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REVIEW PAPER

Oxidative post-translational modifications of cysteine residues in plant signal transduction

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Abstract

In plants, fluctuation of the redox balance by altered levels of reactive oxygen species (ROS) can affect many aspects of cellular physiology. ROS homeostasis is governed by a diversified set of antioxidant systems. Perturbation of this homeostasis leads to transient or permanent changes in the redox status and is exploited by plants in different stress signalling mechanisms. Understanding how plants sense ROS and transduce these stimuli into downstream biological responses is still a major challenge. ROS can provoke reversible and irreversible modifications to proteins that act in diverse signalling pathways. These oxidative post-translational modifications (Ox-PTMs) lead to oxidative damage and/or trigger structural alterations in these target proteins. Characterization of the effect of individual Ox-PTMs on individual proteins is the key to a better understanding of how cells interpret the oxidative signals that arise from developmental cues and stress conditions. This review focuses on ROS-mediated Ox-PTMs on cysteine (Cys) residues. The Cys side chain, with its high nucleophilic capacity, appears to be the principle target of ROS. Ox-PTMs on Cys residues participate in various signalling cascades initiated by plant stress hormones. We review the mechanistic aspects and functional consequences of Cys Ox-PTMs on specific target proteins in view of stress signalling events.

Key words: Cysteine oxidative post-translational modifications, oxidative stress, phytohormone signalling, reactive oxygen species, redox regulation, signal perception.

Introduction

Plants continuously need to adapt their development towards fluctuating environmental conditions in order to survive and aim for optimal fitness (Wituszyńska *et al.*, 2013*a*, *b*). These adaptations require mechanisms that are constantly

Abbreviations: ABA, abscisic acid; Asc, ascorbate; CPK, calcium-dependent protein kinase; Cys, cysteine; DsPTP, double-specificity protein tyrosine phosphatase; Fdx, ferredoxin; Gpx, glutathione peroxidase; Grx, glutaredoxin; GSH, reduced glutathione; GSSG, oxidized glutathione; JA, jasmonate; MAPK/MPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase; Met, methionine; MKK, MAP kinase kinase; OPDA, 12-oxo-phytodienoic acid; Ox-PTMs, oxidative post-translational modifications; PR, pathogenesis-related; Prx, peroxiredoxin; PTP, protein tyrosine phosphatase; PYR/PYL/RCAR, pyrabactin resistance/PYR1-like/regulatory component of ABA receptor; R-SH$^-$, thiolate; R-SH, thiol group; R-SOH, sulfenic acid; R-SO_2H, sulfinic acid; R-SO_3H, sulfonic acid; RNS, reactive nitrogen species; ROS, reactive oxygen species; R-S-SH, persulfide bond; R-S-S-R/R-S-S-R′, intra-/intermolecular disulphide bridge; SA, salicylic acid; Srx, sulfiredoxin; TGA, TGACG sequence-specific binding protein; Trx, thioredoxin.

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monitoring, sensing, and transducing a diverse array of environmental stimuli. Besides changes in phytohormone levels, signalling via perturbed reactive oxygen species (ROS) homeostasis can participate in the necessary steps leading to an efficient cellular and organismal response (Wrzaczek *et al.*, 2013; Gilroy *et al.*, 2014).

