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Review

The function of the NADPH thioredoxin reductase C-2-Cys peroxiredoxin system in plastid redox regulation and signalling

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ABSTRACT

Protein disulphide–dithiol interchange is a universal mechanism of redox regulation in which thioredoxins (Trxs) play an essential role. In heterotrophic organisms, and non-photosynthetic plant organs, NADPH provides the required reducing power in a reaction catalysed by NADPH-dependent thioredoxin reductase (NTR). It has been considered that chloroplasts constitute an exception because reducing equivalents for redox regulation in this organelle is provided by ferredoxin (Fd) reduced by the photosynthetic electron transport chain, not by NADPH. This view was modified by the discovery of a chloroplast-localised NTR, denoted NTRC, a bimodular enzyme formed by NTR and Trx domains with high affinity for NADPH. In this review, we will summarize the present knowledge of the biochemical properties of NTRC and discuss the implications of this enzyme on plastid redox regulation in plants.

1. Introduction

Protein disulphide–dithiol interchange is a reversible post-translational modification affecting the activity of enzymes with regulatory function and, thus, allows the rapid adaptation of cell metabolism to internal or external stimuli. Thioredoxins (Trxs), small proteins of 12–14 kDa with a characteristic folding and a conserved active site –WCGPC–, catalyse protein disulphide reduction, thus playing a central role in redox regulation [1]. Because in these redox reactions the two cysteine residues of the Trx active site become oxidized, a new catalytic cycle will require reduction of this disulphide. Therefore, the maintenance of redox regulation requires reducing power, which is provided by NADPH in a reaction catalysed by NADPH-dependent thioredoxin reductase (NTR) [2]. The two-component redox system formed by NTR and Trx is found in all types of organisms from bacteria to plants and animals and, thus, may be considered universal.

Whilst heterotrophic organisms contain a small number of genes encoding Trxs, plants, as well as other oxygenic photosynthetic organisms, are peculiar in that they contain a large number of Trxs, of which at least 20 are localised in the chloroplast [3,4]. Moreover, chloroplasts have unique properties concerning redox regulation because Trxs are reduced by a ferredoxin (Fd)-dependent Trx reductase, FTR, which is exclusively found in these organelles. Therefore, it has been classically considered that redox regulation in chloroplasts relies on the FTR/Trx system, which is dependent on Fd reduced by the photosynthetic electron transport chain and, thus on light. In contrast, redox regulation in heterotrophic organisms and non-green plant tissues is performed by the NTR/Trx system and uses NADPH as source of reducing power.

The notion that chloroplast redox regulation relies exclusively on reduced Fd was modified by the discovery of a novel type of NTR. This enzyme, called NTRC, is exclusive for oxygenic photosynthetic organisms such as plants, algae and some cyanobacteria [5]. NTRC is a bimodular enzyme formed by an NTR domain, at the N-terminus, and a Trx domain, at the C-terminus and, therefore, represents an NTR/Trx system in a single polypeptide, which in plants and algae is located in the chloroplast. Interestingly, NTRC shows a high affinity for NADPH, thus allowing the use of this source of reducing power for chloroplast redox regulation [6]. During the day, when the levels of reduced Fd are expected to be high, the function of NTRC may be considered complementary to the FTR/Trx pathway. However, during the night, when the levels of reduced Fd and NADPH coming from photochemical reactions are low, NTRC may become the most relevant pathway for redox homeostasis using as source of reducing power NADPH produced

Abbreviations: AGPase, ADP-glucose pyrophosphorylase; Fd, ferredoxin; FNR, ferredoxin:NADP oxidoreductase; FTR, ferredoxin-dependent thioredoxin reductase; NTRC, NADPH-dependent thioredoxin reductase C; OPPP, oxidative pentose phosphate pathway; Srx, sulfiredoxin; Trx, thioredoxin

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from sugars by the oxidative pentose phosphate pathway (OPPP) [7]. An *Arabidopsis* mutant lacking NTRC shows a pleiotropic phenotype of retarded growth and sensitivity to different abiotic stresses [5,8,9], indicating that the function of NTRC is of great importance for plant development and adaptation to environmental changes. This mutant is hypersensitive to prolonged darkness, and its phenotype is more severe when grown under short-day photoperiod [8,9], lending support to the proposal that the function of NTRC becomes more important under conditions of light limitation.

