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Review article

Thioredoxin-mediated redox signalling in plant immunity☆

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ABSTRACT

Activation of plant immune responses is associated with rapid production of vast amounts of reactive oxygen and nitrogen species (ROS/RNS) that dramatically alter cellular redox homeostasis. Even though excessive ROS/RNS accumulation can cause widespread cellular damage and thus constitute a major risk, plant cells have evolved to utilise these molecules as important signalling cues. Particularly their ability to modify redox-sensitive cysteine residues has emerged as a key mechanism to control the activity, conformation, protein-protein interaction and localisation of a growing number of immune signalling proteins. Regulated reversal of cysteine oxidation is dependent on activities of the conserved superfamily of Thioredoxin (TRX) enzymes that function as cysteine reductases. The plant immune system recruits specific TRX enzymes that have the potential to functionally regulate numerous immune signalling proteins. Although our knowledge of different TRX immune targets is now expanding, little remains known about how these enzymes select their substrates, what range of oxidized residues they target, and if they function selectively in different redox-mediated immune signalling pathways. In this review we discuss these questions by examining evidence showing TRX enzymes exhibit novel activities that play important roles in diverse aspects of plant immune signalling.

1. Redox signalling in plant immunity

Plants are continuously exposed to attack by different pathogens and herbivorous insects. These attackers are resisted using a complex network of defence responses that include constitutive preformed structures such as the cell wall and surface waxes, as well as inducible immune signalling that through transcriptional, translational and metabolic changes result in physiological defences and production of antimicrobial compounds [1]. The first line of inducible defence relies on the detection of pathogen-associated molecular patterns by cell surface localised pattern-recognition receptors. Pathogen pattern-triggered immunity is associated with dramatic transcriptional reprogramming to activate immunity, closure of the stomata to prevent pathogen entry into the leaf apoplast, and callose deposition to limit cell penetration [2]. Successful pathogens, however, can overcome pattern-triggered immunity by injecting effector proteins that suppress key immune signalling hubs in the host. In response, plants have evolved intracellular nucleotide-binding leucine-rich repeat (NB-LRR) containing receptors that recognise the presence of pathogen effectors and trigger programmed cell death of the infected cells, thereby effectively isolating the pathogen from healthy uninfected tissues [3].

Activation of pattern- and effector-triggered immunity is associated with the rapid production and accumulation of reactive oxygen and nitrogen species (ROS and RNS) such as superoxide (O_2^-) and its dismutation product hydrogen peroxide (H_2O_2), and nitric oxide (NO). The production of ROS and RNS appears to serve multiple functions. Accumulation of these highly reactive molecules is thought to create a hostile extracellular environment for plant pathogens, contribute to immunity by strengthening the cell wall through cross-linking of glycoproteins or lipid peroxidation, play a local signalling role in inducing programmed cell death, signal systemically within a matter of minutes, and mediate establishment of broad-spectrum systemic acquired resistance [4–9].

2. Redox-based post-translational modifications in plant immunity

Although ROS and RNS have the potential to be highly damaging, eukaryotic cells have also harnessed their reactivity by utilising “sensor” proteins with redox-sensitive cysteine residues that function as activity or signalling switches. Quantitative reactivity profiling of cysteine residues in mammalian proteomes indicated that despite the intrinsic nucleophilic nature of this residue, highly reactive cysteines are a relatively rare phenomenon [10]. Thus, redox-sensitive cysteine residues are ideal activity or signalling switches in proteins that harbour them. Cysteines also provide added potential for signalling

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complexity, as they allow multiple different reversible redox-based modifications, including *S*-nitrosylation (S—NO), *S*-sulfenation (S—OH) and *S*-thiolation (S—S), the latter of which includes disulphide bonds and *S*-glutathionylation (i.e. the covalent attachment of glutathione to cysteine residues [11–14].