The accumulation of ROS has been demonstrated not only for multiple stress conditions like wounding (Orozco-Cardenas and Ryan, 1999), chilling (Wise and Naylor, 1987), excess light (Fryer *et al.*, 2003), and pathogen infections (Apostol *et al.*, 1989; Levine *et al.*, 1994), but also during developmental processes like root growth (Foreman *et al.*, 2003), gravitropism (Joo *et al.*, 2001), extracellular ATP signalling (Song *et al.*, 2006), and pollen-tube growth (Potocký *et al.*, 2007; Kaya *et al.*, 2014) and its rupture (Duan *et al.*, 2014). Core cellular processes intrinsically linked with the production of ROS include mitochondrial respiration (Navrot *et al.*, 2007), photosynthesis (Asada, 2006), peroxisomal metabolism mainly including photorespiration, fatty acid β-oxidation and purine degradation (del Río, 2013), and apoplastic oxidative burst/long-distance signalling mediated by plasma membrane-bound NADPH oxidases and apoplastic peroxidases (Suzuki *et al.*, 2011; Daudi *et al.*, 2012). Over more than three decades, the general concepts of redox regulation in plants have been shaped (Schürmann and Buchanan, 2008; Montrichard *et al.*, 2009). However, it is not yet clear how the majority of stress-related redox stimuli are perceived and transduced. One mechanism to sense ROS and reactive nitrogen species (RNS) is by the modification of proteins on specific cysteine (Cys) and methionine (Met) residues (Roos and Messens, 2011; Jacques *et al.*, 2013). The availability of different oxidation states of sulfur-containing amino acids permits the formation of a diverse palette of oxidative post-translational modifications (Ox-PTMs), including nitric oxide (NO)-mediated *S*-nitrosylation (Yu *et al.*, 2014), hydrogen sulfide-mediated sulfhydration (Mustafa *et al.*, 2009; Álvarez *et al.*, 2012; Paul and Snyder, 2012), and ROS-mediated changes (sulfenylation, R-SOH; formation of intra-/intermolecular disulphide bridges, R-S-S-R/R-S-S-R′; *S*-glutathionylation, R-S-SG; sulfinylation, R-SO_2H; and sulfonylation, R-SO_3H).

The best-studied cases for ROS-mediated PTMs are those on enzymes involved in the Calvin cycle (Schürmann and Buchanan, 2008), sulfur metabolism (Kopriva *et al.*, 2012), and starch metabolism (Glaring *et al.*, 2012). Besides these redox-sensitive metabolic components, Ox-PTMs also emerge as regulatory switches on various signal transduction proteins, including transcription factors (excellently reviewed by Dietz, 2014), kinases, phosphatases, proteases, and RNA-binding proteins. In this review, we focus on hydrogen peroxide (H_2O_2)-mediated thiol-based redox signalling, including kinase modules, proteases, and RNA-binding proteins. Special attention is given to Cys Ox-PTMs that mediate the cross-talk between ROS and signalling pathways initiated by stress-related hormones. In this review, the interplay between ROS and developmental hormones such as auxins, cytokinins, gibberellins, and brassinosteroids will not be discussed. For an overview of redox regulation during plant development, we refer to excellent reviews by Considine and Foyer (2014) and Schmidt and Schippers (2014), complemented by an inventory of redox-sensitive transcription factors implemented in plant growth regulation (Dietz, 2014).

Cys Ox-PTMs in plant ROS control systems

Plants have evolved various strategies to keep ROS levels under a tight control that is governed by enzymatic and non-enzymatic ROS-producing and -scavenging systems (Apel and Hirt, 2004; Mittler *et al.*, 2011; Fig. 1A). Glutathione (GSH) and ascorbate (Asc) are the major non-enzymatic cellular redox systems, with tocopherol and diverse alkaloid, carotenoid, and flavonoid metabolites often listed but sometimes debated as physiologically relevant antioxidants (Apel and Hirt, 2004; Hernández *et al.*, 2009). Maintenance of a reduced glutathione pool (high GSH/GSSG ratio) is crucial for cellular redox homeostasis, since GSH is utilized to regenerate oxidized ascorbate in the glutathione–ascorbate cycle (Fig. 1A; Foyer and Halliwell, 1976; del Río, 2011).

GSH and Asc work hand in hand with glutathione peroxidases (Mills, 1957) and ascorbate peroxidases (Groden and Beck, 1979; Nakano and Asada, 1981), respectively, which together with catalases, superoxide dismutases and peroxiredoxins (Prxs) constitute the major enzymatic classes involved in ROS scavenging (Mittler *et al.*, 2004). Due to its relatively high pK_a (8.9; Van Laer *et al.*, 2013), glutathione is fully protonated at physiological pH, and thereby its reactivity towards disulphides and ROS is rather limited.

