDS-PAGE under reducing conditions, with the anti-SO$_{2}/3$ antibody used as a probe (Fig. 3B), in agreement with previous results (Iglesias-Baena et al., 2010). As previously reported (Kirchsteiger et al., 2009), the level of overoxidized 2-Cys Prxs was much decreased in the ntrc mutant, as shown by the lower content of the monomeric form of the enzyme, which was almost undetectable (Fig. 3A), and
the lower intensity of the band detected with the anti-SO$_{2/3}$ antibody (Fig. 3B). The ntrc-srx double mutant also showed reduced levels of overoxidized 2-Cys Prxs, which were slightly, but consistently, higher than in the ntrc mutant as shown by both the amount of monomer and the anti-SO$_{2/3}$ antibody (Fig. 3A, B). These results suggest that the key step determining the level of 2-Cys Prx overoxidation is the reduction of the disulfide bridge linking the peroxidatic and resolving cysteines of the enzyme, which is highly dependent on NTRC in plant chloroplasts. To test this possibility further, purified recombinant 2-Cys Prxs A and B from Arabidopsis were subjected to treatment with increasing concentrations of hydrogen peroxide. The highest concentration of hydrogen peroxide tested (10 mM) did not cause any overoxidation of oxidized 2-Cys Prxs (Fig. 4). In contrast, when 2-Cys Prxs were pre-reduced with DTT, even low concentrations of hydrogen peroxide caused overoxidation, both 2-Cys Prx A and 2-Cys Prx B showing a similar level of sensitivity (Fig. 4). Therefore, these assays in vitro show that the disulfide form of these enzymes is resistant to overoxidation.

Two components, NTRC-dependent and NTRC-independent, contribute to 2-Cys Prx overoxidation in Arabidopsis chloroplasts

It has been shown that 2-Cys Prx overoxidation in different organisms undergoes circadian rhythmicity (Edgar et al., 2012), though the biochemical basis of this oscillation is not yet known (Stangherlin and Reddy, 2013). Once the effect of NTRC and Srx on the level of chloroplast 2-Cys Prxs overoxidation was established, we tested whether these enzymes are involved in determining any oscillation of the redox status of 2-Cys Prxs in these plant organelles. To address this question we first analysed the pattern of expression of the NTRC and SRX genes in Arabidopsis during a 24-h period...

Fig. 3. The deficiency of NTRC and Srx has opposite effects on chloroplast 2-Cys Prxs overoxidation. (A) Arabidopsis wild-type, ntrc, srx, and ntrc-srx double mutant plants were grown for 10 days under long-day conditions in plates. Protein extracts (12.5 µg) from seedlings were subjected to SDS-PAGE (12% acrylamide), supplemented with 4 M urea under non-reducing conditions, electrotransferred onto nitrocellulose filters, and probed with the anti-2-Cys Prx antibody. (B) The amount of overoxidized 2-Cys Prxs was determined on protein extracts (60 µg) from seedlings of the different lines, which were subjected to SDS-PAGE (12% acrylamide), supplemented with 4 M urea under reducing conditions. Gels were electrotransferred onto nitrocellulose filters, and probed with the anti-SO$_{2/3}$ antibody. For each genotype, band intensity was quantified with the Scion Image analysis software (Scion Corporation) and normalized against that of the indicated band (arrow) in the ponceau S-stained membrane, which is shown as a loading control. Relative densities are referred to the intensity of the wild type, which was set at 1.

Fig. 4. 2-Cys Prxs A and B from Arabidopsis show similar sensitivity to overoxidation. Purified recombinant 2-Cys Prxs A and B (0.125 µg of protein) from Arabidopsis were incubated for 30 min with or without 20 mM DTT; then samples were incubated in the presence of the indicated concentrations of hydrogen peroxide for 30 min. Samples were then subjected to SDS-PAGE (15% acrylamide) under non-reducing conditions, electrotransferred on to nitrocellulose filters and probed with anti-2-Cys Prx or anti-SO$_{2/3}$ antibodies, as indicated. dim, dimer; mon, monomer.