Regulation of the cellular amount of *S*-nitrosothiol-containing proteins (protein-SNO) has been shown to play a particularly important role in establishment of disease resistance. Mutant genotypes with elevated levels of free NO or NO donor molecules accumulate elevated levels of protein-SNO and exhibit impaired immune signalling [9,15,16]. Elevated protein-SNO levels were shown to suppress pathogen-induced accumulation and signalling by the indispensable immune hormone salicylic acid (SA), resulting in severe disease susceptibility. Despite the increasing number of protein-SNO identified and characterized in many eukaryotes, understanding how *S*-nitrosothiols are employed as specific, reversible signalling cues in plants remains a challenging task. Unlike most other post-translational modifications, formation of *S*-nitrosothiols is not directly facilitated by enzymes but rather by local concentrations of NO or NO donors. Nonetheless, an enzyme that turns over *S*-nitrosoglutathione (GSNO), a potent NO donor and cellular reservoir for NO bioactivity, has previously been identified. Knockout of this enzyme, known as GSNO Reductase 1 (GSNOR1), resulted in elevated cellular levels of GSNO and consequently of protein-SNO [15], indicating that GSNOR1 indirectly governs the cellular level of protein-SNO. In this regard, it has been reported that GSNOR1 itself is also regulated by *S*-nitrosylation, providing NO with feedback control over its own signalling pathway [17]. In addition to *S*-nitrosylation, GSNO can also trigger *S*-glutathionylation on receptive thiols. *S*-glutathionylation is an important regulator of protein function and can prevent irreversible thiol oxidation that is often associated with protein dysfunction [18].

S-nitrosothiols have been shown to regulate diverse immune signalling processes [19]. *S*-nitrosylation of cysteines in or near enzymatic active sites appears to play an important role in suppression of plant immune enzymes. *S*-nitrosothiols accumulate upon attempted infection and regulate the activity of enzymes involved in pathogen effector-triggered immunity and programmed cell death [20,21]. *S*-nitrosylation of Peroxiredoxin IIE inhibits its ability to detoxify peroxinitrite ($ONOO^-$), a reaction product of NO and superoxide that accumulates upon activation of effector-triggered immunity and causes oxidative damage in part through tyrosine nitration [22]. Moreover, the cysteine protease activity of Metacaspase 9 (MC9), related to animal programmed cell death executioners known as caspases, was found to be suppressed by *S*-nitrosothiol formation in its active site [23]. Effector-triggered production of superoxide by the NADPH-dependent oxidase RBOHD was also shown to be controlled by *S*-nitrosothiols. *S*-nitrosylation of RBOHD expelled its co-factor FAD, thereby inhibiting the production of reactive oxygen intermediates and restricting programmed cell death [21]. Finally, the SA-binding activity and carbonic anhydrase activity of SABP3, a positive regulator of effector-triggered immunity, was suppressed by site-specific *S*-nitrosylation [24]. Collectively these findings demonstrate that *S*-nitrosothiols exert their effect on immunity through regulation of enzymatic activity.

Cysteine oxidation, including *S*-nitrosylation has also been found to control the activity of transcriptional regulators and co-regulators. The transcription factor MYB30 is a regulator of effector-triggered programmed cell death by facilitating gene expression and synthesis of very-long-chain fatty acids [25]. Two cysteines in the DNA-binding domain of MYB30 are targeted by *S*-nitrosylation, altering its conformation and reducing DNA-binding [26]. Oxidative modifications also induce conformational changes in the SA-responsive master immune coactivator NPR1 (nonexpressor of pathogenesis-related genes 1). In absence of pathogen threat, disulphide bonds covalently link conserved cysteine residues of different NPR1 monomers, forming an inactive cytoplasmic oligomer that is excluded from the nucleus. Upon pathogen challenge an increase in SA levels induces changes in cellular redox that promote the reduction of NPR1 disulphide bonds with subsequent release of NPR1 monomers. Monomeric NPR1 translocates to the nucleus in order to activate numerous SA-responsive immune genes [27,28]. Interestingly, NO is also involved in the regulation of NPR1 oligomer-monomer homeostasis. *S*-nitrosylation of Cys156 of NPR1 promotes formation of NPR1 oligomer, possibly by facilitating the formation of the more oxidized disulphide state at Cys156 or surrounding conserved cysteine residues [29]. Importantly, SA induces transient reductive and oxidative fluctuations, suggesting NPR1 oligomer must be reformed after a reductive phase. Indeed, mutation of Cys156 caused constitutive translocation of NPR1 into the nucleus where eventually it was lost due to proteasome-mediated degradation, demonstrating that *S*-nitrosothiol-mediated oligomerization is critical for long-term NPR1 homeostasis [29,30]. As a nuclear coactivator NPR1 interacts with the bZIP family of TGA transcription factors, some of which are also targeted by oxidative modifications. TGA1 and TGA4 contain conserved cysteine residues, which in case of TGA1 were shown to form an intramolecular disulphide bond that prior to defence activation precludes interaction with NPR1 [31]. The same cysteines are also modified by *S*-nitrosylation and *S*-glutathionylation, which appeared to protect these residues from further oxidation and facilitated DNA binding activity of TGA1 [32]. Taken together these findings clearly indicate an intimate relationship between redox-based cysteine modifications and immune gene expression.