Ox-PTMs of Cys residues

The chemical properties of the sulfur atom (i.e. wide range of oxidation states) make Cys and Met residues the major sites of oxidation within proteins (Davies, 2005). In Cys Ox-PTMs, the thiol group (R-SH) represents the –2 oxidation state of the sulfur atom, which is the fully reduced form. Not all Cys residues in a protein are prone to ROS-mediated modifications, and the reactivity of different thiol-proteins towards ROS varies according to their physiological function and local redox environment. Between individual Cys residues, the reactivity is strongly correlated with their pK_a, i.e. the ability to form the anionic form of the sulfur, called thiolate ($R\text{-}S^-$), which is much more reactive than the thiol. If the pK_a of the sulfur atom is higher than the pH of the solution, the protonated thiol will be the dominant species. However, if the pK_a is lower than the pH, the majority of the thiols will be present as a thiolate (Cys prone to oxidation). The pK_a of Cys residues is largely determined by the local electrostatic environment, i.e. the presence of proximal charged residues or dipoles and the hydrogen bonding between thiols/thiolates and neighbouring residues (Harris and Turner, 2002). Hydrogen bonding has a strong influence on the pK_a of reactive Cys residues. In general, the more hydrogen bonds a Cys-sulfur receives, the lower the pK_a is and the more the thiolate form is stabilized (Roos *et al.*, 2013). The nucleophilicity of the Cys is also an important factor in its reactivity; in some cases, a lower stabilization of the thiolate in Cys residues

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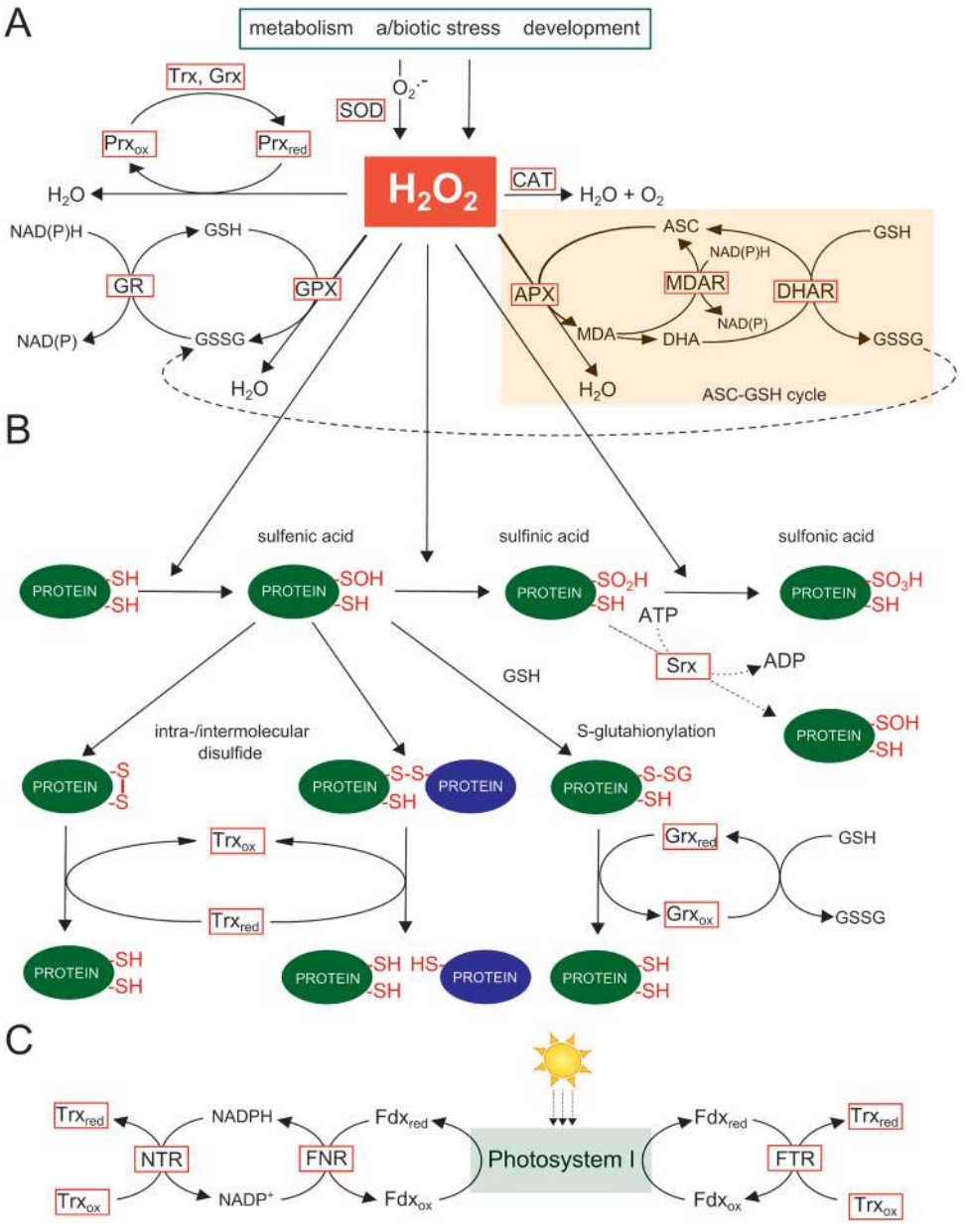

