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REVIEW PAPER

The thioredoxin/peroxiredoxin/sulfiredoxin system: current overview on its redox function in plants and regulation by reactive oxygen and nitrogen species

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Abstract

In plants, the presence of thioredoxin (Trx), peroxiredoxin (Prx), and sulfiredoxin (Srx) has been reported as a component of a redox system involved in the control of dithiol–disulfide exchanges of target proteins, which modulate redox signalling during development and stress adaptation. Plant thiols, and specifically redox state and regulation of thiol groups of cysteinyl residues in proteins and transcription factors, are emerging as key components in the plant response to almost all stress conditions. They function in both redox sensing and signal transduction pathways. Scarce information exists on the transcriptional regulation of genes encoding *Trx/Prx* and on the transcriptional and post-transcriptional control exercised by these proteins on their putative targets. As another point of control, post-translational regulation of the proteins, such as *S*-nitrosylation and *S*-oxidation, is of increasing interest for its effect on protein structure and function. Special attention is given to the involvement of the Trx/Prx/Srx system and its redox state in plant signalling under stress, more specifically under abiotic stress conditions, as an important cue that influences plant yield and growth. This review focuses on the regulation of Trx and Prx through cysteine *S*-oxidation and/or *S*-nitrosylation, which affects their functionality. Some examples of redox regulation of transcription factors and Trx- and Prx-related genes are also presented.

Key words: Peroxiredoxin, redox gene regulation, signalling, *S*-nitrosylation, *S*-oxidation, sulfiredoxin, thioredoxin.

Introduction

Plant cells generate reactive oxygen (ROS) and nitrogen (RNS) species involved in the general metabolism. These reactive species can be harmful for cellular components due to their chemistry, but they are also involved in cellular signalling to promote defence against environmental stress situations (Martí *et al.*, 2011; Foyer and Noctor, 2013; Lázaro *et al.*, 2013). Consequently, plants have evolved a battery of redundant and elaborated mechanisms, including metabolites and enzymes, that are responsible for controlling ROS/RNS levels involved in oxidative and nitrosative insults responding to environmental cues (Noctor *et al.*, 2014; Talbi *et al.*, 2015).

Owing to the reactivity of their thiol groups, some protein cysteine residues are highly sensitive to oxidation by these reactive molecules, which may perturb cellular homeostasis. Thiol reduction is controlled mainly by the thioredoxin (Trx)/peroxiredoxin (Prx) and glutathione (GSH) systems, which respond to stress situations to regulate redox homeostasis. Thioredoxins are small proteins (around 12 kDa) containing two cysteines in the redox active centre present in all life

Abbreviations: ABA, abscisic acid; bZIP, basic leucine zipper; GSH, glutathione; Prx, peroxiredoxin; PTM, post-translational modification; RNS, reactive nitrogen species; ROS, reactive oxygen species; SA, salicylic acid; SNO, *S*-nitrosylated; SNO-Cys, with *S*-nitrosocysteine; Srx, sulfiredoxin; TF, transcription factor; TR, thioredoxin reductase; Trx, thioredoxin.

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forms. They regulate the function of target proteins through oxidoreductase activity. Trx couples with Trx-dependent peroxidases (Prxs) to scavenge hydrogen peroxide (H_2O_2) and peroxinitrite (König *et al.*, 2002; Balmer *et al.*, 2004; Barranco-Medina *et al.*, 2007). In addition, Trx does not simply act as a scavenger of ROS but also as an important regulator of the oxidative stress response through protein–protein interactions (Martí *et al.*, 2011; Zhang *et al.*, 2011). Changes in thiol status of proteins probably play a role in redox signalling under biotic and abiotic stress. Specifically, modifications in thiol-containing enzymes are reported to have an impact on their structure and functionality, and thus in the perception and control of ROS and RNS changes occurring under stress situations (Spoel and Loake, 2011).

An interesting aspect in cell biology is the control that the Trx/Prx system can exert on the transcriptional system, a signalling process occurring in several pathological and environmental stress conditions. Trxs can set the required redox state of the transcription factors (TFs) to be bound to the promoter region of DNA. Prxs, through the H_2O_2 control, may have an impact on the redox-mediated regulation of transcription (Liu *et al.*, 2013). These TFs couple environmental stress to gene expression, and, for some, overexpression has been reported to increase plant stress tolerance (Hu *et al.*, 2006).

In this review, we summarize some representative examples of the regulation of Trx and Prx through cysteine *S*-oxidation and/or *S*-nitrosylation by affecting their functionality in a particular cellular process and/or stress situation. A comparative analysis of plant, animal, and yeast systems is presented. In addition, we consider some examples linking gene and/or TF regulation to redox changes under stress situations. Finally, we describe the emerging knowledge on the transcriptional regulation of Trx and Prx genes.

ROS and RNS drive conformational changes in the Trx/Prx system: functional significance

ROS generated as by-products of cellular metabolism are known to produce reversible oxidative modifications in proteins by altering their activity or increasing their susceptibility to aggregation and degradation. Interestingly, many of the key players involved in the defence against oxidative stress are redox-sensitive proteins containing cysteine (Cys), methionine (Met), or histidine (His) in their amino acid sequence or metals in their active centres. The redox state of these residues is directly involved in the protein structure and functionality (Couturier *et al.*, 2013). Cys is one of the best-conserved amino acids in proteins, although it accounts for only 2% of amino acid content in cells (Lindahl *et al.*, 2011). Cys residues perform important functions in the cell by playing structural roles as metal ligands and because they are susceptible to several post-translational modifications (PTMs). Cysteine SH can also react with neighbouring SH groups to form a disulfide bridge. While as free amino acid the –SH group of Cys has a pK_a of about 8.3, in thiol oxidoreductase proteins, like Trxs and glutaredoxins, reactive Cys possess a lower pK_a (3–7). This pK_a is influenced by the surrounding micro-environment (Mailloux *et al.*, 2014). The presence of adjacent positively charged amino acids like lysine in glutaredoxins (Stroher and Millar, 2012) or the close proximity of a His residue in sulfiredoxin (Srx) (Iglesias-Baena *et al.*, 2010) can decrease the pK_a of the Cys. Thus, at a physiological pH, these Cys residues will appear predominantly as more-reactive thiolates and facilitate different oxidation states in response to redox signals.

