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Update

Redox regulation of chloroplast metabolism

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Abstract

Regulation of enzyme activity based on thiol-disulfide exchange is a regulatory mechanism in which the protein disulfide reductase activity of thioredoxins (TRXs) plays a central role. Plant chloroplasts are equipped with a complex set of up to 20 TRXs and TRX-like proteins, the activity of which is supported by reducing power provided by photosynthetically reduced ferredoxin (FDX) with the participation of a FDX-dependent TRX reductase (FTR). Therefore, the FDX–FTR–TRXs pathway allows the regulation of redox-sensitive chloroplast enzymes in response to light. In addition, chloroplasts contain an NADPH-dependent redox system, termed NTRC, which allows the use of NADPH in the redox network of these organelles. Genetic approaches using mutants of Arabidopsis (*Arabidopsis thaliana*) in combination with biochemical and physiological studies have shown that both redox systems, NTRC and FDX-FTR-TRXs, participate in fine-tuning chloroplast performance in response to changes in light intensity. Moreover, these studies revealed the participation of 2-Cys peroxiredoxin (2-Cys PRX), a thiol-dependent peroxidase, in the control of the reducing activity of chloroplast TRXs as well as in the rapid oxidation of stromal enzymes upon darkness. In this review, we provide an update on recent findings regarding the redox regulatory network of plant chloroplasts, focusing on the functional relationship of 2-Cys PRXs with NTRC and the FDX–FTR–TRXs redox systems for fine-tuning chloroplast performance in response to changes in light intensity and darkness. Finally, we consider redox regulation as an additional layer of control of the signaling function of the chloroplast.

Introduction

As a process that involves the transport of electrons in the presence of oxygen, photosynthesis inevitably produces reactive oxygen species (ROS), which may cause oxidative damage; therefore, photosynthetic performance is tightly linked to antioxidant systems that maintain ROS under non-toxic concentrations. Different environmental conditions lead to an imbalance of ROS production and scavenging causing oxidative stress, which has a negative effect on plant growth. Beside their potential toxic effect, ROS has a very important signaling function acting as systemic signals that affect the

pattern of expression of a large number of genes, hence coordinating plant response to environmental stress conditions (Zandalinas et al., 2020). ROS signaling depends on the capacity to provoke post-translational modifications in proteins, which is emerging as an important mechanism for the adaptability of plants to the environment (Waszczak et al., 2015; Young et al., 2019). In this regard, cysteine, due to the high reactivity of the thiolate group (Ferrer-Sueta et al., 2011), is the target of many of these modifications. Of interest for this review, cysteine thiolate reacts with hydrogen peroxide being oxidized to sulfenic acid ($-SO^-$) which,

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- Plant chloroplasts harbor a complex redox network composed of the FDX–FTR–TRXs pathway, linking redox regulation to light, and NTRC, an NADPH-dependent system required for the activity of TRXs. Both systems adjust chloroplast performance to environmental cues.
- A relevant function of NTRC is redox control of 2-Cys PRXs, which maintains the reductive activity of chloroplast TRXs in the light. The NTRC–2-Cys PRXs redox system helps fine-tune the redox state of chloroplast enzymes thereby adjusting photosynthetic performance to changes in light.
- 2-Cys PRXs participate in the rapid oxidative inactivation of chloroplast enzymes in the dark, mediating the transfer of reducing equivalents from reduced enzymes, via TRXs, to hydrogen peroxide.
- Involvement of redox regulation in chloroplast retrograde signaling modulates early stages of plant development and response to environmental stress.

under oxidizing conditions, can be further oxidized to sulfinic (-SO_2^-) or even sulfonic acid (-SO_3^-). In addition, cysteines can form intra- or intermolecular disulfides that have deep effects on protein conformation and activity.

Thiol–disulfide interchange constitutes the basis of redox regulation, which is a universal regulatory mechanism since redox-sensitive proteins are found in all organisms (Balsera and Buchanan, 2019). While the mechanism of oxidative disulfide formation in vivo has remained poorly understood, the participation of thioredoxins (TRXs) in disulfide reduction is well established. TRXs are small polypeptides of 12–14 kDa with a characteristic folding, the so-called TRX-fold, and an active site, formed by the signature WCG/PPC, localized at the protein surface (Holmgren, 1995). The reductive activation of target enzymes catalyzed by the disulfide reductase activity of TRXs requires reducing power, which in heterotrophic organisms is provided by NADPH with the participation of an NADPH-dependent TRX reductase (NTR; Jacquot et al., 2009). In plant chloroplasts, redox regulation shows relevant differences when compared with non-photosynthetic organisms (Cejudo et al., 2019). The complex set of up to 20 TRX isoforms of chloroplasts (Geigenberger et al., 2017; Nikkanen and Rintamäki, 2019; Zaffagnini et al., 2019) relies on ferredoxin (FDX) reduced by the photosynthetic electron transport chain through the activity of a FDX-dependent TRX reductase (FTR; Schürmann and Buchanan, 2008). Approaches for trapping TRX interacting proteins combined with mass spectrometry have identified more than 400 putative TRX targets including many

chloroplast enzymes (Montrichard et al., 2009), which means that virtually any process occurring in the organelle is under redox regulation.

Beside the FDX–FTR–TRXs pathway, plant plastids harbor an NTR with a joint TRX domain, termed NTRC (Serrato et al., 2004), which uses NADPH to reduce the hydrogen peroxide scavenging enzyme 2-Cys peroxiredoxin (2-Cys PRX; Moon et al., 2006; Pérez-Ruiz et al., 2006; Alkhalfioui et al., 2007), showing that NADPH is also used in the chloroplast redox network (Spínola et al., 2008). Efforts to elucidate the role of NTRC in chloroplast performance, as well as its functional relationship with the classical FDX–FTR–TRX pathway, have led to a new model of chloroplast redox regulation according to which the redox balance of 2-Cys PRXs indirectly modulates TRX-regulated enzymes (Pérez-Ruiz et al., 2017). This model implies key roles for NADPH and hydrogen peroxide, as input and final sink of electrons, respectively. In this review, we update the current knowledge of the relationship of thiol-dependent redox regulation and antioxidant systems and discuss potential mechanisms of redox modulation of the chloroplast signaling function.