Since the first description of NTRC in 2004 [5] significant progress has been made on the biochemical properties of this peculiar enzyme, and also on some of the metabolic pathways the regulation of which seems to be highly dependent on NTRC. Moreover, an in-depth analysis of the pattern of expression of the *NTRC* gene in *Arabidopsis* plants has revealed that this gene is widely expressed in photosynthetic and non-photosynthetic organs and that the protein is localised in any type of plastids [10], thus opening the possibility that redox regulation is operative in plastids from non-green tissues. In this review we will summarize the present knowledge of the biochemical properties of NTRC. In addition, we will discuss the implications of this novel enzyme on our view of plastid redox homeostasis.

2. NTRC, a molecular switch converting reducing power (NADPH) into redox signal (thiols)

NTRC was identified in a search for plant genes encoding NTRs once the sequences of the *Arabidopsis* and rice genomes were available [11]. This search identified two genes, *NTRA* and *NTRB*, encoding the NTRs, termed NTRA and NTRB, which belong to the low-molecular-weight type NTR found in bacteria and fungi, and differed from the high-molecular-weight type enzyme of mammals [12]. In *Arabidopsis* it was shown that NTRA is the predominant form of the enzyme in the cytosol whereas NTRB is the most abundant form in mitochondria [13]. The third gene, termed *NTRC*, encodes a protein with the characteristic features of low-molecular-weight type NTRs including the binding sites for NADPH and FAD, and the conserved active site –CAI/VC– of these enzymes. However, the polypeptide deduced from this gene showed two remarkable and peculiar features, an extension at the N-terminus, which might serve as transit peptide putatively targeting the enzyme to chloroplast or mitochondria, and a putative Trx domain at the C-terminus. Interestingly, the *NTRC* gene is exclusive for oxygenic photosynthetic organisms, plants, algae and some cyanobacteria, all of them having this gene in single copy. Moreover, fractionation experiments and Western blot analysis showed that NTRC is predominantly localised to the chloroplast stroma both in rice and *Arabidopsis* [5]. Initial biochemical analysis of the recombinant full-length enzyme from rice confirmed that NTRC is a flavoprotein showing both NTR activity, determined as NADPH-dependent DTNB reduction, and Trx activity, determined as DTT-dependent insulin reduction. Indeed, truncated polypeptides of the NTR and Trx domains of NTRC showed the expected NTR and Trx activities [5], which was later confirmed for the *Arabidopsis* enzyme [14]. However, the enzyme was unable to catalyse the NADPH-dependent reduction of insulin, which led to the initial proposal of NTRC as a bimodular enzyme able to act either as NTR or Trx, but not as an NTR/Trx system [5].

Soon after this initial proposal, it was established that NTRC is indeed an enzyme able to conjugate both NTR and Trx activities to efficiently reduce typical, dimeric, 2-Cys Prxs [8,14,15]. Indeed, NTRC showed higher catalytic efficiency for in vitro 2-Cys Prx reduction than the chloroplast-induced drought stress protein (CDSP32) [16], and type *x* Trx [17], previously proposed as the reductants of chloroplast 2-Cys Prxs. In vivo analyses of the 2-Cys Prx redox status in an *Arabidopsis* NTRC knockout mutant showed alterations as compared to the wild type, whereas the 2-Cys Prx redox status of a Trx *x* knockout mutant was similar to that of the wild type [18]. These results are consistent with a higher physiological relevance of NTRC as reductant of chloroplast 2-Cys Prxs. Because 2-Cys Prxs are thiol-dependent peroxidases, these results lead to the proposal that NTRC is involved in chloroplast peroxide detoxification.