H_2O_2 is the most relevant ROS for mediating oxidative reactions due to its reactivity with cysteine, its prolonged half-life relative to other ROS, and its capacity to diffuse through membranes. During redox signalling, H_2O_2 oxidizes the thiolate anion of protein Cys residues to the sulfenic form (Cys–SOH) (Fig. 1A). The reversibility of the process can serve as a signal transduction mechanism, ensuring transient signalling and avoiding irreversible overoxidation of the thiol (Brandes *et al.*, 2009). The sulfenic form can also react with another thiolate to form an intra-or inter-molecular disulfide bond. The sulfenic form is estimated to be formed in the nanomolar range of H_2O_2, whereas higher levels oxidize thiolates to a sulfinic (SO_2H) or sulfonic (SO_3H) species, which can be irreversible. Control of H_2O_2 levels must, therefore, be tightly exerted by the protective antioxidant system to avoid overoxidation of functional proteins. During a stress situation, changes in the Cys redox status trigger signalling pathways, influencing the response to the biotic and abiotic stress responsible for the oxidative situation. However, these redox signalling mechanisms are dependent on the extreme specificity for the appropriate substrates.

The modification of Cys by nitric oxide (NO) forming *S*-nitrosothiols occurs through its reaction with thiyl radicals formed from one electron oxidation of thiolates via NO_2. Additionally, NO_2 can react with NO to form N_2O_3, which can combine with cysteine thiolate to form the *S*-nitrosothiol. Another possibility is the transfer to a free thiol group of a haem-bound NO or the *trans*-nitrosylation (transfer of NO) from an *S*-nitrosylated residue to another thiolate or to GSH to form GSNO, the physiological NO transporter and reservoir. This GSNO contributes to protein *S*-nitrosylation and

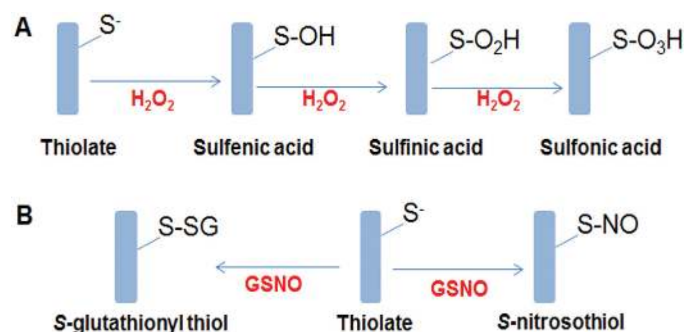

Fig. 1. (A) Different oxidation states of the free thiol side chain on cysteines of regulatory proteins by different levels of H_2O_2. (B) Protein *S*-glutathionylation and *S*-nitrosylation by reaction of the thiolate form with nitrosoglutathione. (This figure is available in colour at *JXB* online.)

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S-glutathionylation, which is thought to be part of the signalling transduction (Astier and Lindermayr, 2012) (Fig. 1B).

Thiol modification of Trx mediated by H_2O_2

Trxs contain a conserved active site, WCG/PPC, which is essential for the redox regulation of specific target proteins. In plants, there are at least 10 families of Trxs, with more than 40 members present in almost all the cellular compartments (see Gelhaye *et al.*, 2005; Martí *et al.*, 2009; Meyer *et al.*, 2012; Traverso *et al.*, 2013). The diversity of isoforms seems to support plants with an additional antioxidant system, compared with mammals, where only two types of Trx have been described: Trx1 and Trx2, in the cytosol and mitochondria, respectively (Lillig and Holmgren, 2007).

As a disulfide reductase, Trx catalyses the reduction of disulfides to dithiol in target proteins at even faster rates than dithiothreitol or GSH. The redox mechanism is based on the reversible oxidation of two Trx cysteine thiol groups to a disulfide, with the transfer of two electrons and two protons (Holmgren, 1995). However, a redox-dependent switch in protein structure and function in response to environmental stress has been described for several Trxs (Chi *et al.*, 2013). *Arabidopsis thaliana* AtTDX (Trx-like protein) composed of two domains, a Trx motif and a tetratricopeptide-repeat motif (Kim *et al.*, 2010), and NTRC (NADPH-Trx reductase, type c), containing an N-terminal thioredoxin reductase (TR) and a C-terminal Trx domain (Pérez-Ruiz *et al.*, 2006), in addition to AtTrx-*h*3, NtTrx *f*, and NtTrx *m*, have all been shown to function as a disulfide reductase, foldase chaperone, and holdase chaperone (Park *et al.*, 2009; Sanz-Barrio *et al.*, 2012; Chae *et al.*, 2013). All presented disulfide reductase and foldase chaperone activities in their low-molecular-weight form, while the holdase activity was presented by the high-molecular-weight complexes. It has been reported that oligomerization status is regulated by heat shock and ROS concentration for AtTDX, which provokes the change to oligomeric complexes and a functional switch to holdase chaperone (Lee *et al.*, 2009). Through this activity, and as a protecting mechanism of protein self-aggregation, overexpressing AtTDX *Arabidopsis* plants presented more resistance to heat-shock stress. Moreover, it seems that the Cys residues in the active site are involved in the disulfide activity, as described for Trx *f* and AtTrx-*h*3 (Park *et al.*, 2009; Sanz-Barrio *et al.*, 2012), but not in the holdase chaperone. However, mutation in non-active-site Cys in Trx *f* has also been found to display a strong influence in the disulfide reductase and chaperone foldase functions. More recently, *Arabidopsis* tetratricopeptide Trx-like (TTL) proteins, which are essential for salt and osmotic stress tolerance, have been shown to be possible co-chaperones that interact with Hsp90 and Hsp70 (Prasad *et al.*, 2010). Through this chaperone-like activity, these proteins could be involved in the stress tolerance.