NADPH and hydrogen peroxide at the crossroad of chloroplast thiol-dependent redox regulatory and antioxidant systems

In plant cells, chloroplasts constitute an important source of ROS (for details see Asada, 2006), which include production of singlet oxygen ($^1O_2$) at photosystem (PS) II and superoxide anion (O_2^-) at PSI (Figure 1). To avoid the oxidative damage of ROS and allow their signaling activity, chloroplasts harbor antioxidant systems, both enzymatic and non-enzymatic. Carotenoids, ascorbate (ASC), and glutathione (GSH) are the main non-enzymatic ROS scavenging systems (Pinnola and Bassi, 2018). Among the enzymatic systems, Cu/Zn- and Fe-superoxide dismutases (SODs) catalyze the conversion of superoxide anion to hydrogen peroxide (Pilon et al., 2011). The generation of hydrogen peroxide at PSI, with electrons derived from water oxidation at PSII, and the reduction of hydrogen peroxide to water constitutes the water–water cycle (Asada, 2000). Hydrogen peroxide, which can generate hydroxyl radicals ($OH^·$) by the Fenton reaction (Khorobrykh et al., 2020), exerts an important signaling function and, thus, its concentration is tightly controlled by the action of ASC peroxidases (APXs) and thiol-dependent peroxidases (TPXs). APXs, of which plastids contain two isoforms, localized at the stroma (sAPX) and the thylakoid (tAPX), use ASC to reduce hydrogen peroxide yielding monodehydroascorbate (MDA), which can be further oxidized to dehydroascorbate (DHA; Maruta et al., 2016; Figure 1). ASC is regenerated by the action of MDA reductase (MDAR) and DHA reductase (DHAR) using GSH, which is thus oxidized (GSSG). Regeneration of GSH is catalyzed by NADPH-dependent GSH reductase (GR; Waszczak et al., 2018; Figure 1).

The other group of hydrogen peroxide scavenging enzymes, TPXs, include PRXs (Dietz, 2011) and GSH
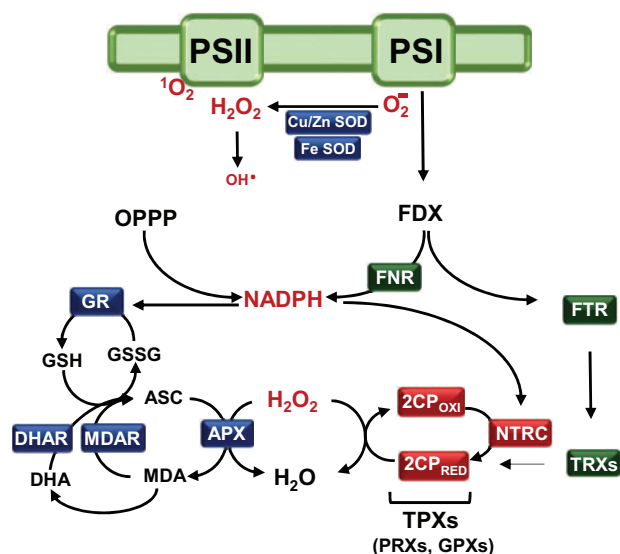
Figure 1 NADPH and hydrogen peroxide at the crossroad of chloroplast antioxidant and thiol-dependent redox regulatory systems. FDX, the final acceptor of the photosynthetic electron transport chain, fuels reducing equivalents to plastid thioredoxins (TRXs) with the participation of FTR. NADPH, which can be produced from sugars by the oxidative pentose phosphate pathway (OPPP), is also generated from FDX by the action of FDX–NADP reductase (FNR). Photosynthesis inevitably produces ROS including superoxide anion (O_2^-), which is converted to hydrogen peroxide (H_2O_2) by Cu/Zn- and Fe-dependent SODs. ASC provides reducing equivalents to scavenge H_2O_2 via ASC peroxidase (APX), producing oxidized intermediates MDA and DHA. Regeneration of ASC via MDAR and DHAR uses reduced GSH hence yielding oxidized GSH (GSSG), which is reduced back to GSH by an NADPH-dependent GR. Plastids harbor an additional group of hydrogen peroxide scavenging enzymes, TPXs, which include PRXs and GPXs (see Box 1). The scheme only represents the most abundant plastid TPX, 2-Cys PRX (2CP), which is predominantly reduced by NADPH-dependent NTRC, and, with lower efficiency (denoted by a thinner arrow), by TRXs.

peroxidases (GPXs; Bela et al., 2015; Box 1). The activity of TPXs relies on the disulfide reductase activity of NTRC, TRXs, and glutaredoxins (GRXs; Collin et al., 2003; Navrot et al., 2006; Pérez-Ruiz et al., 2006; Couturier et al., 2011; Liebthal et al., 2018), thus, linking these hydrogen peroxide scavenging systems with the redox regulatory network of the chloroplast. GRXs, which are more specifically involved in protein deglutathionylation and iron–sulfur cluster incorporation, have important roles in plant development (Rouhier et al., 2015; Zaffagnini et al., 2019). As 2-Cys PRXs, the most abundant chloroplast TPX (Peltier et al., 2006), are preferentially reduced by NTRC, NADPH plays a relevant role supporting this hydrogen peroxide scavenging system (Figure 1). Therefore, NADPH, as the ultimate source of reducing power for all chloroplast enzymes involved in hydrogen peroxide scavenging, and hydrogen peroxide, as the sink of electrons, constitute functional links at the crossroad of chloroplast thiol-dependent redox regulatory network and antioxidant systems.