Chloroplast 2-Cys peroxiredoxin overoxidation | 2961
under a long-day photoperiod (16-h light/8-h dark) followed by a period of 24 h in continuous light. To that end, seedlings were harvested at 4-h time intervals and the content of NTRC and SRX transcripts was determined by RT-qPCR. The accumulation of NTRC transcripts did not show any significant variation during the first 24-h period and, though moderately increased during the period of continuous light, no circadian oscillation was observed (Fig. 5A). The pattern of expression of the SRX gene was very similar to the NTRC gene as the amount of SRX transcripts showed a slight increase during the light periods but no circadian oscillation was observed (Fig. 5A). The expression profile of both NTRC and SRX genes is in clear contrast with the pattern of expression of the CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) gene, which was included in these analyses as a positive control of circadian clock-regulated gene expression (Fig. 5B). Moreover, the circadian expression of the CCA1 gene was not impaired in the ntrc mutant, indicating that the deficiency of NTRC does not exert any significant effect on circadian gene expression.

Once the pattern of expression of the NTRC and SRX genes in Arabidopsis was established, we analysed the day/night cycling and circadian profiling of chloroplast 2-Cys Prx overoxidation. In order to identify patterns of 2-Cys Prx overoxidation, we studied the amount of monomeric form under non-reducing conditions as well as the accumulation of overoxidized enzyme, revealed by the anti-SO_{2/3} antibody. In wild-type plants, the level of 2-Cys Prx overoxidation did not show any significant oscillation during the first 24-h period as shown by the amount of monomeric enzymes (Fig. 6A) or with the anti-SO_{2/3} antibody (Fig. 6B). During the period of continuous light, the amount of monomer increased indicating the accumulation of overoxidized 2-Cys Prxs (Fig. 6A), which was confirmed with the anti-SO_{2/3} antibody (Fig. 6B). These results suggest that the level of 2-Cys Prx overoxidation in chloroplasts responds to light, rather than to circadian oscillation. To further study the effect of light, the redox status of the 2-Cys Prxs was analysed in the NTRC knockout mutant. As mentioned above (Fig. 2), the level of 2-Cys Prx overoxidation in the ntrc mutant was much lower than in wild-type plants. However, when higher amounts of extracts of the ntrc mutant were subjected to SDS-PAGE under non-reducing conditions, the monomeric form of the enzymes could be detected allowing analysis of the effect of light on the level of overoxidation of these enzymes (Fig. 7A, B). In the ntrc mutant, the monomeric form of the 2-Cys Prxs was detected exclusively during the light period, but not during darkness, thus showing a light-dependent contribution to chloroplast 2-Cys Prx overoxidation (Fig. 7A). Furthermore, the level of monomer was increased during the 24-h period under continuous light (Fig. 7A), but remained low when the first 24-h period was followed by a period of continuous darkness (Fig. 7B). Due to the low level of 2-Cys Prx overoxidation in the ntrc mutant, the intensity of the bands detected with the anti-SO_{2/3} antibody was not sensitive enough for an accurate determination and, thus, the level of 2-Cys Prx overoxidation was exclusively based on the amount of monomeric enzymes.

**Discussion**

The inactivation of 2-Cys Prxs by overoxidation has been proposed to be a gain of function change which allows important signalling activity of hydrogen peroxide in eukaryotic organisms (Wood et al., 2003). Moreover, transcription-independent rhythmic oscillation of 2-Cys Prx overoxidation in human red blood cells (O’Neill and Reddy, 2011) and the unicellular alga Ostreococcus tauri (O’Neill et al., 2011) has led to the proposal that the overoxidation of 2-Cys Prxs is a marker of circadian rhythms (Edgar et al., 2012). Overoxidation is a post-translational modification, which occurs under oxidizing conditions, the reversion of which requires the participation of Srx. Therefore, Srx may exert an important function in the rhythmic oscillation of 2-Cys Prx overoxidation. Interestingly,
there are organisms that display oscillation of 2-Cys Prx overoxidation but lack Srx (Stangherlin and Reddy, 2013) and, thus, the actual biochemical mechanism that allows this oscillation remains unknown.