3. Thioredoxins enable reversible signalling with redox-based protein modifications

To utilise post-translational modifications as a cellular signalling switch their removal from proteins is equally as important as their addition. Removal is even more important for redox-based modifications as these are formed non-enzymatically from small reactive molecules in the local environment of the target protein. The superfamily of Thioredoxin (TRX) oxidoreductases is responsible for the reversal of diverse redox-based modifications. Superfamily members include conventional TRX, glutaredoxin (GRX), protein disulphide isomerase (PDI) and nucleoredoxin (NRX) [33]. Especially conventional TRX and NRX enzymes have emerged as critical regulators of redox-based protein signalling during plant immunity and their defence-related actions and functionalities are discussed further in this review.

3.1. Mechanisms of cysteine reduction by TRX

Classical TRX oxidoreductase enzymes have a conserved active site with sequence Cys-Gly-Pro-Cys, which is part of a structural fold found in all members of the TRX superfamily. The first cysteine of the active site is located near the N-terminus of an α-helix, providing it with dipole moment that lowers the pK_a and gives it a nucleophilic character [34]. Thus, the first cysteine is capable of attacking oxidised thiols in substrate targets, resulting in the formation of a covalent mixed disulphide bond between TRX and the substrate. The second cysteine of the TRX active site then resolves the mixed disulphide to render the substrate reduced, while the TRX active site cysteines form a disulphide bridge [33,35]. In order to recycle the oxidized TRX back to its reduced state, a cellular reductant is required and, in plants, two types of TRX reductases have been identified: NAPH-dependent TRX Reductase (NTR) and Ferredoxin-TRX Reductase (FTR). These systems use the reducing power from NADPH and ferredoxin to donate electrons to oxidized TRX, thereby recycling its enzymatic activity [33,36,37]. The activities of TRX-NTR/FTR systems are capable of reducing diverse cysteine modifications, including sulfenic acids and disulphides [38,39].

3.2. Mechanisms of protein denitrosylation by TRX

In addition to the above conventional activities of TRX, more

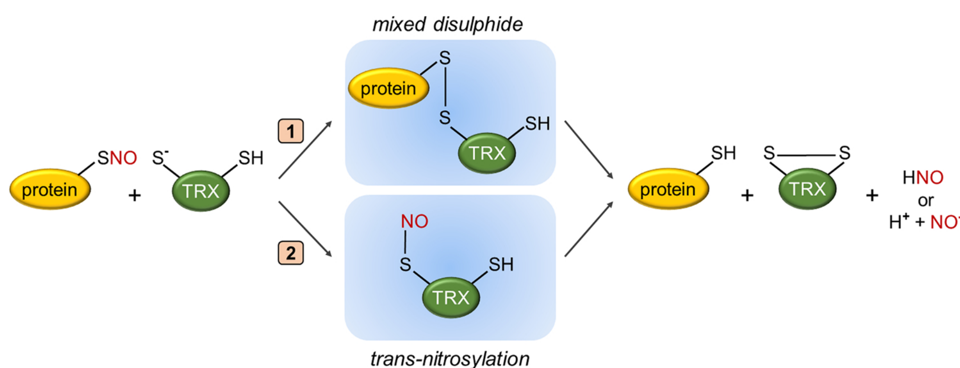

Fig. 1. Molecular chemistry of protein denitrosylation reactions by TRX.
Analogous to its disulphide reduction activity, TRX denitrosylates protein-SNO by forming a mixed disulphide intermediate (reaction 1) or by transferring the NO group to one of its active site cysteines through trans-nitrosylation (reaction 2). In both mechanisms the second resolving cysteine of the active site reduces the first cysteine so that the TRX active site forms an intramolecular disulphide while the substrate is rendered reduced with release of HNO or NO^-.

recently this group of enzymes has also been implicated in regulating protein-SNO levels. Based on the short-lived nature of many stimulus-induced protein-SNO, it seemed likely that protein-SNO reductases that directly promote breakdown of SNO groups may exist [40,41]. Indeed, in mammalian cells the TRX/NTR system was first identified as a bona fide protein-SNO reductase with key roles in apoptosis [40]. Subsequently, the TRX/NTR system in plants and particularly TRX*h*5 were shown to also regulate cellular protein-SNO levels during activation of plant immunity [16,42].