Thiol modification of Prx mediated by H_2O_2

Prxs are thiol-based peroxidases involved in peroxide detoxification and signalling. More recently, it has been demonstrated that Prxs undergo multiple functions as a molecular chaperone, enzyme activator, protein binding partner and redox sensor (Caporaletti *et al.*, 2007; Barranco-Medina *et al.*, 2008; O'Neill *et al.*, 2011). In *Arabidopsis* plants, 10 Prx genes have been classified according to their number and position of the conserved Cys, into four functional categories: 1-Cys Prx, 2-Cys Prx, Type-II Prx, and Prx Q. These are located in different compartments, such as the cytosol, mitochondria, chloroplasts, and nucleus (Rouhier and Jacquot, 2005; Barranco-Medina *et al.*, 2007; Iglesias-Baena *et al.*, 2010; Dietz, 2011). In mammals and humans, six different Prxs (Prx I–VI) are classified and grouped into three types (Rhee *et al.*, 2012). Under the normal catalytic cycle, Prxs are selectively oxidized by H_2O_2 to sulfenic acid (Cys-SOH) at its peroxidatic cysteine (C_P). The C_P in 2-Cys-Prx then reacts with a resolving cysteine (C_R) located in the other subunit of the homodimer to produce an intermolecular disulfide. However, an intramolecular disulfide is formed in the atypical 2-Cys Prxs including hPrx V and plant Prx IIF (Seo *et al.*, 2000; Barranco-Medina *et al.*, 2007). The disulfide or sulfenic acid form of these Prxs is subsequently and specifically converted back to a reduced state by biological thiols, such as Trx or glutaredoxin (Rouhier *et al.*, 2002; Barranco-Medina *et al.*, 2007, 2009; Meyer *et al.*, 2012). The Cys sulfenic form can be hyperoxidized by peroxide to form a more stable sulfinic Cys (Cys-SO_2H). This form can be reduced by Srx via the formation of a phosphoryl intermediate on the sulfinyl moiety, attacked by the catalytic Cys of Srx. Finally, a thiosulfinate intermediate between Prx and Srx is formed (Biteau *et al.*, 2003; Jönsson *et al.*, 2008; Iglesias-Baena *et al.*, 2010) (Fig. 2). However, a hyperoxidized form of the sulfinic form to a sulfonic Cys is irreversible to the best of our knowledge. In pea and *Arabidopsis* plants, Srxs are located in chloroplasts and mitochondria (Rey *et al.*, 2007; Iglesias-Baena *et al.*, 2010). In addition to the well-known function of chloroplastic 2-Cys Prx, the mitochondrial Srx isoform has shown a broader specificity. It is able to retroreduce the sulfinic forms of atypical plant mitochondrial PrxII F and atypical human PrxV (Iglesias-Baena *et al.*, 2011).

Under conditions of extreme oxidative stress, increasing H_2O_2 flux leads to overoxidation of the peroxidatic Cys to sulfinic acid and enzyme inactivation. This probably acts as a dam to H_2O_2 flow or leaves sufficient time for H_2O_2 to mediate signalling (Poole and Nelson, 2008). Prx inactivation via overoxidation may be a way to accumulate H_2O_2 to allow oxidation of other redox proteins (the 'floodgate' hypothesis) (Wood *et al.*, 2003). In this way, Prxs may continuously interpret and report peroxide levels by using their redox and oligomeric states. They could function as highly sensitive peroxide dosimeters that link oxidant metabolism to a variety of redox-dependent processes required for cell cycle re-entry (Phalen *et al.*, 2006).

The different mechanisms in which sensitivity to oxidation of 2-Cys Prxs is involved in its signalling function have been reviewed (Hall *et al.*, 2009). Two of the proposed mechanisms are related to stress response and rely on a gain of function through disulfide exchange with other sensor

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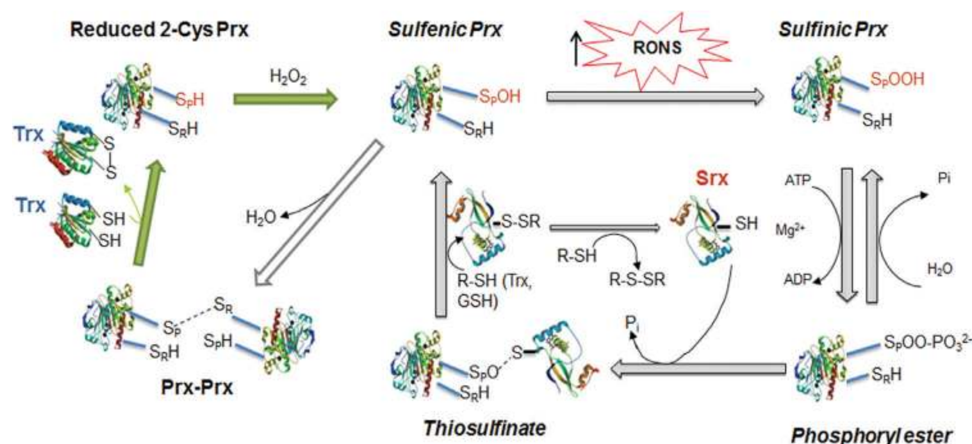