Plant chloroplasts harbor a complex redox network

Redox regulation based on dithiol–disulfide interchange constitutes an essential regulatory mechanism that allows the rapid adaptation of chloroplast metabolism to light. Typical chloroplast TRXs include those of types *m* (four isoforms), *f* (two isoforms), *y* (two isoforms), *x*, and *z* (Geigenberger et al., 2017; Nikkanen and Rintamäki, 2019; Zaffagnini et al., 2019). Initial biochemical analyses identified the redox regulation of enzymes of the Calvin–Benson cycle (CBC) and showed the relevance of *f*-type TRXs in the light-dependent regulation of carbon fixation (Michelet et al., 2013). In addition, the identification of NADP malate dehydrogenase (NADP-MDH) as a target of *m*-type TRXs showed the redox regulation of chloroplast reducing power exchange through the malate valve (Selinski and Scheibe, 2019). Most plastid TRXs are able to reduce TPXs (Box 1), however, as TRXs *x* and *y* were shown to be more efficient reductants of 2-Cys PRXs (Collin et al., 2003, 2004), it was proposed an antioxidant function for these TRXs, in contrast with TRXs *f* and *m*, which were considered to function in redox regulation. A search of genes encoding NTRs in plants identified the *NTRC* gene, which encodes a polypeptide containing both NTR and TRX domains. NTRC is unique to oxygenic photosynthetic organisms (Pascual et al., 2010; Nájera et al., 2017) and, in plants, shows localization in any type of plastid, though it is more abundant in chloroplasts (Serrato et al., 2004; Moon et al., 2006; Kirchsteiger et al., 2012). Truncated polypeptides containing either the NTR or the TRX domain of NTRC showed that this novel enzyme could display both activities (Serrato et al., 2004). The finding of 2-Cys PRX as target of NTRC helped to solve the actual biochemical properties of the enzyme, which is able to conjugate both NTR and TRX activities to efficiently reduce 2-Cys PRXs (Moon et al., 2006; Pérez-Ruiz et al., 2006; Alkhalfioui et al., 2007). Thus, NTRC could be considered as a TRX that bears its own NTR, which might explain the high catalytic efficiency of the enzyme. The catalytically active form of NTRC is a homodimer arranged in a head-to-tail conformation, which interacts with 2-Cys PRXs through the TRX domain (Pérez-Ruiz and Cejudo, 2009), and shows high affinity for NADPH (Bernal-Bayard et al., 2012). Additionally, NTRC could interact with plastid TRXs through its NTR domain; however, this activity of the enzyme remains controversial. Based on the finding that the overexpression of a NTRC variant with a dysfunctional TRX domain partially rescued the growth inhibition phenotype of the *ntrc* mutant, it was proposed that NTRC reduces TRXs, with *f*-type TRX being the most likely candidate (Toivola et al., 2013). Further supporting the NTR activity of NTRC, affinity chromatography trapping experiments identified TRX *z* as a putative target of the enzyme (Yoshida and Hisabori, 2016). However, it is not yet clear the ability of NTRC to reduce TRX *z* in vitro since the poor activity of NTRC as reductant of TRX *z* reported by Bohrer et al. (2012) is in contrast with the higher activity reported by Yoshida and Hisabori (2016).

Box 1 Chloroplast TPXs

TPXs include PRXs and GPXs. In contrast with cofactor-based enzymatic antioxidant systems, TPXs catalyze the reduction of peroxides using the reducing equivalents of either one (1-Cys PRXs) or two (2-Cys PRXs) thiolic groups of cysteine residues at their active sites, termed peroxidatic (Cys_P) and resolving (Cys_R), the latter being absent in 1-Cys PRXs. The reaction mechanism of 2-Cys PRXs can be divided into three steps (Perkins et al., 2015): (i) the peroxide is attacked by Cys_P, which becomes transiently oxidized to sulfenic acid, (ii) this intermediate reacts with Cys_R forming a disulfide and, when the peroxide is hydrogen peroxide, liberating a molecule of water, and (iii) the disulfide is reduced for a new catalytic cycle. 2-Cys PRXs are grouped as typical, homodimeric enzymes in which Cys_P and Cys_R are in different subunits, and atypical, monomeric enzymes with Cys_P and Cys_R in the same polypeptide. Atypical 2-Cys PRXs are classified as PRX Q and type II PRXs of which there are different isoforms: A, B, D, E, and F (Dietz, 2011). PRXs and GPXs are encoded by large gene families in higher plants. For example, Arabidopsis (*Arabidopsis thaliana*) contains 10 *PRX* (Dietz, 2011) and 8 *GPX* genes (Bela et al., 2015), which encode proteins targeted to different subcellular compartments. The chloroplast is by far the organelle with the largest content and types of TPXs. The Arabidopsis chloroplast contains two almost identical 2-Cys PRXs, termed A and B, PRX IIE, PRX Q, and two GPXs, GPX1 and GPX7. Regeneration of these TPXs is catalyzed by NTRC, TRXs, and TRX-like proteins, except Prx IIE, which is preferentially reduced by GRXs (Couturier et al., 2011), as summarized in the figure. Although most chloroplast TRXs are able to reduce 2-Cys PRXs, TRXs of the types *x* (Collin et al., 2003) and *y* (Collin et al., 2004) were identified as the most efficient reductants. Likewise, these TRX isoforms can reduce PRX Q (Collin et al., 2004), whereas GPXs receive reducing power from *y*-type TRXs (Navrot et al, 2006). NTRC, which is also able to reduce PRX Q (Liebthal et al., 2018), is the most efficient reductant of 2-Cys PRXs (Pulido et al., 2010). Based on their high abundance (Peltier et al., 2006) and high catalytic efficiency, 2-Cys PRXs have been considered to play a relevant antioxidant function in plant chloroplasts.
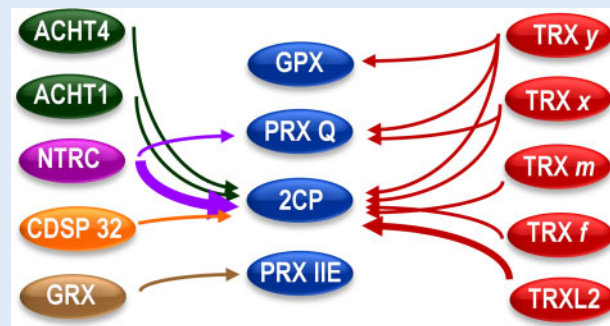
The high efficiency of 2-Cys PRX reduction by NTRC supports the antioxidant function of this enzyme (Pérez-Ruiz et al., 2006). In line with this function, it was shown that Mg-protoporphyrin monomethyl ester cyclase, an enzyme of the chlorophyll biosynthesis pathway, is protected from oxidation by the NTRC-dependent activity of 2-Cys PRXs (Stenbaek et al., 2008). The redox state of 2-Cys PRXs is impaired in the Arabidopsis mutant devoid of NTRC, but not in the mutant devoid of TRX *x*, showing that NTRC is a more efficient reductant of 2-Cys PRXs in vivo than Trx *x* (Pulido et al., 2010), a notion confirmed by in vitro analysis (Yoshida and Hisabori, 2016). Interestingly, NTRC is unable to reduce PRX IIE and shows poor efficiency as a reductant of PRX Q (Yoshida and Hisabori, 2016). Although the capacity of NTRC to reduce GPXs remains to be elucidated, these results indicate that the antioxidant function of NTRC is mainly exerted through the hydrogen peroxide scavenging activity of 2-Cys PRXs, implying that NADPH acts as the ultimate source of reducing power supporting this antioxidant pathway.