The mechanism of TRX-mediated SNO reduction has been a matter of considerable debate. Two biochemical pathways for denitrosylation have been proposed (Fig. 1). Analogous to sulfenic acid and disulphide reduction, the TRX active site may attack the sulphur atom of the SNO moiety, resulting in formation of a mixed disulphide intermediate between TRX and the substrate [40]. Indeed, denitrosylation of mammalian caspase-3 was associated with the formation of a mixed disulphide with the Trx1 active site. Moreover, prevention of disulphide-thiol exchange reactions by mutation of the resolving cysteine residue of the Trx1 active site captured a large repertoire of protein-SNO substrates [43], indicating a mixed disulphide-dependent mechanism for protein-SNO denitrosylation in mammalian cells (Fig. 1, reaction 1). By contrast, the first report describing breakdown of GSNO by *Escherichia coli* TRX measured concomitant release of $NO^{\cdot}$, a reaction product that can only be produced by homeolytic cleavage of an S—NO bond rather than heterolytic cleavage as is predicted to occur during mixed disulphide formation [44]. This alternative mechanism for SNO reduction was further investigated for plant TRX*h*5, which was also capable of reducing GSNO with release of $NO^{\cdot}$ [16]. Importantly, protein-SNO reduction by TRX*h*5 was associated with transient *S*-nitrosylation of its active site, suggesting that SNO reduction occurred through a *trans*-denitrosylation reaction (Fig. 1, reaction 2). Accordingly, only a single active site cysteine was required for successful denitrosylation of protein-SNO while accumulation of mixed disulphides was not detectable [16]. These two distinct mechanisms for protein-SNO reduction perhaps highlight an interesting difference between mammalian TRX on the one hand, and prokaryotic and plant TRX on the other. The determinants of these distinct mechanisms remain unknown but suggest protein denitrosylation is dependent on parameters specified by the SNO reductase, protein-SNO substrate, and accessibility or local environment of the SNO moiety.

3.3. Diverse thioredoxin activities regulate plant immunity

In the last decade it has become increasingly clear that different TRX enzymes play diverse roles in regulation of plant immune signalling. One of the first demonstrated roles of TRX in plant immunity came from studies of the tomato LRR-containing Cf-9 resistance protein that regulates effector-triggered immunity in both tomato and *Nicotiana benthamiana*. Cf-9-interacting TRX (CITRX), now renamed to TRX *z* [45], was found to interact with Cf-9 and its silencing revealed it is a negative regulator of the Cf-9-mediated hypersensitive cell death response. It was proposed that in unchallenged cells, CITRX/TRX *z* inhibits the interaction of Cf-9 with a putative additional protein-interaction partner, thereby preventing Cf-9 from triggering immunity. Upon recognition of pathogen effectors, however, the negative regulation of Cf-9 may be eliminated by downregulation of CITRX/TRX *z* mRNA levels, resulting in accumulation of ROS, kinase signalling, induction of defence-related genes and cell death associated with activation of effector-triggered immunity [46]. Nonetheless, it has remained unclear if Cf-9 or downstream signalling components are reversibly oxidised and true substrates of CITRX/TRX *z*. Moreover, tomato Cf-9 and CITRX/TRX z were found to interact in the cytoplasm, while in Arabidopsis TRX z is localised to the chloroplast where it is thought to play important roles in chloroplast transcription and development [45,47]. Thus, it remains unclear if and how CITRX/TRX *z* and Cf9 come into close proximity to each other during pathogen infection.

TRX activity also plays a role in local and systemic immune signalling by the immune hormone SA. Initial studies showed that TRX*h*5 gene expression was highly induced in response to pathogenic elicitors as well as wounding, abscission and senescence. Interestingly, although the TRX-*h* group consists of 8 members, only TRX*h*5 was upregulated in response to each of these stresses [29,48,49]. Subsequently, TRX*h*5 was found to promote SA-induced immune gene expression by regulating the conformational state of the SA-responsive coactivator NPR1. As described above, NPR1 is retained to the cytoplasm as a high molecular weight oligomer formed through disulphide linkages between conserved cysteine residues. It was reported that upon activation of SA signalling, the immune-inducible TRX*h*5 and constitutively expressed TRX*h*3 enzymes facilitated the reduction of NPR1 disulphide bonds, thereby releasing NPR1 monomer for translocation into the nucleus where it activates SA-responsive immune genes (Fig. 2) [29]. TRX*h*5 not only counteracts NPR1 oligomer formation by retrospectively reducing NPR1 disulphides, it also suppresses the stimulatory effect of Cys156 *S*-nitrosylation on formation of disulphide-linked NPR1 oligomer. TRX*h*5 was demonstrated to be a direct protein-SNO reductase of *S*-nitrosylated NPR1. Consequently, enhanced accumulation of NPR1 oligomer in the cytoplasm of mutant genotypes with elevated levels of free NO or GSNO was reversed by expression of TRX*h*5 [16,29,50]. These findings signify the importance of different TRX activities in the direct regulation of redox-based post-translational modification of key immune signalling proteins.