The analysis of the quaternary structure of the recombinant His-tagged NTRC from rice revealed that the enzyme shows a tendency to form oligomers in an oxidant environment, but dimerizes in the presence of NADPH or DTT [19]. The possibility of reconstituting the NTRC-2-Cys Prx system in vitro, in combination with the use of mutant variants of both proteins has allowed the proposal of a reaction mechanism for NTRC [20]. According to this proposal, the catalytically active form of NTRC is the homodimer, the formation of which is induced in the presence of NADPH. The homodimer would be arranged in head-to-tail conformation so that NADPH transfers electrons to the FAD cofactor at the NTR domain of one of the subunits, thus reducing the disulphide at the active site of this NTR domain. Then intersubunit electron transfer from this active site to the Trx domain of the other subunit would take place, as depicted in Fig. 1. The sensitivity of the oligomerization/dimerization interconversion of the rice enzyme to NADPH suggested a possible mechanism of regulation of NTRC activity based on the availability of NADPH at the chloroplast stroma, the enzyme being more active under reducing conditions, when it adopts the dimeric conformation. However, cryo-electron microscopy analysis showed that NTRC from barley adopts tetrameric and oligomeric forms, which in contrast to the rice enzyme are insensitive to the presence of NADPH, the enzyme showing activity both as tetramer and as oligomer [21]. Moreover, NTRC of cyanobacterial origin, from *Anabaena*, shows high similarity with the plant enzymes but was exclusively detected as dimer without any tendency to form oligomers [22], suggesting that the ability to oligomerize is exclusive of NTRC from eukaryotes. Therefore, the NTRC enzymes so far analysed show similar biochemical properties concerning their activity as 2-Cys Prx reductants, but differ in their propensity

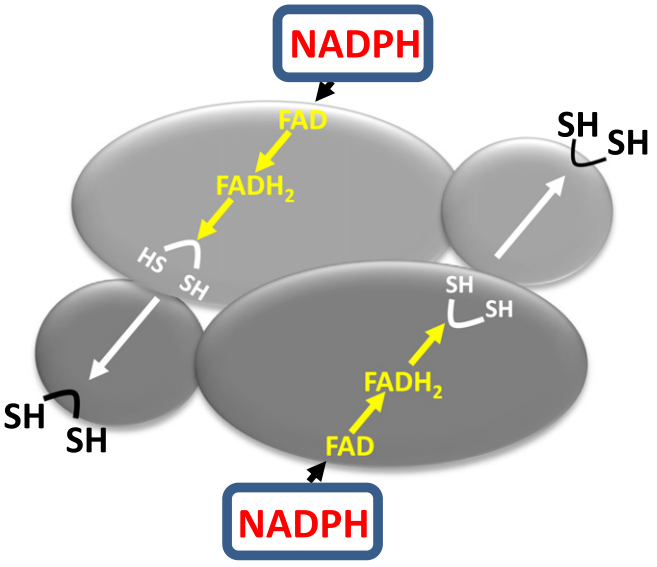

Fig. 1. NTRC acts as a molecular switch. According to the reaction mechanism proposed for NTRC [20] the basic catalytic form of the enzyme is a homodimer. NADPH donates electrons to the FAD cofactor at the NTR domain of each subunit and electrons are transferred to reduce the cysteine residues at the active site of the Trx domain of the other subunit. Thus reducing power (NADPH) is converted into redox signal (thiols).

to form oligomers, the sensitivity of the oligomers to redox conditions and the effect of oligomerization on activity. Thus, to establish whether oligomerization is an important aspect of NTRC function will require studies with more enzymes from different sources, and to advance in determining the structure of the protein.

Regardless of the effect of the quaternary structure of NTRC on activity, the biochemical analyses clearly establish the preference for NADPH as source of electrons, which are ultimately transferred to the cysteine residues at the active site of the Trx domains of the enzyme. Thus, as shown in Fig. 1, it may be considered that NTRC acts as a molecular switch able to convert a signal in the form of reducing power, NADPH, into a redox signal, the thiol groups of the cysteine residues at the active site of the Trx domains. In this way, the NADPH/$NADP^+$ ratio in the chloroplast would determine the reductant capacity of NTRC and, thus, the redox regulation of target proteins. This may be a mechanism for regulating the activity of these targets and the metabolic pathways where they are involved. It will be discussed below the progress that has been made to elucidate which are the processes regulated by NTRC. However, to have a complete view of the function of this enzyme in maintaining plastid redox homeostasis and the impact of this on plant development and response to the environment, it will be required the identification of proteins interacting with NTRC.