NTRC and 2-Cys PRXs modulate the activity of chloroplast TRXs

In recent years, the use of Arabidopsis mutants has led to a significant progress in the understanding of the functional relationship of the chloroplast redox regulated processes and antioxidant systems. As mentioned above, the FDX–FTR–TRXs pathway allows the light-dependent regulation of chloroplast metabolism (Figure 1). In support of the important function of this pathway, Arabidopsis mutants with decreased contents of the catalytic subunit of FTR (FTRc), hence with impaired fuel of electrons into the pathway, show severe chlorosis in leaf sectors near the petiole (Wang et al., 2014), similar to the phenotype of mutant plants with

lower FTR activity (Hashida et al., 2018). In contrast, the deficiency of individual TRXs has low effect on growth phenotype indicating functional redundancy of the different plastid TRXs (Cejudo et al., 2019), except TRX z since mutants lacking this TRX show an albino phenotype (Arsova et al., 2010).

Unlike mutants defective in individual TRXs, the Arabidopsis mutant devoid of NTRC displays a severe growth retard phenotype, which is more pronounced under light limitation (Serrato et al., 2004; Lepistö et al., 2009). Moreover, the Arabidopsis *ntrc* mutant is highly sensitive to both biotic (Ishiga et al., 2012, 2016) and abiotic stress (Serrato et al., 2004; Chae et al., 2013). Since these stresses enhance ROS production, these findings are in line with the antioxidant function proposed for NTRC. However, the lack of NTRC also causes impairment of metabolic processes apparently unrelated with the antioxidant activity of the enzyme such as the redox regulation of ADP-glucose pyrophosphorylase (AGPase), a key regulatory enzyme of starch biosynthesis (Michalska et al., 2009; Lepistö et al., 2013). Similarly, NTRC participates in the redox regulation of chlorophyll biosynthesis enzymes such as Mg-protoporphyrin IX methyltransferase (CHLM), glutamyl-tRNA reductase (GluTR; Richter et al., 2013), and the CHLI subunit of Mg-chelatase (Pérez-Ruiz et al., 2014). Moreover, it was also shown that NTRC affects photoprotection mechanisms through non-photochemical quenching (NPQ; Thormählen et al., 2015; Carrillo et al., 2016; Naranjo et al., 2016).

Altogether, the above-mentioned results show the participation of NTRC in different processes previously known to be under the regulation of TRXs. Intriguingly, these processes are more severely impaired in the *ntrc* mutant than in plants lacking individual TRXs, hence raising the question of the functional relationship of NTRC with the FDX–FTR–TRXs redox system. This issue has been addressed by the analysis of Arabidopsis mutants combining the deficiencies of NTRC and individual TRXs. The simultaneous lack of NTRC and TRX *f1* (Thormählen et al., 2015), TRXs *f1* and *f2*, TRX *x* (Ojeda et al., 2017), or TRXs *m* (Da et al., 2017) causes severe aggravation of growth inhibition. Moreover, the light-dependent redox regulation of CBC enzymes was severely impaired in these mutants (Thormählen et al., 2015; Ojeda et al., 2017), suggesting that NTRC and the FDX–FTR–TRXs redox systems act concertedly. One possibility to explain this concerted action is that both redox systems share common targets. The interaction of NTRC and fructose bisphosphatase (FBPase; Nikkanen et al., 2016) lends support to this notion; however, in vitro assays showed the inability of NTRC to reduce FBPase (Yoshida and Hisabori, 2016; Ojeda et al., 2017), suggesting that the activity of NTRC is exerted without direct redox interaction with the targets.

The question arising is thus the mechanism of action of NTRC. Intriguingly, Arabidopsis mutants with decreased contents of 2-Cys PRXs in the *ntrc* background recovered a growth phenotype similar to the wild type, indicating that decreased contents of 2-Cys PRXs suppress the *ntrc* phenotype. Furthermore, the overexpression of 2-Cys PRXs, which has no significant effect in wild-type plants, resulted in further growth impairment in the *ntrc* background (Pérez-Ruiz et al., 2017), showing that the severity of the *ntrc* phenotype depends on 2-Cys PRXs levels. Based on these results, it was proposed that NTRC maintains the redox state of 2-Cys PRXs, thus avoiding drainage of electrons from the pool of TRXs and, consequently, maintaining their targets reduced, hence active during the day (Figure 2). The accumulation of oxidized 2-Cys PRXs in the absence of NTRC would increase drainage of reducing equivalents from the pool of TRXs impairing the redox regulation of TRX-dependent processes. Further supporting the role of 2-Cys PRXs in chloroplast redox homeostasis, the very severe growth inhibition phenotypes of mutants combining the deficiencies of NTRC and TRXs *f* or *x* were also rescued by decreasing the contents of 2-Cys PRXs (Pérez-Ruiz et al., 2017; Ojeda et al., 2018a). This
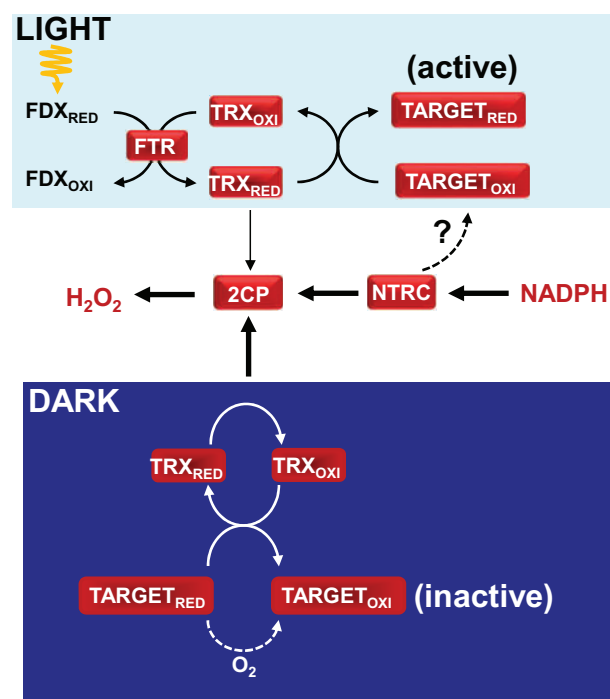