In addition to canonical TRX enzymes the TRX superfamily also contains members with unusual domain structures. Members of the subfamily of Nucleoredoxin (NRX) enzymes typically contain multiple TRX folds and active sites that could have a very different substrate range from canonical TRX. Nonetheless, NRX enzymes show typical disulphide reduction capacities and at least in *Arabidopsis*, NRX1 was shown to also be recycled by the activity of NTR isoform A (NTRA) [51–53]. Whilst other members of the TRX superfamily protein have been the focus of attention of many studies in plants, the role of NRX enzymes and their importance in plant immunity has only just begun to

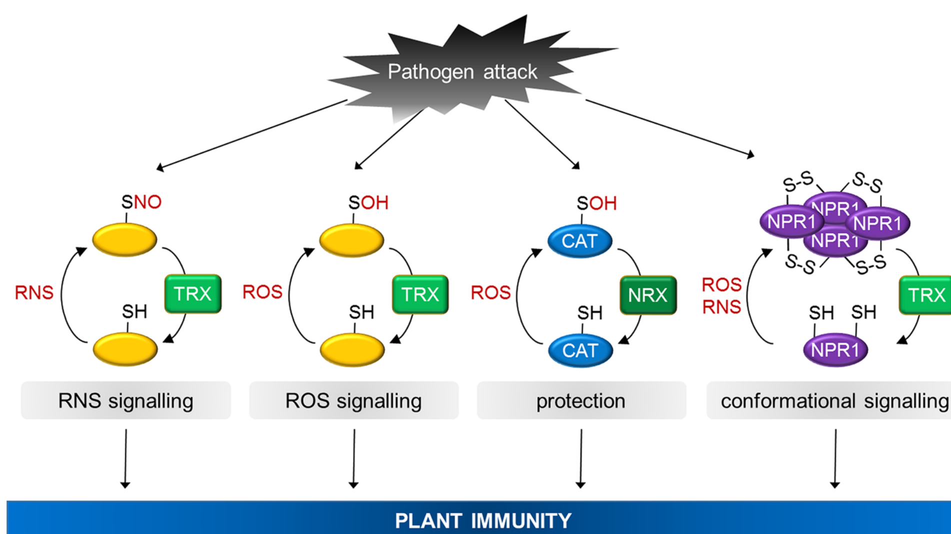

Fig. 2. Signalling and protective functions of TRX enzymes in plant immunity.
Pathogen challenge induces the accumulation of ROS and RNS, resulting in oxidative modification of reactive free thiols (−SH) to form *S*-nitrosothiols (−SNO), *S*-sulfenic acids (−SOH) and dilsulphides (S−S) in signalling proteins and antioxidant enzymes such as catalase (CAT). TRX and NRX are thought to reduce these thiol modifications to enable RNS signalling, ROS signalling, protection of antioxidant enzyme activities, and conformational signalling. Although NRX1 has been shown to reduce oxidised CAT, note that the presence of a sulfenic acid group in CAT is hypothetical. The combined action of these TRX/NRX-regulated pathways establishes plant immunity.

be uncovered. A recent study on the response of wilt-resistant island cotton (*Gossypium barbadense*) to *Verticillium dahliae* showed that most proteins upregulated in the root apoplast secretome were related to ROS metabolism and defence response [54]. One of these induced proteins included *Gb*NRX1, which was subsequently shown to contribute to apoplastic ROS scavenging. Silencing of *Gb*NRX1 resulted in high cellular ROS accumulation and reduced wilt resistance, suggesting that simultaneous ROS production and scavenging by *Gb*NRX1 is necessary for the correct orchestration of plant immune responses. In addition to *Gb*NRX1, *Arabidopsis thaliana* NRX1 was also shown to play a role in plant immune signalling [53]. Both *NRX1* gene expression and enzyme oxidoreductase activity were highly induced upon pathogen attack, and mutant *nrx1* plants displayed enhanced disease resistance. Proteomic analyses of NRX1 substrates indicated these were mostly involved in electron transport and energy pathways that usually involve oxidative reactions, and that many contained enzymatic activities. These findings suggest that pathogen-induced NRX1 regulates or protects a diverse set of proteins involved in cellular redox reactions during plant immune responses.