3. NTRC exerts functions dependent on and independent of 2-Cys Prx reduction

3.1. The antioxidant function of the NTRC/2-Cys Prx system

For the analysis of the function of NTRC on chloroplast redox homeostasis the identification of an *Arabidopsis* T-DNA insertion line, which is knock out for the enzyme, has been of great aid. The *ntrc* mutant is hypersensitive to different abiotic stresses [5] and shows characteristic phenotypic features, such as retarded growth, pale green leaves, irregular distribution of mesophyll cells, alterations of chloroplast structure, and lower CO_2 fixation activity, among others [8,9]. Interestingly, the phenotype of this mutant is highly dependent on the photoperiod, being more severe under short-day conditions [8,9]. As stated above, the localization of NTRC in the chloroplast stroma, the high affinity for NADPH as source of reducing power, and the high catalytic efficiency for 2-Cys Prxs reduction led us to propose that NTRC might form part of the antioxidant machinery of the chloroplast. This system would operate both during the day and the night, since NADPH is generated either from the photochemical reactions or from sugars, by the two initial reactions of the OPPP, catalysed by glucose 6-phosphate and 6-phospho gluconate dehydrogenases [8] (Fig. 2).

Evidence for the protective function of the NTRC-2-Cys Prx antioxidant mechanism was obtained by the analysis of the chlorophyll biosynthesis pathway in the *Arabidopsis ntrc* mutant. This mutant shows lower content of chlorophyll than wild type plants, and it is known that chlorophyll biosynthesis is sensitive to ROS [23], thus suggesting that chlorophyll deficiency in the mutant might be due oxidative damage of some of the enzymes involved in this pathway. Stenbaek et al. [24] showed that aerobic cyclase activity in vitro was enhanced in the presence of NTRC and 2-Cys Prx, which acted as a hydrogen peroxide scavenging system, thus protecting this enzymatic activity of the pathway of chlorophyll biosynthesis.

3.2. Functions of NTRC independent of 2-Cys Prx reduction

Different lines of evidence suggested that NTRC might exert redox regulatory functions not related with the antioxidant activity