In this study we analysed the control of 2-Cys Prx overoxidation taking the Arabidopsis chloroplast as a model system. The Arabidopsis chloroplast harbours two almost identical 2-Cys Prxs, termed A and B, which undergo overoxidation (Kirchsteiger et al., 2009), thus behaving as eukaryotic-type enzymes. It is well known that NTRC, which is an efficient reductant of 2-Cys Prxs, exerts an important role in determining the redox status of these proteins (Pulido et al., 2010).

Moreover, Srx, which is a chloroplast-localized enzyme in plants (Liu et al., 2006; Iglesias-Baena et al., 2011), effectively reduces the sulfinic form of plastidial 2-Cys Prxs (Iglesias-Baena et al., 2010), indicating that this enzyme might also affect the redox status of 2-Cys Prxs in these organelles (Fig. 8).

To establish the interaction of NTRC and Srx in the control of the redox status of chloroplast 2-Cys Prxs, we generated an Arabidopsis double knockout mutant lacking NTRC and Srx. Comparative analysis of the phenotypes of the ntrc-srx double mutant with those of the respective ntrc and srx single mutants clearly indicated the similarity of the double mutant and the ntrc mutant, in contrast with the phenotype of the srx mutant, which resembles the wild-type plants (Figs 1 and 2). These results show that the deficiency of NTRC has a dominant effect over the deficiency of Srx on plant phenotype, and point to the reduction of the disulfide bridge linking the peroxidatic and resolving cysteines of the 2-Cys Prxs as a key step determining the level of overoxidation of these enzymes. The dominant effect of NTRC was confirmed by analysis of the redox status of plastidial 2-Cys Prxs in these mutants. The deficiency of Srx provoked the expected higher level of overoxidized 2-Cys Prx (Fig. 3), in agreement with previous results (Iglesias-Baena et al., 2010). Interestingly, impairment of the redox status of the 2-Cys Prx caused by Srx deficiency has almost no phenotypic effect since srx mutant plants show...
Fig. 8. The control of the redox status of chloroplast 2-Cys Prxs. Typical chloroplast 2-Cys Prxs are homodimeric thiol peroxidases containing a peroxidatic (S\(\cdot\)H) and resolving (S\(\cdot\)H) cysteine residue in each monomer. The thiol form of the peroxidatic cysteine attacks the peroxide and becomes transiently oxidized as sulfenic acid (-S\(\cdot\)OH). This intermediate is then attacked by the resolving cysteine of the other subunit releasing a molecule of water and rendering both cysteines linked by a disulfide bridge. Alternatively, the sulfenic intermediate may be overoxidized to sulfenic acid (-S\(\cdot\)O\(\cdot\)H\(_2\)), which is then reduced by Srx.

no visible phenotypic alterations when grown under standard non-stressful conditions (Fig. 1B, C), or in response to treatments of prolonged darkness (Fig. 2B). The impairment of the redox status of the chloroplast 2-Cys Prxs caused by the deficiency of Srx may be relevant under environmental stresses. However, the function of Srx in the response to stress is not yet clear since it has been reported that the srx mutant shows susceptibility to oxidative stress (Liu et al., 2006; Igleisas-Baena et al., 2010), but also a higher level of tolerance to photooxidative stress (Rey et al., 2007).

As previously reported (Kirchsteiger et al., 2009; Pulido et al., 2010), the ntrc mutant shows a clear deficit of 2-Cys Prx overoxidation (Fig. 3), confirming the primary effect of NTRC on the redox status of these enzymes. However, the finding that the ntrc-srx double mutant shows a slightly higher level of 2-Cys Prx overoxidation compared with the ntrc mutant (Fig. 3) suggests that there is an NTRC-independent component contributing to 2-Cys Prx overoxidation. A more in-depth analysis of the pattern of 2-Cys Prxs overoxidation in the ntrc mutant showed increased overoxidation during the day and remained low during a further 24-h period of continuous darkness (Fig. 7A, B). This result reveals the existence of at least one NTRC-independent redox component contributing to 2-Cys Prx overoxidation. Because the activity of this component was detected exclusively under illumination, it is probably due to the chloroplast FTR/Trx system, which uses photosynthetically reduced ferredoxin as a source of reducing power (Schürmann and Buchanan, 2008). In this regard, it should be mentioned that 2-Cys Prxs are reduced in vitro by Trx x (Collin et al., 2003) and CDSP32 (Broin et al., 2002). Moreover, analyses in vivo showed impairment of the redox status of 2-Cys Prxs in potato plants lacking CDSP32 (Broin and Rey, 2003), and rice Trx m knockdown plants (Chi et al., 2008). Therefore, Trx x and m, as well as the Trx-like protein CDSP32, are candidates to account for the NTRC-independent reduction of 2-Cys Prxs, a prerequisite for the subsequent overoxidation of these enzymes, but determining the contribution of these Trxs still requires further work.