Figure 2 Chloroplast redox regulatory pathways in the light and the dark. The NTRC-2-Cys PRX (2CP) redox system constitutes a redox relay. During the day (LIGHT), the pool of TRXs is maintained reduced by photosynthetically reduced FDX. The redox state of 2-Cys PRXs is predominantly maintained by NTRC and, to a lesser extent, by TRXs (denoted by thick and thin arrows, respectively). Thus, the activity of NTRC avoids the drainage of electrons from TRXs, hence maintaining downstream targets reduced and active. Additionally, NTRC may directly interact with other chloroplast redox-regulated enzymes. During the night (DARK), input of reducing equivalents via reduced FDX ceases and TRXs mediate the transfer of reducing equivalents from reduced targets to 2-Cys PRXs and hydrogen peroxide, while targets consequently become oxidized and inactive. Reoxidation of redox regulated enzymes by molecular oxygen may also occur in the aerobic environment of the chloroplast.

model of chloroplast redox regulation implies that NTRC, by controlling the redox balance of 2-Cys PRXs, affects the redox regulation of any TRX-dependent process in the organelle, providing an explanation for the fact that the activity of this enzyme affects such a variety of processes yet with no direct interaction with the TRX-regulated targets. However, as mentioned above, NTRC interacts with different chloroplast enzymes; thus, the question of whether NTRC acts by directly regulating specific enzymes remains an open issue (Figure 2).

Initial studies of light-dependent regulation of chloroplast metabolism established the reductive activation of enzymes in the light, and also their rapid oxidative inactivation in the dark. While most efforts have been devoted to identifying the mechanism of reductive activation, the mechanism of oxidation has remained poorly understood. Based on the rapid inactivation of FBPase upon oxygen addition to chloroplasts kept under anaerobic conditions, it was proposed that the participation of oxygen in the process of enzyme inactivation occurs either directly or through the formation of superoxide and hydrogen peroxide (Leegood and Walker, 1980). Accordingly, Knuesting and Scheibe (2018) proposed that light-dark regulation of chloroplast enzymes would be operated by a redox switch; in the aerobic environment of the chloroplast, redox-sensitive enzymes are continuously oxidized by oxygen and re-reduced by reducing equivalents provided by the photosynthetic electron transport chain in the light, with the enzymes remaining oxidized in the dark when the electron flow stops. In contrast, Schürmann and Buchanan (2008) proposed the participation of TRXs, which would transfer reducing equivalents either directly to oxygen or, via PRX, to hydrogen peroxide, however, experimental evidence in support of the participation of these enzymes in the process of oxidation was lacking. The participation of 2-Cys PRXs in the maintenance of chloroplast redox homeostasis suggested their involvement in the process of enzyme oxidation, a notion confirmed by the recent finding that Arabidopsis mutants deficient in the two chloroplast 2-Cys PRXs (A and B) display delayed enzyme oxidation in the dark (Ojeda et al., 2018b; Vaseghi et al., 2018; Yoshida et al., 2018; Figure 2). It is known that 2-Cys PRXs interact with different proteins in the chloroplast stroma (Cerveau et al., 2016a; Liebthal et al., 2020) and, thus, could act as an oxidant relay by direct interaction with these targets, as proposed in mammalian cells (Stocker et al., 2017). However, in vitro experiments showed that oxidation of chloroplast enzymes is mediated by TRXs (Ojeda et al., 2018b; Vaseghi et al., 2018), with TRX-like2 (TRX L2) showing higher efficiency (Yoshida et al., 2018). Therefore, these data support the notion that 2-Cys PRXs transfer reducing equivalents to hydrogen peroxide, which thus acts as a final sink of electrons from reduced enzymes in the dark. It should be emphasized that the two models, the participation of oxygen and the participation of 2-Cys PRXs, are not mutually exclusive. Indeed, albeit delayed, dark-triggered enzyme oxidation still occurs in mutant plants lacking 2-Cys PRXs, which indicates the participation of additional mechanism(s).

Furthermore, specific metabolites such as substrates and products of stromal reactions influence redox interconversions of chloroplast enzymes (reviewed in Knuesting and Scheibe, 2018), highlighting the molecular complexity of thiol–disulfide interchange in the organelle.

The participation of 2-Cys PRXs in chloroplast redox regulation (Figure 2) has important implications. First, hydrogen peroxide has the key function of acting as a sink for reducing equivalents from redox regulated enzymes; moreover, since NADPH is the source of reducing power for NTRC, the NADPH/NADP$^+$ ratio would be an important signal determining the rate of biosynthetic pathways of the organelle. In this regard, the level of NADPH increases upon illumination and rapidly decreases in the dark (Lim et al., 2020). Previously, it was proposed that there exists a photosynthetic control that allows the coordination of photochemical reactions, synthesis of ATP and NADPH, and the operation of chloroplast metabolic pathways in order to optimize the use of excitation energy at low irradiance and to dissipate the excess energy at high irradiance (Foyer et al., 1990). The water–water cycle, which consumes electrons in production and further reduction of hydrogen peroxide (Asada, 2000), has long been considered as a mechanism to dissipate excess reducing equivalents, though it was found not to be relevant when CO_2 assimilation is restricted (Driever and Baker, 2011). The transfer of reducing equivalents to hydrogen peroxide catalyzed by 2-Cys PRXs, which accelerates the oxidation of stromal enzymes in the dark, may serve as an additional mechanism to dissipate excess reducing equivalents under high irradiance.

Furthermore, it is well established that chloroplast 2-Cys PRXs, like their counterparts in other eukaryotes (Wood et al., 2003; Perkins et al., 2015), undergo overoxidation, which causes the inactivation of the peroxidase activity of the enzyme (Kirchsteiger et al., 2009; Puerto-Galán et al., 2013). It was found that 2-Cys PRXs overoxidation shows circadian oscillation and based on these results, it was proposed that redox cycles of 2-Cys PRXs constitute a universal marker for circadian rhythms (Edgar et al., 2012). In plant chloroplasts, NTRC, which affects the level of 2-Cys PRXs overoxidation (Puerto-Galán et al., 2015), might affect endogenous circadian rhythms (Box 2), but more work is needed to establish the role of NTRC and 2-Cys PRXs in the circadian clock. Intriguingly, the lack of 2-Cys Prxs causes only a modest inhibition of growth at the adult stage, at least under standard growth conditions (Awad et al., 2015; Ojeda et al., 2018b). It is worth emphasizing that plant chloroplasts are unique systems concerning redox regulation because the redox state of these organelles is rapidly adjusted in response to changes in light intensity, which in nature occur continuously and unpredictably. The Arabidopsis mutant devoid of 2-Cys PRXs shows higher sensitivity than wild-type plants to high light and oxidative stress (Awad et al., 2015), however, this mutant shows better growth performance than the wild type when grown under short fluctuating light pulses, which was attributed to the inefficient inactivation of redox-regulated