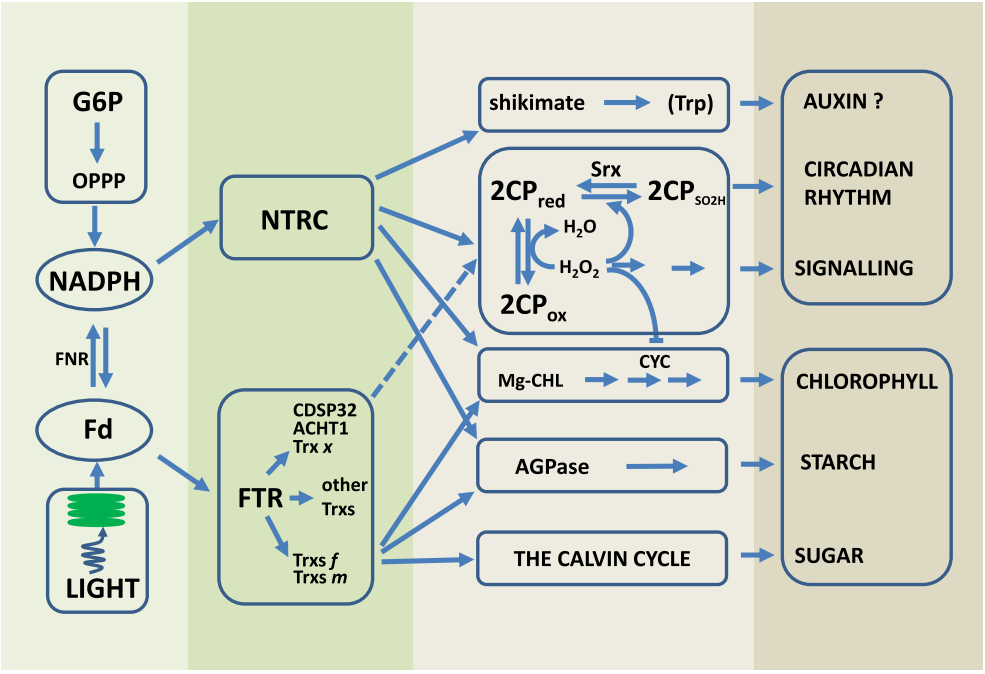

Fig. 2. A summary of the function of the NTRC and FTR/Trx pathways on redox regulation in chloroplasts. Reducing power for redox regulation in the chloroplast is provided by ferredoxin (Fd) reduced by the photosynthetic electron transfer chain and by NADPH produced by the oxidative pentose phosphate pathway (OPPP) from glucose 6-P (G6P). Both, NADPH and Fd are interconvertible by the action Fd-NADP-oxidorreductase (FNR). Redox regulation is performed by two pathways, Fd-dependent thioredoxin reductase (FTR), which maintains the redox status of the complex set of chloroplast Trxs. Of these, the scheme only represents Trxs *f* and *m* involved in redox regulation of Calvin cycle enzymes (sugar biosynthesis), ADP-glucose pyrophosphorylase (AGPase) (starch biosynthesis) or Mg-chelatase (Mg-CHL) (chlorophyll biosynthesis). The NTRC pathway plays a complementary function for redox regulation of starch and chlorophyll biosynthesis, though the regulation of Mg-CHL by NTRC is still a matter of debate. Several Trxs and Trx-like proteins, CDSP32, ACHT1 and Trx *x*, have been proposed to act as 2-Cys Prx (2CP) reductants (dashed arrow), but NTRC is probably the most important 2CP reductant in vivo. The peroxidase activity of 2CP reduces hydrogen peroxide and, thus, protects the Mg-protoporphyrin monomethyl ester cyclase (CYC) and has a positive effect on chlorophyll biosynthesis. Hydrogen peroxide acts as second messenger in signaling and favours 2CP overoxidation ($2CP_{SO2H}$), which has been proposed to be involved in circadian rhythmicity. Finally, NTRC is involved in redox regulation of aromatic amino acid biosynthesis such as Trp, which is a precursor of auxin synthesis.

of 2-Cys Prxs. Chloroplasts are equipped with several antioxidant systems, both enzymatic and non-enzymatic, which exert a compensatory effect on detoxification [25,26]. Therefore, it is not expected that the phenotype of the *ntrc* mutant is exclusively due to the imbalance of the antioxidant machinery produced by the deficiency of 2-Cys Prx reduction. In this regard, *Arabidopsis* transgenic plants expressing an antisense 2-Cys Prx gene and, thus, with reduced levels of the protein, showed impairment of photosynthetic parameters only at early phases of seedling development [27,28]. A more in-depth analysis of the pathways for 2-Cys Prx reduction, as well as the effect of these peroxidases on plant growth and response to abiotic stresses, was performed by the study of different mutants in *Arabidopsis*. A Trx *x* knock out mutant showed a phenotype very similar to the wild type plants, in contrast to the severe phenotype of the *ntrc* mutant [18]. Moreover, the redox status of the 2-Cys Prx, as analysed by Western blots under non-reducing conditions, was similar in *trxx* mutant and wild type plants, whereas it was clearly impaired in the *ntrc* mutant, suggesting that NTRC is the most relevant reductant of 2-Cys Prxs in vivo. The 2-Cys Prx A-2-Cys Prx B double mutant, which has severely reduced levels of 2-Cys Prxs, showed only mild alterations of growth and photosynthetic parameters, as compared with the *ntrc* mutant [18], clearly showing that NTRC may exert additional functions not related with its activity as 2-Cys Prx reductant. As mentioned above, the identification of targets of NTRC will be required to establish these functions; however, some progress showing NTRC regulation of starch, aromatic amino acids and chlorophyll biosynthesis has been made during the last years.

Starch is the most important storage carbohydrate in plants. It is accumulated during the day and mobilized during the night and, thus, constitutes an important factor integrating the regulation of plant growth [29]. The key regulatory step of starch biosynthesis is catalysed by ADP-glucose pyrophosphorylase (AGPase), an enzyme subjected to allosteric and redox regulation. In leaves, AGPase is redox-activated in response to light and sugars [30]. While light-dependent activation is highly dependent of Trx *f1* [31], sugar-dependent activation in the dark relies essentially on NTRC, which has been shown to reduce, and activate, AGPase in vitro [32]. Additional evidence of the involvement of NTRC on starch synthesis, in this case in response to microbial volatiles was reported [33]. However, Li et al. [34] found a moderate NTRC-dependent reduction of AGPase in response light when plants were grown under photo-oxidative conditions, which suggest that redox regulation of AGPase may be influenced by environmental conditions.