Fig. 1. The impact of H_2O_2 on plant cells. (A) Production and enzymatic scavenging of H_2O_2. SOD, superoxide dismutase; Prx, peroxiredoxin; CAT, catalase; GR, glutathione reductase; GPX, glutathione peroxidase; APX, ascorbate peroxidase; MDAR, monodehydroascorbate reductase; DHAR, dehydroascorbate reductase, Srx, sulfiredoxin. (B) Oxidative post-translational modifications of Cys residues. Adapted from *Free Radical Biology and Medicine* 51. Roos G and Messens S. Protein sulfenic acid formation: from cellular damage to redox regulation, 314–326. Copyright (2011), with permission from Elsevier. (C) Light-dependent reduction of thioredoxins within the chloroplast. Fdx, ferrodoxin; FTR, ferrodoxin–thioredoxin reductase; NTR, NADPH-dependent thioredoxin reductase, FNR, ferrodoxin-$NADP^+$ reductase.

increases its nucleophilicity, while a highly stabilized thiolate needs a larger amount of energy to reach the transition state (Ferrer-Sueta *et al.*, 2011). Another important factor that controls the reactivity of Cys residues is their steric accessibility within the three-dimensional structure of the protein (Marino and Gladyshev, 2010).

The first step in ROS-dependent signalling involves the reversible oxidation of reactive Cys residues to sulfenic acid (R-SOH; Fig 1B). Unless stabilized within its protein environment, this modification is highly unstable and leads to further modifications (Claiborne *et al.*, 1993). An excess concentration of oxidant can lead to further oxidation to sulfinic acid ($R\text{-}SO_2H$), and subsequently to irreversible sulfonic acid ($R\text{-}SO_3H$; Roos and Messens, 2011). The reversion of the $R\text{-}SO_2H$ modification is catalysed by an ATP-dependent sulfiredoxin enzyme (Srx) that is capable of reducing $R\text{-}SO_2H$ to R-SOH in *Arabidopsis* (Rey *et al.*, 2007). However, thus far, $R\text{-}SO_2H$ reduction is rather exceptional with the only two known substrates of AtSrx: the chloroplast 2-Cys Prx (Rey *et al.*, 2007; Iglesias-Baena *et al.*, 2010) and mitochondrial PrxIIF (Iglesias-Baena *et al.*, 2011). Alternatively, R-SOH can react with free protein thiols to form intra- or intermolecular disulphide bonds (R-S-S-R/R-S-S-R′) or is modified by low-molecular-weight thiols (like GSH in plants), leading to Cys *S*-glutathionylation (Fig 1B). *S*-Glutathionylation events were initially considered to serve as a protective