Fig. 2. Prxs share a common catalytic mechanism where, by reducing peroxide, the catalytic active cysteine is oxidized to a sulfenic acid (SOH), which then forms a disulfide bond with a resolving cysteine that is reduced by the Trx system. Under severe oxidative stress, Prxs are overoxidized to the sulfinic form (SOOH), inactivating the peroxidase activity but gaining a chaperone activity. The sulfinic acid is phosphorylated through a reversible step in the presence of Srx and ATP. The phosphoryl ester (Prx–SOO–PO_3^{2-}) is converted into a thiosulfinate with Srx (Prx–SO–S–Srx), and inorganic phosphate (Pi) is released. A reducing agent (R-SH) (Trx, GSH) reduces this complex to release the sulfenic Prx and the complex Srx-reducing agent that is subsequently reduced to active Srx-SH. (This figure is available in colour at *JXB* online.)

proteins or on a switch to a chaperone activity. The third mechanism relies on a loss of function as peroxidase, thus allowing localized H_2O_2 build-up as described in the flood-gate model, although this mechanism is not clearly proven. Any structural change in Prxs as a consequence of oxidation could affect their redox state, oligomeric structure, and/or interaction with other proteins and have a significant impact on the cascade of signalling events. In pea plants, PsPrxIIF has been shown to interact *in vitro* and *in vivo* with the mitochondrial PsTrx*o*1 (Barranco-Medina *et al.*, 2008), and both proteins probably protect mitochondria from oxidative stress in saline conditions (Martí *et al.*, 2011). *In vitro* interaction of PsPrxIIF–PsTrx*o*1 was dependent on the catalytic residues, redox state, and conformational changes of the protein. This interaction has been observed in isolated pea mitochondria in both oxidant and reducing conditions (Barranco-Medina *et al.*, 2008).

The acquirement of a new function of Prxs as molecular chaperone has been demonstrated in plants and animals. In plants, a functional switch from peroxidase to chaperone activity of the 1-Cys Prx and 2-Cys Prx from Chinese cabbage was induced by oxidative stress, and was accompanied by both conformational and oligomeric changes. However, another 2-Cys Prx (CPrxII) showed the highest peroxidase activity but no chaperone activity, as observed in the other two isotypes. The capacity of both Prxs to switch between roles as peroxidases and chaperones is probably involved to some extent in the response under different environment conditions (Kim *et al.*, 2012). More recently, a collection of 2-CysPrx variants was studied to establish an unequivocal link between conformation and function (König *et al.*, 2013). In these variants, depending on the amino acid substituting at Cys, tyrosine or phenylalanine, the peroxidase and/or chaperone functions were strongly influenced, as was the hyperoxidation sensibility. The authors underlined the importance of the advance in our understanding of multifunctional properties of 2-Cys Prx, which is probably involved in the observed moderate tolerance to oxidative and temperature stresses when overexpressed (An *et al.*, 2011). In *Pseudomonas*, the observed functional switch seems to be regulated by an additional non-active Cys112 residue. Mutated variants with an additional Cys increased the peroxidase activity, while their chaperone activities decreased compared with that of the wild type. Thus, the additional residues seemed to induce a structural change of the protein that favoured its dual function (An *et al.*, 2011).

Other interesting recent studies have proposed that the redox status of Prxs constitutes a rhythmic biomarker that exhibits circadian oscillations in cells from humans, mammals, and plants, most likely reflecting an endogenous rhythm in the generation of ROS (O'Neill and Reddy, 2011). In fact, in *Arabidopsis* plants, hyperoxidized Prx has been shown to exhibit oscillations in constant light conditions (Edgar *et al.*, 2012). This emerging, unexplored mechanism influences its redox state.

Thiol modification of Trx mediated by NO

S-Nitrosylation may have a significant role as a protective reversible mechanism against irreversible oxidation of thiol groups in proteins during oxidative and nitrosative stress. Specifically, *S*-nitrosylation of Trx has been studied extensively in the human Trx1, which contains three structural Cys, in addition to the two active-site ones. Inconsistent results have been reported for the specificity of the GSNO-dependent nitrosylation of the three non-active-site Cys residues. Cys62 was described as *S*-nitrosylated from crystal structure analysis, despite being a buried residue, which after oxidation is exposed and therefore might be *S*-nitrosylated (Weichsel *et al.*, 2010). However, in other studies, Cys69 or Cys73 are reported as primary sites of *S*-nitrosylation. Barglow *et al.* (2011) reported that human reduced Trx1 was nitrosylated at Cys62 faster than the oxidized Trx1 form, which presented only Cys73 as modified,

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while no modification was observed in other Cys residues. Moreover, *S*-nitrosylation occurred only at high concentrations of GSNO (1 mM), while at biological concentrations, Trx1 was *S*-nitrosylated only in a single Cys (Cys62 or Cys73). The redox state of the Cys residues, the concentration of GSNO used, and the experimental conditions employed by the different groups could all be responsible for the contradictory results described. While oxidized Trx1 was reported to be *S*-nitrosylated in Cys73, this modification was not detectable in reduced Trx1 (Wu *et al.*, 2010). These authors also showed a *trans*-nitrosylating activity for Trx1, as described previously by Hashemy and Holmgren (2008). Moreover, Prx1 was identified among 47 new Trx1 *trans*-nitrosylating target proteins, and the *S*-nitrosylation prevented its H_2O_2-induced overoxidation, thus protecting HeLa cells from apoptosis (Wu *et al.*, 2010). In fact, the *S*-nitrosylation of Trx1 on Cys73 only occurred after the disulfide bond formation between Cys32 and Cy35, and the *S*-nitrosylated protein was able to *trans*-nitrosylate caspase-3, a main executor in the apoptotic signalling pathway. All these results point to oxidative and nitrosative alternating modifications of specific Cys residues in Trx as key regulating events in the protein functionality. As indicated previously, stress situations can regulate the redox state of the different Cys, resulting in a change in structure and in the reduction of *trans*-nitrosylating or denitrosylating activities of the protein.