The unusual oxidoreductase NTRC was recently also linked to plant immune responses. NTRC contains both an NADPH-dependent TRX reductase domain and a TRX domain in a single polypeptide. NTRC is localised to the chloroplast and is a central player of the chloroplast redox detoxification system. Plants lacking NTRC showed enhanced disease susceptibility to the bacterial pathogen *Pseudomonas syringae*, which was accompanied by elevated accumulation of ROS [55]. Furthermore, whilst there were no significant differences in pathogen-induced SA accumulation between the wild type and *ntrc* mutants, elevated accumulation of the developmental and immune hormone jasmonic acid was observed in *ntrc* plants, suggesting NTRC suppresses chloroplast-generated ROS that may act to promote JA signalling.

While the above data demonstrate that the TRX system is widely employed in different plant immune signalling steps, this system may also be hijacked by plant pathogens. The plant pathogen *Ralstonia solanacearum* produces the effector protein RipAY, a putative γ-glutamyl cyclotransferase involved in the degradation of glutathione. Interestingly *R. solanacearum* utilised the host TRX system to activate the glutathione degrading activity of RipAY, resulting in inhibition of pattern-triggered immune responses [56]. Moreover, RipAY was specifically activated only by cytosolic TRX-*h* family members but not by chloroplastic TRX members in vitro, indicating a level of substrate specificity. However, promotion of RipAY activity was independent of the TRX-*h* active site, suggesting that TRX-*h* enzymes also have thiol-independent signalling functions.

The necrotrophic fungus *Cochliobolus victoriae* causes Victoria blight on common oat and its pathogenicity is closely associated with production of victorin, a toxin that induces programmed cell death in susceptible plants. To do this *C. victoriae* is thought to exploit the cell death-inducing activity of the NB-LRR resistance protein LOV1 (*Locus Orchestrating Victorin effects1*), which confers sensitivity to victorin [57,58]. It was recently found that victorin binds to TRX*h*5 at active site Cys39, which inhibits its activity and activates a LOV1-dependent resistance response that includes an oxidative burst and programmed cell death [59]. Although programmed cell death limits the growth of biotrophic pathogens that rely on live host tissues [60], necrotrophic pathogens that feed on dead tissues are likely provided with an advantage [61]. Thus, as a necrotrophic pathogen, *C. victoriae* is thought to hijack a TRX*h*5-dependent NB-LRR resistance pathway that leads to programmed cell death and exploits this response to confer host susceptibility [57,59].

4. Substrate specificity and selective signalling

By contrast to other eukaryotic genomes, plant genomes encode for numerous TRX enzymes. Mutational and knock out analyses of TRX genes has had limited success as the majority of mutants do not exhibit obvious phenotypes [33]. Although this is indicative of substantial genetic redundancy between TRX enzymes, the dramatic genetic expansion of the TRX family in the plant kingdom suggests that each of these enzymes has evolved to support a specific function within the plant cell. So how is substrate specificity and selective signalling by TRX enzymes achieved and how can specific TRX-regulated pathways be dissected in future?

4.1. Cellular localisation and redistribution upon stress

Plant TRXs are located in nearly all cellular compartments and the largest proportion is localised to plastids [33,39]. For example, members of the subfamilies *m*, *f*, *y*, *x* and *z* reside in the chloroplast, whereas members of the *o* and *h* as well as NRX subfamilies display various localisation patterns to the mitochondria, cytoplasm or nucleus [33,37,51,62]. Chloroplast TRX were initially identified as regulators of the Calvin-Benson cycle [33,63–65], but it is now well established that they have numerous other functions in the regulation of diverse cellular processes and metabolic pathways. In this respect, TRX *m* and *f* family members interact with protein targets involved in processes such as starch metabolism or lipid synthesis, while TRX *x* and *y* are thought to play a role in antioxidant systems, as they were found to be highly efficient reducers of diverse peroxiredoxins [63,66]. Although chloroplast TRX and their substrates have been extensively studied at the

biochemical, structural and functional level, less in known about the function of mitochondrial TRX enzymes. Nonetheless, these enzymes have been shown to target, amongst others, substrates for ATP synthase, components of the electron transport chain, enzymes of the TCA cycle and alternative oxidase, thereby controlling physiological and redox-related signalling pathways [62,67,68]. Cytoplasmic TRX-*h* enzymes have been linked to developmental pathways including auxin signalling and function as backup for glutathione reductase, which maintains glutathione homeostasis [69–71]. The large diversity in TRX enzymes together with their wide distribution throughout cell types and cellular compartments suggests that localisation could play an important role in determining substrate specificity.