Another metabolic alteration in the *ntrc* mutant is found in the shikimate pathway leading to increased levels of aromatic amino acids, tyrosine, tryptophane and phenylalanine. These amino acids show increased levels in the mutant in particular when grown under short-day conditions [9]. The altered content of Trp might be related with the reduction of the content of auxin in the mutant, as will be discussed below, but the reason for this imbalance is not yet understood.

Finally, it has been suggested that NTRC may be important for the regulation of the chlorophyll biosynthesis pathway [9], not only by the antioxidant effect on the aerobic cyclase activity discussed above, but through direct redox regulation of the pathway. The first step specific for the pathway of chlorophyll biosynthesis, the insertion of Mg^{2+} in protoporphyrin IX, is catalysed by Mg-chelatase, a complex enzyme formed by three types of subunits, denoted CHLH, CHLD and CHLI [35]. Subunits H and I show conserved cysteine residues, which are essential for activity [36], and Trx *f*-mediated redox regulation of the ATPase activity of CHLI has been shown [37]. In addition, Stenbaek and Jernsen [38] mentioned the possibility that NTRC might be involved in redox regulation of Mg-chelatase activity, though Luo et al. [39] analysing the Mg-chelatase activity from pea found a positive effect of Trxs *f* and *m*, but not of NTRC. Therefore, whilst the function of NTRC on chlorophyll biosynthesis by protecting the aerobic cyclase from oxidative damage, as depicted in Fig. 2, is well established, the possibility that NTRC might exert any direct regulation of Mg-chelatase activity is still issue of debate. It should be taken into account that imbalanced chlorophyll biosynthesis modifies chloroplast signalling thus affecting the expression of chloroplast proteins encoded by nuclear genes, the so-called retrograde signalling [40]. Therefore, the involvement of NTRC on redox regulation of chlorophyll biosynthesis, in particular at the level of Mg-chelatase, may have implications on retrograde signalling between chloroplast and nucleus [41]. However, much work is still required to precisely establish the function, if any, of NTRC in retrograde signalling.

4. The effect of NTRC on 2-Cys Prx overoxidation and signalling

4.1. 2-Cys Prxs sensitive and insensitive to overoxidation

The previous discussion of the activity of NTRC as the major reductant of chloroplast 2-Cys Prxs was focussed on the antioxidant function of this system and the beneficial effects that scavenging of hydrogen peroxide has on chlorophyll biosynthesis. This function is indeed relevant because despite the primordial function of photosynthesis as source of organic material and oxygen to maintain life on earth, it is a process that inevitably produces reactive oxygen species (ROS) and, thus, chloroplasts are among the organelles with a highest production of ROS, including hydrogen peroxide [42]. However, it is important to remark that beside this toxic effect, hydrogen peroxide has an important function as second messenger [43]. The dual effect of hydrogen peroxide, as toxic agent and second messenger, implies that its intracellular concentration needs to be tightly regulated. In this regard, two strategies seem to have evolved: while prokaryotic organisms are equipped with very efficient antioxidant systems that allow a rapid detoxification of peroxides with the central objective of avoiding their harmful effects, eukaryotic organisms allow controlled increases of hydrogen peroxide, which may be potentially dangerous, but allows its function as second messenger with signalling purposes.

Wood et al. [44] proposed that the different signalling activity of hydrogen peroxide in eukaryotes and prokaryotes is due to the different structural properties of 2-Cys Prxs in these organisms. Typical 2-Cys Prxs are homodimeric, thiol-based peroxidases, the reaction mechanism of which is based on the action of two conserved cysteine residues, termed peroxidatic and resolving [45]. To initiate a catalytic cycle both cysteine residues need to be reduced. The thiol group of the peroxidatic cysteine reacts with the peroxide and becomes transiently oxidized to sulfenic acid (–SOH), which is then attacked by the resolving cysteine producing a molecule of water, and the cysteine residues become oxidized to form a disulphide bridge. Under oxidising conditions, the transient sulfenic acid intermediate may become overoxidized to sulfinic ($–SO_2H$) or even sulfonic ($–SO_3H$) acids. This modification causes the inactivation of the 2-Cys Prx [46] and, as consequence, the increase of the level of hydrogen peroxide, which may then function as second messenger, as proposed in the so-called "floodgate hypothesis" [44] (Fig. 2). According to this hypothesis 2-Cys Prxs from eukaryotic organisms are sensitive to overoxidation, whereas 2-Cys Prxs from prokaryotic organisms are insensitive.