Box 2 **2-Cys PRX overoxidation and the circadian clock**

The catalytic cycle of 2-Cys PRXs correlates with two stable conformations of the enzyme, fully folded (FF) and locally unfolded (LU; see the figure). The attack of the peroxide by the thiolate group of the peroxidatic cysteine (Cys_P), which forms the sulfenic intermediate, occurs in the FF conformation. The enzyme must undergo a conformational change to the LU conformation to allow the reaction of the sulfenic intermediate with the resolving cysteine (Cys_R), which results in the formation of a disulfide bond (Wood et al., 2003). 2-Cys PRXs from eukaryotes contain two well-conserved motifs, GGLG and YF, which bury apart Cys_P and Cys_R, stabilizing the FF conformation and favoring overoxidation of the sulfenic intermediate to sulfinic acid under oxidizing conditions. Overoxidation, which can be recovered by the action of sulfiredoxin (SRX), has deep effects on the conformation and catalytic activity of 2-Cys PRXs. The overoxidized enzyme forms aggregates that lack peroxidase activity and display chaperone function (Puerto-Galán et al., 2013), hence allowing the local accumulation of hydrogen peroxide, which can then exert signaling function according to the floodgate hypothesis (Wood et al., 2003). Moreover, overoxidation of 2-Cys PRX from different organisms, including plants, shows circadian oscillation leading to the proposal that these enzymes are universal markers of metabolic circadian rhythms (Edgar et al., 2012; see the figure). Remarkably, most prokaryotic 2-Cys PRXs lack the GGLC and YF motifs being thus insensitive to overoxidation; therefore, this post-translational modification is considered a gain-of-function of eukaryotic 2-Cys PRXs that favor the signaling activity of hydrogen peroxide in these organisms. Nevertheless, 2-Cys PRX from photosynthetic prokaryotes, such as the filamentous cyanobacteria *Anabaena*, is sensitive to overoxidation (Pascual et al., 2010), indicating that filamentous cyanobacteria, considered the ancestors of present chloroplasts, have a eukaryotic-type strategy to cope with hydrogen peroxide. Similarly, chloroplast 2-Cys PRXs are sensitive to overoxidation (Kirchsteiger et al., 2009), which peaks in the early morning after the night–day transition (Edgar et al., 2012; Cerveau et al., 2016b), further supporting the relevant signaling activity of hydrogen peroxide produced in the organelle. Interestingly, Arabidopsis mutants lacking NTRC show severely diminished 2-Cys PRXs overoxidation (Puerto-Galán et al., 2015), indicating the participation of NTRC in the control of 2-Cys PRX overoxidation and, consequently, in the activity of these enzymes as markers of metabolic rhythms. These results suggest a functional relationship of the redox state of the chloroplast and the circadian clock, an issue that deserves future attention.
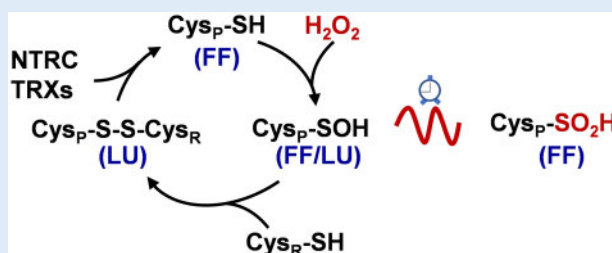
chloroplast enzymes in the absence of 2-Cys PRXs (Vaseghi et al., 2018). In line with these observations, the Arabidopsis mutant lacking GPX7 displays better growth of rosette leaves and higher density of lateral roots than the wild type (Passaia et al., 2014). The better growth of mutants lacking 2-Cys PRXs, in short fluctuating light pulses, and GPX7 suggests that the imbalance of the redox-regulated enzymes, which would be more reduced and, thus, more active in these mutants, may have beneficial effects of plant growth.

Redox modulation of chloroplast sensor activity

Except for a small number of proteins encoded by the chloroplast genetic information, most of the proteins of the organelle are encoded by the nuclear genome. Thus, the transcriptional activity of both genomes must be tightly coordinated via a bi-directional communication, from nucleus-to-chloroplast (anterograde signaling), and from chloroplast-to-nucleus (retrograde signaling). Retrograde signals informing of the chloroplast state are operative in the process of chloroplast biogenesis (biogenic control) and in mature chloroplasts in response to environmental cues (operational control; Chan et al., 2016a). Therefore, besides the primary function of chloroplasts as a source of metabolic intermediates, these organelles have an important signaling activity as sensors of environmental conditions. As discussed above, the redox state of chloroplast enzymes reflects the metabolic performance of the organelle. In this section, we discuss possible mechanisms of redox regulation of chloroplast sensor activity.

Mature chloroplasts emit a variety of signaling molecules that act as operational retrograde signals affecting the expression of plastid redox-associated nuclear genes (PRANGs) in response to environmental cues (Chan et al., 2016a). One such signaling molecule is 3′-phosphoadenosine-5′-phosphate (PAP), the concentration of which increases in response to abiotic stresses such as drought and high light; hence, it was proposed to function as an operational retrograde signal (Estavillo et al., 2011). PAP concentration is maintained by the SAL1 phosphatase, a redox-regulated enzyme that catalyzes the dephosphorylation of PAP to AMP and Pi. Interestingly, the *ntrc* mutant shows lower SAL1 activity than the wild type in response to high light (Chan et al., 2016b), suggesting a function for NTRC in the redox regulation of this enzyme. Although the molecular basis of this effect of NTRC on SAL1 activity remains to be elucidated, it may be one of the targets connecting the operational retrograde signaling with the redox state of the chloroplast (Figure 3). An additional operational retrograde signaling pathway in response to high light involves the translocation of triose phosphate from the chloroplast via a triose phosphate translocator (TPT), the activation of kinase MPK6 and transcription factors APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF; Vogel et al., 2014). As stated above, the CBC, which is the source of triose phosphate, is under redox regulation which, thus, may be an additional layer of modulation of the TPT/MPK6 operational retrograde signaling pathway (Figure 3).