Both the genetic and biochemical analyses reported here indicate that a pre-requisite for the overoxidation of chloroplast 2-Cys Prxs is the reduction of the disulfide bridge linking the peroxidatic and resolving cysteine residues at the active site of these enzymes. This is in line with studies showing that structural motifs limiting the formation of this disulfide bridge, as occurs in enzymes from eukaryotic organisms, provoke sensitivity to overoxidation (Wood et al., 2003). The analysis of 2-Cys Prx 3 from human mitochondria, which is resistant to overoxidation, confirmed that disulfide formation protects it from inactivation (Haynes et al., 2013). Furthermore, the endoplasmic reticulum-localized Prx IV shows a low sensitivity to overoxidation, which is due to the lack of a robust Prx recycling system in this cellular compartment (Cao et al., 2014). Thus, the reduction of the disulfide bridge allows a new catalytic cycle of the enzyme with the formation of the sulfenic intermediate, which may be overoxidized to sulfenic acid (Fig. 8). The severe decrease of the level of 2-Cys Prx overoxidation in the ntrc and the ntrc-srx mutants (Fig. 3) supports the relevance of the disulfide reduction step and, thus, of NTRC in determining the redox status of these enzymes. Once the 2-Cys Prxs become overoxidized, the reversion step catalysed by Srx may be important for adjusting the level of overoxidized enzymes. However, the low phenotypic impact of the deficiency of Srx indicates that this component of the redox regulation of 2-Cys Prxs is less relevant than the disulfide reduction step, at least under standard non-stressful conditions. In this regard, it should be noted that the fraction of overoxidized 2-Cys Prxs was small even in the srx mutant (Fig. 3). Since reversion of overoxidation is so far considered to be exclusively catalysed by Srx, the de novo synthesis of plastidial 2-Cys Prxs may exert a relevant function in the maintenance of the level of overoxidation of these enzymes.

Based on the results reported here, we established the hypothesis that NTRC and Srx might exert key roles in any oscillation of chloroplast 2-Cys Prx overoxidation. However, neither the analysis of the redox status of the 2-Cys Prxs by non-reducing gels (Fig. 6A) nor the identification of overoxidized 2-Cys Prxs with the anti-SO\(\cdot\)x antibody (Fig. 6B) showed rhythmic oscillation of chloroplast 2-Cys Prx overoxidation, in contrast with previous results (Edgar et al., 2012). Our data, however, are in line with the notion that 2-Cys Prx overoxidation may reflect the oxidant status of the chloroplast. According to this, the low variation of the level of expression of the NTRC gene, which does not show circadian oscillation (Fig. 5A), would allow a basal level of 2-Cys Prx reduction and overoxidation, as detected in wild-type plants (Fig. 6A, B). During the day, light would allow additional reduction of the 2-Cys Prxs and, thus, of the level
of overoxidation, most probably through the light-dependent FTR/Trx system. The fact that this component is detected exclusively in the absence of NTRC (Fig. 7A, B) suggests that it has a low contribution to the total level of 2-Cys Prx overoxidation. The pattern of expression of the SRX gene, which is slightly increased under illumination (Fig. 5A), might be a mechanism to equilibrate the excess of overoxidation of 2-Cys Prxs produced during the light period. It should be emphasized that light promotes hydrogen peroxide release from the chloroplast (Mubarakshina et al., 2010), which might be an important component of the signalling activity of this organelle. Moreover, 2-Cys Prxs have been proposed to play a central role determining the signalling activity of the chloroplasts (König et al., 2012). Thus, the mechanisms described here allowing the precise tuning of 2-Cys Prx overoxidation in chloroplasts may be central for the signalling activity of these organelles in response to environmental factors such as light or darkness.