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mechanism on active-site Cys residues, preventing over-oxidation and subsequent permanent protein damage. Only recently was the role of *S*-glutathionylation in redox signalling recognized (Zaffagnini *et al.*, 2012). The reduction of disulphide bonds and deglutathionylation are controlled by glutaredoxins (Grxs) and thioredoxins (Trxs), respectively (Fig. 1B). Compared with prokaryotes and animals, plants are equipped with a much more complex Trx/Grx network. The *Arabidopsis* genome encodes 44 Trx/Trx-like and 50 Grx/Grx-like proteins (Meyer *et al.*, 2012). Depending on the subcellular localization, Trxs utilize multiple sources of reducing equivalents to perform the reduction of intra-/intermolecular disulphide bonds. In chloroplasts, light reactions reduce ferredoxin (Fdx), which in turn reduces ferredoxin–thioredoxin reductase (FTR), which ultimately regenerates the Trx sulfhydryl groups (Fig. 1C; Schürmann and Buchanan, 2008). Another general source of reducing equivalents, common in the Trx and Grx systems, is NADPH, which after oxidation to $NADP^+$ is reduced by Fdx:$NADP^+$ reductase within the chloroplast stroma, as well as during the oxidative pentose phosphate pathway. While Trxs are directly reduced by NADPH-dependent thioredoxin reductases, Grxs engage in a two-step reaction involving GSH and NADPH-dependent glutathione reductases (Fig. 1B, C).

Cross-talk between ROS and phytohormone signalling mediated by Cys Ox-PTMs

Abscisic acid (ABA) signalling

Thus far, the best described cross-talk between ROS and ABA occurs during guard-cell movement (Fig. 2; Pei *et al.*, 2000; Kwak *et al.*, 2003). In response to stress conditions (e.g. pathogen attack, drought), the rising concentration of ABA within guard cells triggers a signalling cascade, which ultimately results in stomatal closure and reduced transpiration. Perception (binding) of ABA by PYRABACTIN RESISTANCE/PYR1-LIKE/REGULATORY COMPONENT OF ABA RECEPTOR (PYR/PYL/RCAR) receptor proteins (Ma *et al.*, 2009; Park *et al.*, 2009) results in conformational changes within their protein structure, leading to binding and inhibition of PROTEIN PHOSPHATASE 2C (PP2C) family members that act as constitutive repressors of the SnRK2.6/OST1 kinase (Cutler *et al.*, 2010). Upon derepression, SnRK2.6/OST1 achieves full activation by autophosphorylation and further activates (phosphorylates) its target proteins such as the NADPH oxidase RbohF, leading to apoplast-localized ROS production (Kwak *et al.*, 2003; Sirichandra *et al.*, 2009). This oxidative burst activates Ca^{2+} influx channels (Pei *et al.*, 2000). In parallel, SnRK2.6/OST1 activates the anion channel SLOW ANION CHANNEL-ASSOCIATED 1 (SLAC1; Lee *et al.*, 2009; Vahisalu *et al.*, 2010), which has a central role in guard cells. Another ion channel targeted by SnRK2/OST1 is the KAT1 potassium channel, which loses its activity upon phosphorylation (Sato *et al.*, 2009). The rise in cytoplasmic Ca^{2+} concentration activates multiple calcium-dependent protein kinases CPK3, CPK6 (Mori *et al.*, 2006), CPK4, CPK11 (Zhu *et al.*, 2007), CPK5 (Dubiella *et al.*, 2013), CPK21, and CPK23 (Geiger *et al.*, 2010). Among them, CPK21 activates SLAC1 (Geiger *et al.*, 2010) and SLAC1 homologue 3 (SLAH3; Geiger *et al.*, 2011; Demir *et al.*, 2013). Together, these signalling steps lead to the coordinated action of membrane channels that control the levels of osmotically active ions within guard cells.

Two members of the PP2C family ABA INSENSITIVE 1 (ABI1) and ABI2, which act as negative regulators of ABA signalling (Merlot *et al.*, 2001), were shown to undergo H_2O_2-dependent inhibition (Fig. 2). Oxidizing conditions led to the