Denitrosylation has been described as fundamental for the reversibility of the modification, and Trx1/TR are involved in this process in mammalian cells, with direct activity towards *S*-nitrosylated (SNO) proteins. This denitrosylating activity is carried out via the catalytic Cys32 and Cys35 (Sengupta and Holmgren, 2013). SNO-Trx1 is able to *trans*-nitrosylate caspase-3, thus preventing apoptosis, while reduced Trx1 denitrosylates SNO-caspase-3. The last function has also been described for mitochondrial Trx2 on stimulation of the death receptor Fas (Sun *et al.*, 2013). Thus, SNO-protein denitrosylation/*trans*-nitrosylation could imply an additional function for the Trx system as a key regulating point in the apoptotic process. In fact, a decrease in *trans*-nitrosylation could facilitate cell death in medical disorders, while increasing *S*-nitrosylation of caspase-3 could lead to higher survival rates of damaged cells.

Research on the role of *S*-nitrosylation on Trx in plants is limited. Plant Trx*m*5 has been detected as *S*-nitrosylated protein together with glyceraldehyde 3-phosphate dehydrogenase (also identified in *Arabidopsis* after nitrosylating treatments; Lindermayr *et al.*, 2005). However, the *S*-nitrosylation was found only in a *nitric oxide excess1* (*noe1*) mutant of rice and not in control plants. Recently, in *Brassica juncea* seedlings under cold stress, Trx*h* was shown to be a target of *S*-nitrosylation, probably as a result of the NO produced (Sehrawat and Deswal, 2014).

The *S*-nitrosylation/denitrosylation process appears to be influenced by several NO species, redox proteins, enzymatic denitrosylase activities such as GSNO reductase (GSNOR), the Cys residue environment of the involved proteins, and the subcellular location of all of these components. The complexity can again be shown in the mechanism involving redox signals through cytosolic Trxs and GSNO in plants. Both are involved in the oligomer–monomer exchange of NPR1 (non-expressor of pathogenesis-related gene 1) on pathogen challenge in *A. thaliana*. Without pathogen infection, NPR1 is located in the cytosol as an inactive oligomer maintained through intermolecular disulfide bonds between solvent-exposed Cys residues. After pathogen infection, salicylic acid (SA) induces the reduction to monomers, which are translocated to the nucleus, promoting the transcriptional activation of SA-dependent defence-related genes (Mou *et al.*, 2003). However, additional results have been reported related to the regulation of NPR1 through the opposing action of GSNO and Trx in the NO-mediated *S*-nitrosylation. It has been shown that GSNO *S*-nitrosylates NPR1 at Cys156 and may facilitate its oligomerization through disulfide linkage between some of the NPR1 monomers, preventing NPR1 translocation to the nucleus (Tada *et al.*, 2008). Moreover, Trx*h*5 is required for *in vivo* SA-induced monomer release, probably preventing oligomerization. However, data obtained in GSNO-treated *Arabidopsis* protoplasts by Lindermayr *et al.* (2010) showed that the treatment produced *S*-nitrosylation of both NPR1 and TGA1 [TGACG motif binding factor 1, a basic leucine zipper (bZIP) protein]. This favoured NPR1 translocation into the nucleus and enhanced TGA1 binding activity to the promoter regions of defence-related genes. The fact that oligomerization mediated by *S*-nitrosylation did not inhibit NPR1 translocation implies that the mechanism of oligomer–monomer exchange regulating nuclear translocation remains unclear. Likewise, due to the possible glutathionylating effect of GSNO used in the experiments, the type of modification responsible for the increased DNA-binding activity of TGA1 is not completely elucidated. These authors even reported oligomerization induced by *S*-nitrosylation as a step prior to monomer accumulation compatible with the results of Tada *et al.* (2008). The revealed complexity of the mechanism could explain the apparently contradictory experimental data. Interestingly, the involvement of Trx as a denitrosylase in this process was recently reported by Kneeshaw *et al.* (2014), demonstrating that Trx*h*5 was able to denitrosylate TGA1 in the plant immune response. This is very interesting given the scarce knowledge about the involvement of plant Trxs in denitrosylation processes. The Trx*h*5/NTRA system was shown to be able to denitrosylate a variety of plant proteins in both *in vitro* and *in vivo* experiments, suggesting that Trx*h*5 facilitates monomerization and nuclear translocation. Moreover, the SNO-Trx*h*5 intermediate was detected in the process of *trans*-denitrosylation of Trx*h*5 to SNO-protein targets, using a single active-site Cys. However, the detailed mechanism is not completely elucidated. This is in agreement with the finding of Trx*h*3 as an *S*-nitrosylated protein in *Arabidopsis* leaves only after challenge by the pathogen *Pseudomonas syringae*, which probably represents a link between gene regulation and redox changes triggered by pathogens (Maldonado-Alconada *et al.*, 2011). Moreover, the Trx*h*/NTR system has been shown to be unable to denitrosylate *S*-nitrosylated glyceraldehyde

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3-phosphate dehydrogenase *in vitro* (Zaffagnini *et al.*, 2013), pointing to a selective process, at least in *in vitro* systems.