**Conclusion**

It is well established that NTRC and Srx exert a great influence on chloroplast 2-Cys Prx overoxidation; the relationship between these activities in the control of the level of 2-Cys Prx overoxidation, however, is as yet unknown. Here, we analysed this relationship by the generation of a double mutant of Arabidopsis, ntrc-srx, deficient in both NTRC and Srx. The dominant effect of the deficiency of NTRC over the deficiency of Srx suggests that the reaction catalysed by NTRC, i.e. the reduction of the disulfide-linking peroxidatic and resolving cysteine residues, is a pre-requisite for 2-Cys Prx overoxidation. This conclusion was further supported by the finding that this disulfide protects the peroxidatic cysteine from overoxidation in vitro. Analyses of plants grown under a long-day photoperiod followed by a 24-h period of continuous light or continuous darkness revealed no circadian oscillation of 2-Cys Prx overoxidation in Arabidopsis chloroplasts. In line with these observations, neither the NTRC nor the SRX genes show circadian regulation of expression. The analysis of 2-Cys Prx overoxidation in the ntrc mutant uncovered an NTRC-independent component contributing to 2-Cys Prx overoxidation in Arabidopsis chloroplasts. The fact that this component is light dependent, together with the increase of oxidized 2-Cys Prxs in response to continuous light, lends further support to the notion that light, rather than the circadian clock, promotes 2-Cys Prx overoxidation. In addition, these findings suggest that the light-dependent Fd/FTR/Trx redox system may contribute to the overoxidation of chloroplast 2-Cys Prxs. Research to identify the Trx(s) involved is now under way.

**Supplementary material**

**Supplementary Table S1.** Oligonucleotides used for genotyping the srx and ntrc mutants.

**Supplementary Table S2.** Oligonucleotides used for qRT-PCR analysis.

**Funding**

This work was supported by European Regional Development Fund-cofinanced grants from the Spanish Ministry of Economy and Competitiveness (MINECO) (BIO2013-43556-P) and Junta de Andalucía (BIO-182 and CVI-5919). A post-doctoral Juan de la Cierva contract from MINECO to J.M.P.-R., and a pre-doctoral fellowship from Junta de Andalucía (Spain) to M.G. are deeply appreciated.

**References**

Alkhalfioui F, Renard M, Vensel WH, Wong JH, Tanaka CK, Hurkman WJ, Buchanan BB, Montrichard F. 2007. Thioredoxin-linked proteins are reduced during germination of Medicago truncatula seeds. Plant Physiology 144, 1559–1579.

Alonso JM, Stepanova AN, Leisze TJ, et al. 2003. Genome-wide insertional mutagenesis of Arabidopsis thaliana. Science 301, 653–657.

Biteau B, Labarre J, Toledano MB. 2003. ATP-dependent reduction of cysteine-sulphenic acid by S. cerevisiae sulphiredoxin. Nature 425, 980–984.

Broin M, Cuiné S, Eymery F, Rey P. 2002. The plastidic 2-cysteine peroxiredoxin is a target for a thioredoxin involved in the protection of the photosynthetic apparatus against oxidative damage. The Plant Cell 14, 1417–1432.

Broin M, Rey P. 2003. Potato plants lacking the CDS32 plastidic thioredoxin exhibit overoxidation of the BAS1 2-cysteine peroxiredoxin and increased lipid peroxidation in thylakoids under photooxidative stress. Plant Physiology 132, 1335–1343.

Cao Z, Subramaniam S, Bulleid NJ. 2014. Lack of an efficient endoplasmic reticulum-localized recycling system protects peroxiredoxin IV from hyperoxidation. The Journal of Biological Chemistry 289, 5490–5498.

Chi YH, Moon JC, Park JH, et al. 2008. Abnormal chloroplast development and growth inhibition in rice thioredoxin m knock-down plants. Plant Physiology 148, 808–817.