In support of the above notion, biotic and abiotic stresses have been reported to result in cellular translocation of TRX enzymes or entire TRX/NTR systems, suggesting that these enzymes are recruited to specific sites for their signalling or protective functions. For example, in unchallenged cotton plants GbNRX1 is present in the cytosol, whereas challenge inoculation with *V. dahlia* leads to its translocation to peripheral regions of the cell and secretion into the apoplast where it dissipates ROS [54]. Furthermore, in early stages of developing wheat seeds TRX-*h* enzymes are localised to maternal tissues and the endosperm, whereas in latter stages when water content strongly reduces and creates a cellular environment that may promote oxidative stress, TRX-*h* enzymes accumulated in the aleurone and scutellum cells. The onset of oxidative stress in aleurone and scutellum cells was also associated with predominant localisation of these TRX-*h* to the nucleus rather than cytoplasm [72,73]. Likewise, NTR also co-localised with TRX-*h* to seed cells that experienced oxidative stress [74]. These examples illustrate that TRX enzymes can be spatially reorganised by cell type and nucleocytoplasmic distribution according to the oxidative stress encountered.

Although the nucleus does not appear to have its own exclusive TRX system, several more TRX enzymes have been shown to partially reside there. In pea plants a mitochondrial TRX-*o* was also shown to localise to the nucleus but no specific relation to nuclear oxidative stress or signalling was uncovered [75]. Fluorescence microscopy of TRX*h*5 transiently expressed in *Arabidopsis* protoplasts that may experience a moderate level of oxidative stress, showed that this enzyme localised to both the cytoplasm and nucleus in agreement with movement of its nucleocytoplasmic substrate NPR1 [16]. Moreover, as their name suggests, NRX enzymes have been shown to localise to the nucleus albeit not exclusively [52,76]. Together with the finding that NTRA is also partially localised to the nucleus, this suggests that a complete NRX/NTRA system is present in this cellular compartment [51]. Future studies may further elucidate how TRX enzymes move between and within cells to achieve a level of specificity in cellular redox signalling.

4.2. Substrate selection and pathway specificity

Although cellular localisation will establish a level of specificity, how TRX enzymes within a single cellular compartment differ in function remains largely unknown. A key possibility is that TRX enzymes have distinct affinities for different substrate repertoires. This notion is supported by findings that chloroplast TRX enzymes act selectively on substrates located in this organelle [63,66]. While TRX *f*, and possibly TRX *m* as a backup, regulate Calvin-Benson cycle enzymes, TRX *x*, *y* and *z* are ineffective in this respect. By contrast, these latter enzymes are part of an important antioxidant system that reduces various peroxiredoxins. So how do TRX enzymes select their substrates? While the answer to this question is still largely unknown, it was recently demonstrated that TRX enzymes recognize oxidised substrates with much higher selectivity compared to already reduced substrates [77]. This was found to be due to conformational restrictions imposed upon the substrate by a disulphide bond, which correlated with a low-entropy state. TRX preferentially associated with this low-entropy conformation of the substrate and by reducing the disulphide, converted the substrate into a non-restrictive or open conformational state with high entropy and low affinity for TRX. These data indicate that favourable solvation entropy may be the driving force for substrate selection by TRX enzymes. Although this discovery finally provides an explanation for how TRX enzymes recognise disulphide-containing substrates, it remains to be seen if this is a rule of thumb that extends to other oxidative modifications such as *S*-nitrosothiols and *S*-sulfenic acids, which may not be associated strongly with conformational restraints on the substrate. Understanding further the physical and chemical parameters that dictate oxidised over reduced substrate recognition will help comprehend how TRX enzymes remain efficient in context of the complex cellular protein soup.

Although genetic analyses of TRX mutants has only provided limited information on pathway specificity and substrate selection, a new approach taken by our laboratory has proven more successful. To analyse the SNO reductase activity of immune-induced TRX*h*5, advantage was taken of two redox-compromised genetic backgrounds: *gsnor1* mutants that accumulate elevated levels of the NO donor GSNO and *nox1* mutants that exhibit enhanced levels of free NO due to over-accumulation of the NO precursor L-arginine [15,21,78]. Both *gsnor1* and *nox1* mutants accumulate elevated levels of protein-SNO, display strongly reduced activation of SA-dependent immune genes and are immune compromised [15,16,50]. Because these mutants lacked SA-induced activation of endogenous *TRXh5* gene expression, TRX*h*5 was expressed from a constitutive promoter. While expression of TRX*h*5 failed to restore immunity in *gsnor1* mutants, it was able to rescue immunity in *nox1* mutants. Further analyses indicated that TRX*h*5 did not alter protein-SNO levels in *gsnor1* mutants, while it lowered protein-SNO levels in the *nox1* genetic background, thereby reinstating SA-responsive immune gene expression and immunity [16]. These findings suggest that TRX*h*5 selectively targets protein-SNO derived from free NO but not from GSNO. Another recent study further supports this notion that two distinct, albeit partly overlapping sets of protein-SNO exist. Overexpression of GSNOR1 complemented the *gsnor1* mutant phenotype but failed to rescue disease resistance in *nox1* mutants, suggesting it was ineffective in reducing NO-derived protein-SNO levels [50]. Moreover, compared to single mutants, the *gsnor1 nox1* double mutant exhibited both elevated levels of pathogen-induced protein-SNO and enhanced disease susceptibility, indicating the existence of NO- and GSNO-derived protein-SNO pools that function additively in plant immunity.