2-Cys Prxs sensitive to overoxidation are characterised by the presence of two motifs, GG(L/V/I)G and YF, at the C-terminus of the enzyme [44,47]. In these enzymes there is a distance of 14 Å separating the peroxidatic and resolving cysteines, which slows

down disulphide formation during the catalytic cycle, so that the sulfenic acid intermediate is prone to overoxidation. It was proposed that the structural determinants that make 2-Cys Prxs sensitive to overoxidation are exclusive to enzymes of eukaryotic organisms, which according to the floodgate hypothesis use hydrogen peroxide in signalling [44]. In contrast, 2-Cys Prxs from prokaryotic organisms lack these motifs, the two catalytic cysteines being more proximal, thus facilitating the formation of the disulphide, which protects the peroxidatic cysteine from overoxidation.

4.2. Chloroplast 2-Cys Prxs undergo overoxidation

The different sensitivity of 2-Cys Prxs from eukaryotes and prokaryotes to overoxidation proposed in the floodgate hypothesis indicated that the enzymes from eukaryotes have evolved to become sensitive [48]. This raised the question of the sensitivity to overoxidation of 2-Cys Prxs from chloroplasts, organelles of eukaryotic organisms, but of cyanobacterial, that is prokaryotic, origin. The two almost identical 2-Cys Prxs, termed 2-Cys Prx A and B, localised in the *Arabidopsis* chloroplast undergo overoxidation, thus behaving as eukaryotic type enzymes [49]. An extensive search of the GG(L/V/I)G and YF motifs, characteristic of sensitive enzymes, revealed the presence of these motifs in 2-Cys Prxs of all eukaryotic organisms, but also in enzymes from some prokaryotes notably including cyanobacteria such as *Anabaena* and *Synechocystis*. Indeed, further analyses showed that the 2-Cys Prx from *Anabaena* was sensitive to overxoidation, thus behaving as eukaryotic enzymes, whereas the enzyme from *Synechocystis* was not, thus behaving as a prokaryotic enzyme [47]. Moreover, it was shown that these two cyanobacterial strains show different strategies to cope with hydrogen peroxide, while *Anabaena* is highly sensitive, *Synechocystis* is not. This difference is most probably due to the high catalase activity and insensitive 2-Cys Prx of *Synechocystis*, which allows an efficient detoxification of the peroxide, in contrast to the strategy of *Anabaena*, based on low catalase activity and sensitive 2-Cys Prx and, thus, inefficient detoxification of peroxides [47]. These results show that 2-Cys Prx sensitivity to overoxidation is more common than initially predicted in the floodgate hypothesis. Moreover, the strategy adopted by plant chloroplasts to cope with hydrogen peroxide is similar to the *Anabaena* strategy. This is a genuine eukaryotic-type strategy based in allowing hydrogen peroxide accumulation, which may be harmful but also can be used as second messenger for signalling. This is in contrast to the prokaryotic-type strategy represented by *Synechocystis*, based on rapid detoxification of the peroxide, which avoids any harmful effects but, in consequence, does not allow any further signalling function of hydrogen peroxide.

4.3. NTRC and sulfiredoxin (Srx) control 2-Cys Prx overoxidation in chloroplasts

Although 2-Cys Prx inactivation by overoxidation was initially considered an irreversible process, it was later shown to be reversible in a reaction catalysed by Srx, which requires ATP and Mg^{2+} [50,51]. Plant Srx is localised in the chloroplast [52] and is involved in the response of the plant to oxidative stress [53,54]. Moreover, the *Arabidopsis* NTRC knock out plants showed almost no 2-Cys Prx overoxidation [18,49], in agreement with the idea that the disulphide between the peroxidatic and resolving cysteines, the reduction of which is severely impaired in this mutant, protects the former from overoxidation. Therefore, the level of 2-Cys Prx overoxidation in chloroplasts seems to be essentially controlled by NTRC and Srx (Fig. 2).

Overoxidation has profound effects on 2-Cys Prx structure and activity. While the homodimeric enzyme shows peroxidase activity, overoxidation induces the oligomerization of the enzyme, which then displays chaperone activity [55]. Based on these properties, it has been proposed that 2-Cys Prxs define a regulatory hub in the chloroplast [56], which may have important implications for the signalling function exerted by these organelles. In this regard, it is interesting to note that 2-Cys Prx overoxidation has been proposed to act as a transcription-independent circadian rhythmicity marker in the eukaryotic alga *Ostreococcus tauri* [57].