Collin V, Issakidis-Bourguet E, Marchand C, Hirasawa M, Lancelin J-M, Knaat DB, Miginiac-Maslow M. 2003. The Arabidopsis plastidial thioredoxin. New functions and new insights into specificity. The Journal of Biological Chemistry 278, 23747–23752.

Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible WR. 2005. Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. Plant Physiology 139, 5–17.

Dietz K-J. 2003. Plant peroxiredoxins. Annual Review of Plant Biology 54, 93–107.

Dietz K-J. 2011. Peroxiredoxins in plants and cyanobacteria. Antioxidants and Redox Signaling 15, 1129–1159.

Dietz K-J, Jacob S, Oelze M-L, Laxa M, Tognetti V, Nunes de Miranda SM, Baier M, Finkemeier I. 2006. The function of peroxiredoxins in plant organelle redox metabolism. Journal of Experimental Botany 57, 1697–1709.

Edgar RS, Green EW, Zhao Y, et al. 2012. Peroxiredoxins are conserved markers of circadian rhythms. Nature 485, 459–464.

Haynes AC, Qian J, Reisz JA, Furdui CM, Lwthwer WT. 2013. Molecular basis for the resistance of human mitochondrial 2-Cys peroxiredoxin 3 to hyperoxidation. The Journal of Biological Chemistry 288, 29714–29723.

Iglesias-Baena I, Barranco-Medina S, Lázaro-Payo A, López-Jaramillo FJ, Sevilla F, Lázaro JJ. 2010. Characterization of plant sulfiredoxin and role of sulphenic form of 2-Cys peroxiredoxin. Journal of Experimental Botany 61, 1509–1521.

Iglesias-Baena I, Barranco-Medina S, Sevilla F, Lázaro JJ. 2011. The dual-targeted plant sulfiredoxin retroreduces the sulfenic form of atypical mitochondrial peroxiredoxin. Plant Physiology 155, 944–955.

Jang HH, Lee KO, Chi YH, et al. 2004. Two enzymes in one; two yeast peroxiredoxins display oxidative stress-dependent switching from a peroxidase to a molecular chaperone function. Cell 117, 625–635.
Kirksteiger K, Ferrández J, Pascual MB, González M, Cejudo FJ. 2012. NADPH thioredoxin reductase C is localized in plastids of photosynthetic and nonphotosynthetic tissues and is involved in lateral root formation in Arabidopsis. The Plant Cell 24, 1534–1548.

Kirksteiger K, Pulido P, González MC, Cejudo FJ. 2009. NADPH thioredoxin reductase C controls the redox status of chloroplast 2-Cys peroxiredoxins in Arabidopsis thaliana. Molecular Plant 2, 298–307.

König J, Muthuramalingam M, Dietz K-J. 2012. Mechanisms and dynamics in the thiol/disulfide redox regulatory network: transmitters, sensors and targets. Current Opinion in Plant Biology 15, 261–268.

Laloi C, Stachowiak M, Pers-Kamczyc E, Warzych E, Murgia I, Apel K. 2007. Cross-talk between singlet oxygen- and hydrogen peroxide-dependent signaling of stress responses in Arabidopsis thaliana. Proceedings of the National Academy of Sciences, USA 104, 672–677.

Lepistö A, Kangasjärvi S, Luomala EM, Brader G, Sipari N, Keränen M, Keinänen M, Rintamäki E. 2009. Chloroplast NADPH-thioredoxin reductase interacts with photoperiodic development in Arabidopsis. Plant Physiology 149, 1261–1276.

Liu XP, Liu XX, Zhang J, Xia ZL, Liu X, Qin HJ, Wang DW. 2006. Molecular and functional characterization of sulfiredoxin homologs from higher plants. Cell Research 16, 287–296.

Moon JC, Jang HH, Chaeb HB, et al. 2006. The C-type Arabidopsis thioredoxin reductase ANTR-C acts as an electron donor to 2-Cys peroxiredoxins in plastids. Biochemical and Biophysical Research Communications 348, 478–484.