Although it remains unknown how TRX*h*5 selects particular protein-SNO targets, the above findings suggest it can discriminate between different SNO groups depending on the type of NO donor and accepting thiol group. Specificity and efficiency of *S*-nitrosylation may be determined by the pK_a of thiols, stereochemistry of NO donors or the conformational state of target proteins [79,80]. While GSNO is thought to generate protein-SNO through a trans-nitrosylation mechanism, it has been postulated that free NO mediates protein *S*-nitrosylation through distinct oxidative and radical-mediated pathways [80]. These mechanistic differences in SNO generation may explain NO donor specificity for particular protein thiols and by extension could be associated with conformational states that are accessible or attractive to selected TRX enzymes. This is supported by reports that although the TRX-*h*/NTR system denitrosylates many substrates in vitro [16,42], it cannot target all protein-SNO such as *S*-nitrosylated glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in *Arabidopsis*, which is denitrosylated by reduced glutathione instead [81]. Taken together, we hypothesize that the observed selectivity in TRX*h5*-mediated protein-SNO could extend to other oxidative modifications, thus placing diverse TRX family members at the heart of oxidative thiol signalling (Fig. 2).

5. Protective versus signalling roles of thioredoxins

Pathogen infection is characterised by the cellular and apoplastic accumulation of ROS and RNS. While these molecules are important in

cell signalling, their accumulation can also lead to nitro-oxidative stress with formation of undesired oxidative thiol modifications [6,7,82–84]. These hostile cellular conditions prompt the activation of different antioxidant mechanisms including catalase, ascorbate or glutathione peroxidases (APX and GPX, respectively), monodehydroascorbate reductases (MDAR), dehydroascorbate reductases (DHAR) or peroxiredoxins (PRX), that all function in H_2O_2 scavenging pathways [8,83]. Nevertheless, these antioxidant systems themselves are susceptible to ROS/RNS-induced thiol modifications. For instance, the activities of MDAR and DHAR are suppressed by cysteine oxidation, while contrasting reports exist on the effect of cysteine oxidation on APX [85]. Moreover, surface exposed cysteine residues in catalases were reported to undergo oxidation, which inhibited H_2O_2 scavenging activity [53,86]. Thus, quite surprisingly antioxidant enzymes themselves may experience detrimental oxidative damage in the ROS/RNS rich environments they function in. A growing body of evidence now suggests that TRX enzymes may be involved in protecting antioxidant enzymes from becoming inactivated by thiol oxidation. Antioxidant enzymes have been identified in several in vitro proteomic screens for potential substrates of cytosolic and mitochondrial TRX family members from various land plants and green algae [87–93]. A recent proteomic study reported that NRX1 targets major enzymes of the H_2O_2 scavenging pathway, including ascorbate peroxidase (APX) and all three *Arabidopsis* catalases. Substrate interaction with NRX1 resulted in reduction of catalase thiol oxidation and was associated with enhanced H_2O_2 scavenging activity [53]. Thus, in analogy to NRX1, other TRX family members may not only function as cell signalling switches, but also as protectors of antioxidant enzymes that guard plant cells from oxidative protein damage (Fig. 2).

6. Conclusions and perspectives

Although TRX enzymes have been studied for many decades, recent years have placed this evolutionary conserved group of antioxidant enzymes at the centre of many developmental and stress signalling pathways. Understanding the full complement of their roles in regulating plant immune signalling is still in its infancy but the discovery of new thiol reductase activities as well as functions in thiol signalling and protection indicates they may be intimately involved in many aspects of immunity (Fig. 2). Many challenges remain, not least to precisely define the in vivo substrate repertoires of each cellular TRX. Understanding how TRX enzymes select their substrates and limit their activities to specific pathways (e.g. specific immune sectors) will also be vital to fully appreciating their role in cellular signalling. With the advent of new genetic approaches and advances in biochemical and proteomic techniques such as activity-based protein profiling, however, answers to the above challenges are now within reach and should provide for exciting new discoveries in TRX biology in near future.

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