5. Redox homeostasis in plastids of non-photosynthetic tissues

As mentioned above, NTRC is involved in redox regulation of AGPase and, thus, of starch synthesis in chloroplasts of photosynthetic tissues. Interestingly, redox regulation of AGPase was also impaired in roots in the *ntrc* mutant [32]. This result implied that NTRC, which was considered exclusively localised in chloroplasts, might be also present in non-photosynthetic plant tissues. An in-depth analysis of *NTRC* gene expression in *Arabidopsis* based on qPCR and Western blots, as well as transgenic plants expressing the GUS reporter gene under the control of the *NTRC* gene promoter, confirmed the wide pattern of expression of the *NTRC* gene in photosynthetic and non-photosynthetic organs, though expression is higher in green tissues. Moreover, *Arabidopsis* plants expressing NTRC–GFP fusion protein revealed the localization of NTRC in any type of plastids [10]. These results, in conjunction with the identification of the complete FTR-Trx system in wheat amyloplasts [58], suggested that, far from being exclusive to chloroplasts, redox regulation occurs in any type of plant plastid.

As depicted in Fig. 2, redox homeostasis of chloroplasts of photosynthetic tissues relies on reducing power provided by Fd reduced by the photosynthetic electron transport chain, through the FTR-Trx system, and by NADPH produced as the final acceptor of the photosynthetic electron transport chain, during the day, or from sugars, by the OPPP, during the night. However, plastids of non-photosynthetic tissues have no photochemical reactions so that the source of reducing power for redox regulation in these organelles is exclusively NADPH generated by the OPPP from sucrose imported from photosynthetic tissues. In these plastids, it is expected that NTRC, which is able to directly use NADPH as source of reducing power (Fig. 1), would exert a central role in redox regulation.

The notion that redox regulation is operative in all types of plastids raised the question of the existence of possible mechanisms for coordinating their function. In order to address this question we have generated *Arabidopsis* transgenic plants with redox regulation exclusively restituted in leafs or in roots, by expression of NTRC in the *ntrc* mutant background under the control of the *RbcS* promoter or the *Pht1* promoter, respectively. The analysis of these plants showed that chloroplast redox homeostasis was necessary and sufficient for leaf and root growth, whereas restoration of redox homeostasis exclusively in roots had little effect on either leaf or root growth [10]. Moreover, the root phenotype of lateral root deficiency of the *ntrc* mutant was recovered in plants expressing NTRC exclusively in leaves, but not in plants expressing NTRC exclusively in roots. These results point to a primordial function of chloroplasts in coordinating the growth of the different organs during plant development. To play this central role, the chloroplast acts as source of photosynthetic assimilates to support the growth of other plant organs. In addition, this organelle may have a signalling function to allow the harmonisation of the growth and development of photosynthetic and heterotrophic organs. In this regard, it should be mentioned that the *ntrc* mutant has deficient levels of auxins [9], which might explain the lateral root deficiency phenotype of this mutant. Thus, it is tempting to speculate on the possibility that chloroplast redox homeostasis is required for coordinating leaf and root development in particular at early stages of development.

6. Concluding remark

In summary, the biochemical properties of NTRC allow the use of NADPH as source of reducing power for redox regulation in any type of plastids, both photosynthetic and non-photosynthetic. Based on our present knowledge, NTRC seems to be the most relevant physiological reductant of 2-Cys Prxs. In this way NTRC might be an important component of the antioxidant machinery of the plant to control the levels of hydrogen peroxide, hence avoiding its potential harmful effects. However, hydrogen peroxide has been proposed as an important second messenger in eukaryotes, an activity in part dependent of the sensitivity of 2-Cys Prxs to overoxidation. Therefore, the activity of NTRC as reductant of 2-Cys Prxs may be also relevant for the important signaling function of the chloroplast. In addition, NTRC is involved in the redox regulation of different metabolic pathways such as starch, aromatic amino acids or chlorophyll biosynthesis, all of them of primary importance for plant growth and adaptation to environmental conditions. These findings are opening exciting possibilities on how plastid redox regulation affects plant development.

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