Mubarakshina MM, Ivanov BN, Naydov IA, Hillier W, Badger MR, Kirchner-Müller F, Ströher E, Kandlbinder A, Dietz K-J. 2010. Overoxidation of 2-Cys peroxiredoxin in prokaryotes: cyanobacterial 2-Cys peroxiredoxins sensitive to oxidative stress. The Journal of Biological Chemistry 285, 43821–43827.

O'Neill JS, van Ooijen G, Dixon LE, Troein C, Corellou F, Bouget Stangherlin A, Reddy AB. 2011. Multiple functions of peroxiredoxins: peroxidases, sensors and regulators of the intracellular messenger H_{2}O_{2} and protein chaperones. Antioxidants and Redox Signaling 15, 781–794.

Puerto-Galán L, Pérez-Ruiz JM, Ferrández J, Cano B, Naranjo B, Nájera VA, González M, Lindahl AM, Cejudo FJ. 2013. Oxidation of chloroplast 2-Cys peroxiredoxins: balancing toxic and signaling activities of hydrogen peroxide. Frontiers in Plant Science 4, 310.

Puerto-Galán L, Papadopoulou A, Barrault MT, Rumeau D, Havaux M, Biteau B, Toledano MB. 2007. The Arabidopsis thaliana sulfiredoxin is a plastidic cysteine-sulfenic acid reductase involved in the photooxidative stress response. The Plant Journal 49, 505–514.

Rhee SG, Woo HA. 2011. Multiple functions of peroxiredoxins: peroxidases, sensors and regulators of the intracellular messenger H_{2}O_{2} and protein chaperones. Antioxidants and Redox Signaling 15, 781–794.

Rhee SG, Woo HA, Kil IS, Bae SH. 2012. Peroxiredoxin functions as a peroxidease and a regulator and sensor of local peroxides. The Journal of Biological Chemistry 287, 4403–4410.

Schürmann P, Buchanan BB. 2008. The ferredoxin/thioredoxin system of oxygenic photosynthesis. Antioxidants and Redox Signaling 10, 1235–1274.

Serrato A, Pérez-Ruiz JM, Spinola MC, Cejudo FJ. 2004. A novel NADPH thioredoxin reductase, localized in the chloroplast, which deficiency causes hypersensitivity to abiotic stress in Arabidopsis thaliana. The Journal of Biological Chemistry 279, 43821–43827.

Stangherlin A, Reddy AB. 2013. Regulation of circadian clocks by redox homeostasis. The Journal of Biological Chemistry 288, 26505–26511.

Vandenameele S, Van Der Keelen K, Dat J, et al. 2003. A comprehensive analysis of hydrogen peroxide-induced gene expression in tobacco. Proceedings of the National Academy of Science, USA 100, 16113–16118.

Vanderauwera S, Zimmermann P, Rombauts S, Vandenameele S, Langebartels C, Gruissem W, Inzé D, Van Breusegem F. 2005. Genome-wide analysis of hydrogen peroxide-regulated gene expression in Arabidopsis reveals a high light-induced transcriptional cluster involved in anthocyanin biosynthesis. Plant Physiology 139, 806–821.

Ventriglia T, Kuhn ML, Ruiz MT, Ribeiro-Pedro M, Valverde F, Ballicora MA, Preiss J, Romero JM. 2008. Two Arabidopsis ADP-glucose pyrophosphorylase large subunits (APL1 and APL2) are catalytic. The Journal of Biological Chemistry 283, 300, 26505–26511.

Wood SA, Poole LB, Karplus PA. 2003. Peroxiredoxin evolution and the regulation of hydrogen peroxide signalling. Science 300, 653–656.

Woodson JD, Perez-Ruiz J, Schmitz RJ, Ecker JR, Chory J. 2013. Sigma factor-mediated plastid retrograde signals control nuclear gene expression. The Plant Journal 73, 1–13.

Yang KS, Kang SW, Woo HA, Hwang SC, Chae HZ, Kim K, Rhee SG. 2002. Inactivation of human peroxiredoxin I during catalysis as the result of the oxidation of the catalytic site cysteine to cysteine-sulfenic acid. The Journal of Biological Chemistry 277, 38029–38036.