Thiol modifications of Prx mediated by NO

Besides the involvement of NO and associated *S*-nitrosylation in the above signalling processes related to Trxs, several studies have shown that Prxs from animals and plants are targets of *S*-nitrosylation under normal and stress conditions. Prx2, the most abundant Prx in mammalian neurons, is a target of *S*-nitrosylation in human Parkinson's disease brains. The reaction of NO with two critical Cys residues (Cys51 and Cys172) (Fang *et al.*, 2007) reduced enzyme activity and prevented its overoxidation by exogenous H_2O_2. Mammalian Prx1 is also *S*-nitrosylated, causing oligomeric structural and functional alterations and loss of peroxidase activity. In this sense, treatment with *S*-nitrosocysteine (SNO-Cys) modulated the Trx/Prx/TR system through *S*-nitrosylation of TR and Prx1. Trx-mediated regeneration of oxidized Prx1 was blocked by SNO-Cys, probably through a competition mechanism with Prx1 for the Trx system (Engelman *et al.*, 2013).

Recently, the application in plants of exogenous NO donors like *S*-nitrosylating agents and the proteome-wide analysis upon biotic and abiotic stressors have identified a growing list of *S*-nitrosylated candidate proteins. In *Arabidopsis* leaves, cytosolic PrxIIB and a chloroplast PrxIIE are proteins that are specifically *S*-nitrosylated during the plant immune response (Lindermayr *et al.*, 2005; Romero-Puertas *et al.*, 2007). *S*-Nitrosylation in PrxIIE was demonstrated to be a specific time-dependent response, inhibiting its peroxidase and peroxynitrite reductase activity. A model was suggested in which this PTM regulates the transduction of $NO^{\bullet}$- and ROS-linked signals during infection by *P. syringae*, highlighting a key role for PrxIIE in controlling the endogenous level of $ONOO^-$ (Romero-Puertas *et al.*, 2007, 2008). In *Brassica juncea* extracts treated with GSNO, 1-Cys Prx was identified among the *S*-nitrosylated proteins, although it did not appear as differentially *S*-nitrosylated under cold stress (Abat and Deswal, 2009). Also, in wild rice and in an *noe1* rice mutant subjected to H_2O_2-induced leaf cell death, a chloroplastic PrxII E was found as target of *S*-nitrosylation (Lin *et al.*, 2012).

Recent evidence has shown an interplay between oxidative and nitrosative stress through the carbonylation and *S*-nitrosylation of proteins in plants subjected to salt stress (Tanou *et al.*, 2009). The *S*-nitrosylation of specific active Cys residues leads to a conformational change that possibly prevents their carbonylation and thus the irreversible loss of protein function under salt stress. In this study, Prx is described as one of the proteins suffering carbonylation and *S*-nitrosylation, suggesting a fine regulation of protein PTMs under oxidative and nitrosative stress, which could define their abundance, function, and localization.

In the response to salt stress, the mitochondrial pea Prx was specifically *S*-nitrosylated in a time-dependent manner over a long but not a short time period, possibly responding to increased NO in part associated with mitochondria under long-term salt stress (Camejo *et al.*, 2013). Subsequently, we have demonstrated that recombinant mitochondrial PrxIIF is modified by GSNO and sodium nitroprusside dehydrate treatments resulting in a conformational change and a functional switch. Thus, the peroxidase activity of the *S*-nitrosylated PrxIIF was reduced and a novel function as *trans*-nitrosylase activity has been reported recently when incubated with citrate synthase protein, thus preventing its thermal aggregation (Camejo *et al.*, 2015). It would be interesting to know whether this *trans*-nitrosylating activity also occurs *in vivo* in salt-stressed plants in which mitochondrial pea Prx appeared as *S*-nitrosylated.

Redox regulation of gene transcription under stress

As a result of the increased cellular ROS production in response to environmental stress, organelle signals enter the nucleus to modulate gene expression. However, the actual sensors and second messengers of the retrograde signalling cascade are not well understood (Foyer and Noctor, 2013). The rapid increase in ROS may alert a sensor molecule that transduces the signal binding to an effector, such as the responsive elements in DNA, thus allowing gene expression. Reactive thiol proteins appear as good candidates to act as sensors for the ROS transmission of the information to the transcription system. A link between gene regulation and redox changes has been reported under the stress response (Astier *et al.*, 2011), and the slow return to the Prx sulfenic state by Srx has been reported recently as being a crucial property for a direct or indirect oxidative stress sensor (Wood *et al.*, 2003; Lázaro *et al.*, 2013).

ROS-responsive genes related to Trxs and Prxs

Transcriptional profiling of *Arabidopsis* plants subjected to several abiotic stress conditions has identified several *cis*-regulatory elements associated with ROS (Allu *et al.*, 2014). Some of these ROS-responsive elements have the G-box element, CACGTG, involved in the response to abiotic stress and hormone signalling, particularly abscisic acid (ABA). A W-box (TTGAC/T) is also recognized by WRKY (from the WRKY sequence at the N terminus), a TF induced under biotic and abiotic stress (Cheng *et al.*, 2012). As one example, it has been suggested that WRKY6 is a positive regulator on cytosolic AtTrx*h*5 expression, which is also mediated by ROS under oxidative stress conditions (Laloi *et al.*, 2004). Moreover, *AtTRXh5* was upregulated in plants overexpressing *WRKY6*. This regulation is specific to the Trx *h* family.