Biogenic retrograde signals coordinate multiple processes leading to the differentiation of a functional chloroplast at early stages of plant development. Interestingly, Arabidopsis mutants deficient in components of the chloroplast redox regulatory network show defective seedling development. This is the case of mutants lacking NTRC and TRXs *f*, which show defective chloroplast ultrastructure in cotyledons and high mortality at the seedling stage (Ojeda et al., 2017), suggesting that plastid redox regulation may also modulate biogenic retrograde signaling. Chloroplast biogenesis requires the activation of the transcriptional machinery of the organelle, which is performed by two RNA polymerases, nuclear-encoded (NEP) and plastid-encoded (PEP; Jarvis and López-Juez, 2013). Full PEP functionality depends on the correct assembly of a multi-protein complex formed by the PEP core subunits, sigma factors, and PEP-associated proteins (PAPs), which include TRX *z* (Pfannschmidt et al., 2015). Unlike the other plastidial TRXs, Arabidopsis mutants lacking TRX *z* develop albino cotyledons due to defects in chloroplast biogenesis (Arsova et al., 2010), which is in line with the seedling lethal phenotype of mutant plants deficient in other PAPs (Pfannschmidt et al., 2015). In principle, the presence of TRX *z* as part of the PEP complex suggested the redox control of chloroplast gene expression, however, the finding that the phenotype of the *trxz* mutant could be restored by redox-insensitive variants of TRX *z* pointed to a minor role of the redox activity of the enzyme in PEP-dependent transcription (Wimmelbacher and Börnke, 2014).
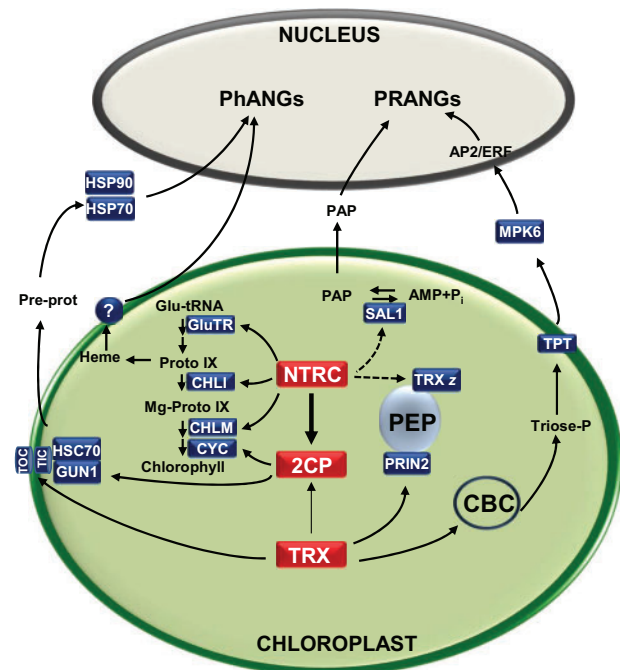

Figure 3 The chloroplast redox network may modulate retrograde signaling through several potential mechanisms. Retrograde signaling coordinates the activity of nuclear and plastid genomes during the process of chloroplast biogenesis (biogenic control), affecting the expression of PhANGs, and in mature chloroplasts in response to environmental cues (operational control), affecting PRANGs. The redox state of the chloroplast may constitute an additional layer of regulation that modulates biogenic and operational retrograde signaling. The NTRC–2-Cys PRX redox system controls tetrapyrrole biosynthesis enzymes such as GluTR, Mg-protoporphyrin IX methyltransferase (CHLM), the CHLI subunit of Mg-chelatase, and the Mg-protoporphyrin IX methylester cyclase (CYC). Biogenic retrograde signaling may thus be affected by the redox control of tetrapyrrole metabolism, as heme synthesis in the chloroplast promotes the expression of PhANGs. Import of pre-proteins (Pre-prot) may be affected by redox modifications of components of the translocons at the outer (TOC) and inner (TIC) chloroplast membranes or the interaction of 2-Cys PRX (2CP) with the GUN1–HSC70 complex, which triggers retrograde signaling mediated by cytosolic chaperones HSP90 and HSP70. Redox regulation of PEP-dependent transcription may be exerted by NTRC-dependent regulation of TRX z and PRIN2, which can also be regulated by TRXs. The redox state of phosphatase SAL1, which affects the levels of operational retrograde signal PAP, might be influenced by the NTRC–2-Cys PRX system. Operational retrograde signaling in response to high light mediated by kinase MPK6 and transcription factors APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) might be modulated by TRXs that regulate the Calvin–Benson cycle (CBC), the source of triose phosphate, which is translocated from the chloroplast via a TPT. Dashed arrows indicate indirect or putative interactions.

Nevertheless, the redox regulation of PEP transcription and, thus, of chloroplast biogenesis, via TRX *z* remains as a likely possibility, which deserves future attention (Figure 3). Another candidate target of the redox regulation of plastid transcription is plastid redox insensitive 2 (PRIN2), which is essential for full PEP activity (Kindgren et al., 2012; Hernández-Verdeja et al., 2020). PRIN2 undergoes redox

regulation by the interconversion of a dimeric (inactive) form, linked by a disulfide bridge, and a monomeric (active) form produced upon light-triggered reduction of the disulfide (Díaz et al., 2018). Although TRX z and TRX ƒ1 reduce PRIN2 in vitro, it is not yet clear whether these TRXs participate in the process. Based on the notion that the NTRC-2-Cys PRX redox system controls the reducing capacity of the pool of plastid TRXs (Pérez-Ruiz et al., 2017), the redox state of PRIN2 and, thus, the activity of the PEP machinery may also by affected by this redox system (Figure 3).