It is becoming increasingly evident that ROS (H_2O_2) production and transcriptional regulation of redox-regulated genes are controlled by the circadian clock. These findings point to a possible functional link between ROS signalling and circadian output, which provides a mechanistic link for the plant response to oxidative stress. In fact, *CCA1* (*CIRCADIAN CLOCK-ASSOCIATED 1*) seems to be a

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central regulator for the co-ordinate relationship between the ROS-driven transcription, ROS production, and scavenging in response to oxidative stress (Lai *et al.*, 2012). Among these genes, the circadian regulation of both chloroplastic *f* and *m*1 pea Trxs through control of the CCA1 TF was apparent, probably through its direct interaction with an evening element of the promoter region of these genes. This circadian regulation of Trxs can provide an additional regulatory mechanism that might be related to their redox regulation of light-dependent protein targets (Barajas-López *et al.*, 2011). Transcript data reported that the abundance of *AtTrx-like5* mRNA (WCRRC Thioredoxin1) and *AtTrxf2* responded to oscillatory regulation (Harmer, 2009). Besides light, the expression of *PsTrxf* and *m1* is regulated by sugar, probably through a DOF 7 TF. However, the redox signalling pathways leading to changes in gene expression remain elusive (Barajas-López *et al.*, 2012).

Many plant hormones and cellular redox agents are of great importance in the regulation of gene transcription under plant acclimation to stress. A number of excellent reviews have been published on the interplay between the signalling pathways (e.g. Astier *et al.*, 2011; Bartoli *et al.*, 2013). Thus, this interaction is not considered further here. As a representative example, osmotic stresses (drought, salinity) induce the expression of AZF1 and AZF2 (*Arabidopsis* zinc-finger proteins) via ABA-dependent or -independent pathways (Kodaira *et al.*, 2011). When an amount of AZF1 and AZF2 TFs accumulates under stress, many ABA-repressive genes are suppressed. AZF1 and AZF2 interact with the promoter regions of several small auxin-up RNA (SAUR) genes. Both TFs regulate ABA and auxin signalling in plant responses to osmotic stress. Recently, we described AZF2 as one of the putative TFs regulating *AtTrxo1* gene expression (Ortiz-Espín *et al.*, 2013). Further work may allow us to assess whether and how this redox-regulated TF operates *in vivo* to influence *Trxo1* expression, which is also involved in redox regulation under plant development and/or the response to stress conditions (Martí *et al.*, 2011).

The transcriptional regulation of the *At2CysPrx A* gene (*2CPA)* responds to ABA and chloroplast signals. This gene is induced by peroxides and photo-oxidative stress and repressed by ABA, and the regulation serves as a model to study redox and hormonal signalling pathways independently (Baier *et al.*, 2004; Hiltscher *et al.*, 2014). Moreover, *2CPA* expression is highly sensitive to ascorbate, is light-dependent, and seems to need the co-existence of the TFs Rap2.4 and RCD1 (Shaikhali *et al.*, 2008; Shaikhali and Baier, 2010) (see below).

ROS-responsive TFs

Until the late 1980s, no evidence had been found that sulfenic acids, in addition to their role in catalysis and protection against ROS, can act directly as signalling molecules triggering the cell response to oxidative stress via TFs (Zheng *et al.*, 2001) and allowing acclimation to specific stress conditions (Locato *et al.*, 2010).

The DNA-binding activity of numerous TFs in microorganisms and yeast, such as OxyR, OhrR, AP1, and CrtJ, is regulated by the primary formation of a sulfenic acid, which is often transformed into a disulfide bond (Zheng *et al.*, 1998; D'Autreaux and Toledano, 2007) or modified by *S*-nitrosylation. *Escherichia coli* OxyR was shown to undergo different oxidative modifications in Cys199 such as -SOH, -SSG and -SNO, which differently affected its DNA-binding capacity (Kim *et al.*, 2002). Similarly, CrtJ was modified on Cys420 after exposure to H_2O_2 or oxygen (Cheng *et al.*, 2012). In mammals, the nuclear erytroid 2 related bZIP TF (Nrf2) in its inactive form is bound to the protein Keap1, the redox sensor of the complex that promotes its ubiquitination. H_2O_2 oxidizes reactive Cys of Keap1 and changes its conformation, breaking the complex and releasing Nfr2 in its active form. After phosphorylation, Nrf2 is translocated to the nucleus, where it binds to the antioxidant responsive elements of DNA and promotes the expression of antioxidant proteins such as Trx, TR, Prxs, glutaredoxins, and Srxs, which in turn eliminate the redox signalling (Flohé and Flohé, 2011). In yeast, the Prx Tpx1 has been shown to be necessary for the H_2O_2-dependent activation of the bZIP TF Pap1 in a concentration-dependent manner (Schippers *et al.*, 2012). This provoked its translocation to the nucleus and the activation of antioxidant genes. Moreover, the recycling of Tpx1 by the cytosolic-reduced Trx1 is required (Calvo *et al.*, 2013). However, to date, few examples of such signalling functions are known in plants, although some redox-regulated TFs under retrograde regulation have been described under the stress response (Gupta and Luan, 2003).

As well as the above-described involvement of Trx*h*5 in the redox regulation of NPR1, allowing its translocation to the nucleus to activate redox-sensitive TGA TFs (Mou *et al.*, 2003), sugarcane Trx*h*1 has been identified as an interacting partner of the redox-regulated TF SsNAC23. It is a member of the plant-specific NAC TF family, with roles in the development and response to cold stress (Jensen *et al.*, 2010). The regulation seems to affect disulfide bridges between some of the three cysteine residues in the TF. Thus, Trx*h*1 might act by changing the structure of SsNAC23, with implications for its interactions with other proteins (Jensen *et al.*, 2010). However, the exact molecular mechanism is not clearly described.

As another example, the redox-sensitive Rap2.4a, an AP2-type transcription factor, was identified as the first signal transduction component involved in redox regulation of the chloroplast *2CPA* gene promoter mentioned above. Upon moderate redox imbalances, Rap2.4a protein dimerizes and binds to the promoter region activating *2CPA* transcription. Under severe oxidative stress, this TF oligomerizes, loses its DNA affinity, and thus decreases transcription levels of the gene. Rap2.4 also orchestrates the activation of other genes encoding chloroplast antioxidant enzymes, including stromal ascorbate peroxidase (sAPX) and Cu,Zn-SOD (Shaikhali *et al.*, 2008). The TF RCD1 (RADICAL-INDUCED CELL-DEATH 1) protein interacts with Rap2.4a and potentiates its effect in the transcriptional activation of genes encoding

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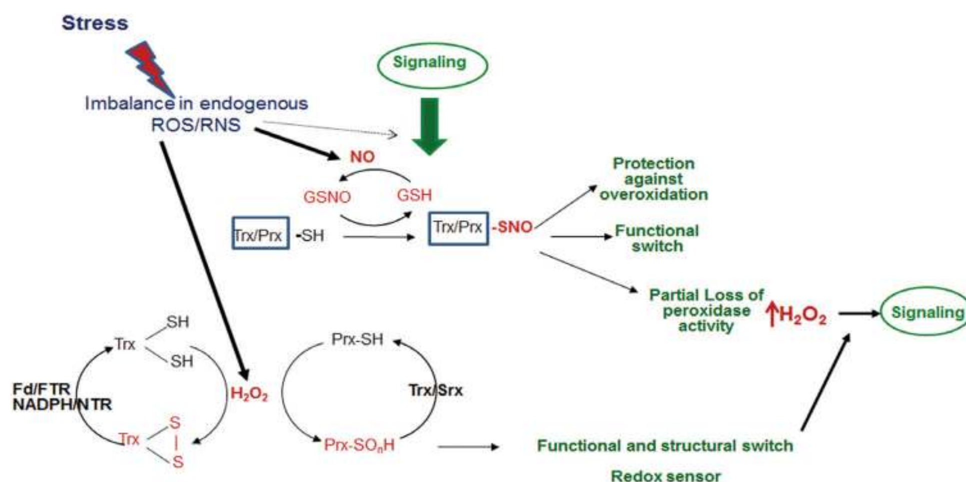

Fig. 3. Under nitrosative or oxidative stress in the plant cell, Trx and Prx can be *S*-nitrosylated to protect them from overoxidation and to allow signalling. On the other hand, Prx can be overoxidized to the sulfinic form to function as a redox sensor. This form can be regenerated to the reduced one by the action of Srx and Trx. Prxs share a common catalytic mechanism where, by reducing peroxide, the catalytic active Cys is oxidized to a sulfenic acid (SOH), which then forms a disulfide bond with resolving Cys that is reduced by the Trx system. Under severe oxidative stress, Prxs overoxidize to the sulfinic form (SOOH), inactivating the peroxidase activity but gaining a chaperone activity. Ferredoxin/Trx system: Fd/FTR (ferredoxin/ferredoxin-dependent Trx reductase); NADPH/Trx system: NADPH/NTR (NADPH-dependent Trx reductase). (This figure is available in colour at *JXB* online.)

chloroplast antioxidant enzymes (Hiltscher *et al.*, 2014). RCD1 also interacts with DREB2A TF in drought responses (Vainonen *et al.*, 2012).

Redox control of eukaryote MYB TFs is through the two conserved Cys residues, which, under oxidizing conditions, form an intramolecular disulfide bond that alters its domain structure, preventing DNA binding. For clusters of MYB proteins in *Arabidopsis* presenting only one Cys residue, an alternative mechanism that involves cysteine *S*-nitrosylation may control and negatively influence their DNA-binding activity (Dubos *et al.*, 2010). Other redox-regulated TFs in plants are heat-shock factors (Hsfs), which bind to the conserved heat-shock elements. *Arabidopsis* HsfA1a, directly senses heat stress and H_2O_2 via redox state (Liu *et al.*, 2013). This TF has two Cys residues that, when substituted for Ser, suppressed the expression of its target gene (Jung *et al.*, 2013). The expression of *HsfA2* and its target genes, *APX2* and heat-shock protein *18.1-C1*, is low under non-stressed conditions but is strongly induced by heat shock and ROS in *Arabidopsis* (Nishizawa *et al.*, 2006; Shigeoka and Maruta, 2014). Another example is the TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR1 (TCP), which participates in plant cell proliferation and growth. It contains a Cys sensitive to redox conditions and is inhibited under oxidative stress. The inhibition can be reversed by Trx and Trx reductase (Viola *et al.*, 2013). An additional role of the modification of class I TCP proteins by redox agents may be their protection from permanent inactivation under oxidative stress conditions, considering that disulfide bond formation and modification by GSH, H_2O_2, or NO are reversible processes.

In conclusion, the above results suggest that redox changes operate *in vivo* to influence the activity of different TFs within plant cells. These redox-dependent modifications have an impact on downstream events regulated by each specific TF. This mechanism is thought to be one of the most important ways for the plants to respond and adapt to stress conditions.

Future perspectives

ROS and NO play a pivotal role in regulating numerous responses to biotic and abiotic stresses in plants. The involvement of the crosstalk between ROS and NO in the regulation of protein activity and function and the effect on signalling affecting gene transcription in response to different stresses is beginning to be elucidated. While there has been important progress in the signalling role of the redox state of thiol in Trxs and Prxs through PTMs such as protein oxidation, *S*-glutathionylation, and *S*-nitrosylation/denitrosylation, many challenges remain regarding thiol specificity. When and how they are co-ordinated to allow specific proteins to respond and their repercussions in the regulation of targets proteins and/or specific genes is a challenging task (Fig. 3). In addition, some Trxs could promote *trans*-nitrosylation and reduce *S*-nitrosothiols; thus, the characterization of plant Trxs as denitrosylases is important to establish their role in NO signal transduction. The introduction in plants of new Trx/Prx/Srx variants mimicking these PTMs in specific thiols may help to unravel the role of these proteins as redox sensors and in the signalling process under normal and stress conditions.

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