Disruption of chloroplast development at the early seedling stage by treatment with the carotenoid synthesis inhibitor norflurazon (NF) or the plastid translation inhibitor lincomycin provokes the repression of photosynthesis-associated nuclear genes (PhANGs). These inhibitors were used to isolate the *genomes uncoupled* (*gun*) mutants, defective in biogenic retrograde signaling (Susek et al., 1993). Five of the six *gun* mutants identified (*gun2-6*) are defective in the metabolism of tetrapyrroles, chlorophyll, and heme (Larkin, 2016). Several steps of the tetrapyrrole biosynthesis pathway are redox regulated (Brzezowski et al., 2015), hence, the levels of intermediates of the pathway, and their function as retrograde signals, may be modulated by the redox state of the chloroplast. The first specific reaction of the chlorophyll branch of the pathway, the insertion of Mg^{2+} into protoporphyrin IX, is catalyzed by Mg-chelatase, a heterotrimeric enzyme composed of subunits CHLD, CHLH, and CHLI (Tanaka et al., 2011). The finding that the *gun5* mutant is defective in the CHLH subunit (Mochizuki et al., 2001), together with the regulatory role of GUN4 on CHLH (Larkin et al., 2003; Peter and Grimm, 2009) uncovered the key role of this complex in retrograde signaling. NTRC affects the redox regulation of CHLI (Pérez-Ruiz et al., 2014), GluTR, and CHLM (Richter et al., 2013), whereas 2-Cys PRX protects Mg-protoporphyrin monomethyl ester cyclase (Stenbaek et al., 2008). These results show that the NTRC–2-Cys PRXs redox system controls the stability of tetrapyrrole biosynthesis enzymes (Richter et al., 2018) and, therefore, may modulate biogenic retrograde signaling (Figure 3).

Of the *GUN* genes initially identified, the function of *GUN1* has remained elusive. Recently, immunoprecipitation experiments in de-etiolating seedlings showed that GUN1 interacts with cpHSC70-1, a chloroplast chaperone that participates in protein import into the organelle. Moreover, in the *gun1* mutant or in chemically damaged plastids, the accumulation of unimported preproteins in the cytosol acts as a retrograde signal leading to the induction of PhANGs via the accumulation of the cytosolic chaperone HSP90 (Wu et al., 2019). These relevant findings led to the proposal that GUN1 functions in protein import during chloroplast biogenesis, uncovering that folding stress in the cytosol triggers a retrograde signal (Wu et al., 2019). Interestingly, together with cpHSC70, 2-Cys PRX A was identified among the candidate partners of GUN1 during early chloroplast biogenesis (Wu et al., 2019), suggesting that redox signals may affect import-related retrograde communication. This possibility is compatible with the observation that the import of several photosynthetic preproteins is affected by the redox status of the organelle (Balsera et al., 2010). Moreover, a different study found that both 2-Cys PRX isoforms, A and B, were among the GUN1-interactors identified by coimmunoprecipitation experiments performed in adult leaves, which include chloroplast factors involved in protein synthesis and homeostasis (Tadini et al., 2016). Although the participation of 2-Cys PRX in retrograde signaling is unclear, its interaction with GUN1 during chloroplast biogenesis and in rosette leaves suggests that these enzymes may contribute to both biogenic and operational control of chloroplast function. In support of this possibility, cpHSC70 was identified in protein complexes, which also contain 2-Cys PRXs, purified from Arabidopsis plants expressing a tagged version of NTRC (González et al., 2019). Altogether, these findings suggest that protein homeostasis and, thus, retrograde signaling might be under redox regulation via the interaction of NTRC and/or 2-Cys PRX with the GUN1–cpHSC70 complex (Figure 3).

Concluding remarks

By their sessile lifestyle, plants must cope with continuous and unpredictable changes of environmental conditions, this probably being the reason for the complex redox network of plant chloroplasts. The FDX–FTR–TRXs redox pathway, discovered about 50 years ago (recently reviewed by Buchanan, 2016), links the regulation of photosynthetic metabolism to light in contrast to redox regulation in non-photosynthetic organisms, which rely on NADPH. However, the discovery of NTRC, a plastid-localized enzyme with high affinity for NADPH, showed that the chloroplast redox network also uses NADPH. Intriguingly, the deficiency of NTRC has severe phenotypic effects, in contrast with the minor effects caused by the deficiency of individual plastid TRXs, which indicates a central function of the NADPH-dependent redox system in chloroplast performance. Genetic analyses led to the proposal that NTRC and the FDX–FTR–TRXs pathway are integrated by the redox balance of 2-Cys PRXs. The role of 2-Cys PRXs modulating chloroplast redox homeostasis was further extended by the participation of these enzymes in the oxidative inactivation of chloroplast enzymes in the dark. The redox balance of 2-Cys PRXs is maintained by NTRC, the most relevant reductant of the enzyme, avoiding drainage of reducing equivalents from the pool of TRXs, which allows the activation of downstream TRX targets during the day and its oxidative inactivation during the night. This model, however, does not exclude the previously proposed role of molecular oxygen in the oxidation of redox-regulated enzymes in the aerobic environment of the organelle. The functional relationship of thiol-dependent antioxidant systems and the redox regulatory network of the chloroplast raises several questions that should be addressed in the future (see the "Outstanding Questions"). A central issue to advance in the understanding of chloroplast redox regulation is the use of sensor probes for accurately

OUTSTANDING QUESTIONS

- How is the NTRC–2-Cys PRX redox system regulated to avoid the futile loss of NADPH in vivo?
- What is the interplay between enzymatic and non-enzymatic mechanisms governing the redox state of chloroplast enzymes?
- What is the physiological relevance of circadian oscillation of 2-Cys PRXs overoxidation?
- How do the dynamic changes of NADPH and H_2O_2 affect chloroplast performance?
- Which additional mechanism(s), beside 2-Cys PRXs, participate in the process of chloroplast enzyme oxidation in the dark?
- What is the role of individual chloroplast TRXs in enzyme oxidation in the dark?
- How do chloroplast redox signals modulate biogenic and operational retrograde signaling pathways?

determining the in vivo concentrations of NADPH and hydrogen peroxide. An additional aspect to be taken into consideration is that the NTRC–2-Cys PRXs system oxidizes NADPH to reduce hydrogen peroxide in vitro. Thus, to avoid futile oxidation of NADPH in vivo, the activity of the NTRC–2-Cys PRXs system may be tightly controlled. It is well established that chloroplast 2-Cys PRXs undergo overoxidation, which might affect endogenous circadian rhythms. This mechanism and/or additional post-translational modifications may meet the regulatory requirements of the system, but more work is needed to establish the biochemical regulation of the NTRC–2-Cys PRXs redox system. Finally, chloroplasts have important functions as sensors of environmental conditions and as a source of retrograde signals that coordinate the growth of plants at different developmental stages. Chloroplast redox state might be an additional layer of regulation to modulate the sensor activity of the organelle, thus the connection between redox transmitters and signaling pathways is an issue that deserves further exploration.

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