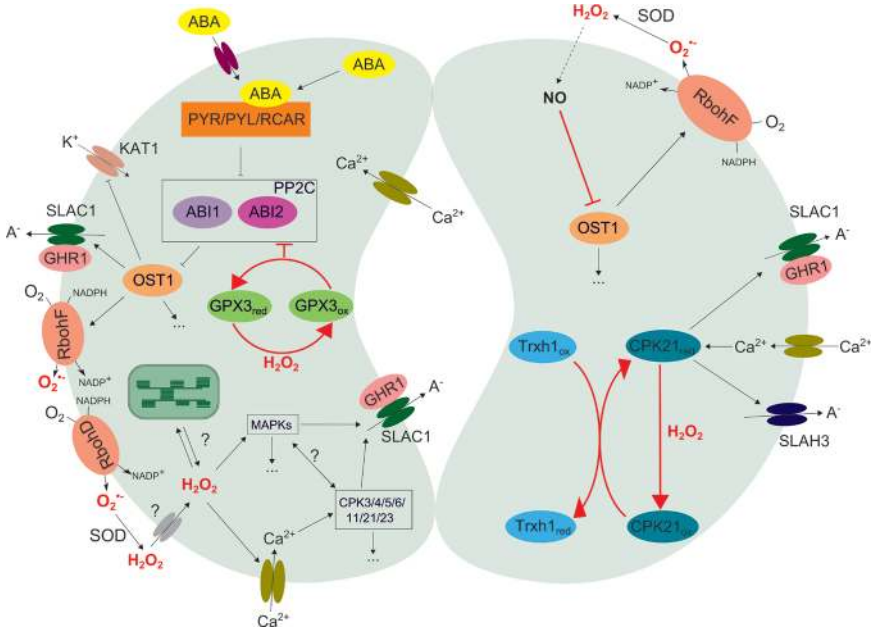

Fig. 2. Schematic representation of Cys post-translational modifications in guard-cell signalling. Red arrows indicate reactions involving Ox-PTMs of Cys residues. For description of abbreviations and specific regulatory events, we refer to the main text.

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inactivation of phosphatase activities *in vitro*, presumably via formation of an intramolecular disulphide bond (Meinhard and Grill, 2001; Meinhard *et al.*, 2002). This oxidative inactivation amplifies ABA signalling and effects in guard cells. *In planta*, ABI2 oxidation is mediated by GLUTATHIONE PEROXIDASE 3 (GPX3). GPX3 acts as a sensor protein that, upon perception of increased ROS levels, relays the oxidizing equivalents to ABI2 via thiol–disulphide exchange to inhibit its phosphatase activity (Miao *et al.*, 2006). Similar scenarios have been described as well in yeast and mammalian cells exposed to oxidative stress (Delaunay *et al.*, 2002; Gutscher *et al.*, 2009). In yeast, oxidation of the H_2O_2 sensor Oxidant Receptor Peroxidase 1 (ORP1/Gpx3) triggers a thiol–disulphide relay mechanism that ultimately leads to nuclear accumulation of the yeast AP-1 (YAP1) transcription factor (Delaunay *et al.*, 2002) and subsequent induction of H_2O_2-responsive genes (Lee *et al.*, 1999). Together with their strong sequence similarities within kingdoms (Margis *et al.*, 2008), these notions indicate that thiol peroxidases act as widespread redox sensors.

As well as the GPX3-mediated inhibition of ABI1/2, the activity of CPK21 is also redox dependent (Fig. 2). CPK21 undergoes H_2O_2-dependent inhibition that is linked to the formation of an intramolecular disulphide bond (Cys97–Cys108) and is reduced by Trx-h1 (Ueoka-Nakanishi *et al.*, 2013).

Among the late events that follow the quick ABA-dependent signalling cascade leading to stomatal closure is oxidative burst-dependent accumulation of NO (Bright *et al.*, 2006; Zhang *et al.*, 2007). Recently, Wang *et al.* (2015) demonstrated that an increased concentration of NO inhibits the SnRK2.6/OST1 protein kinase via *S*-nitrosylation of Cys137 following 30 min of ABA exposure (Fig. 2). The authors suggested that this mechanism serves to restrict and desensitize the ABA signalling pathway. This notion is in line with an earlier study (Yun *et al.*, 2011) describing a negative impact of NO on the activity of NADPH oxidases. The activity of RbohD is negatively regulated via *S*-nitrosylation of Cys890, thus compromising its ability to generate ROS (Yun *et al.*, 2011). Both examples provide evidence for a cross-talk between NO and ROS within the ABA signalling pathway.

Recent evidence suggests that ANNEXIN1 (AtANN1) mediates the ROS-dependent Ca^{2+} fluxes in *Arabidopsis* roots (Laohavisit *et al.*, 2012). ANNEXIN1 binds to lipid membranes in a Ca^{2+}-dependent manner and stimulates Ca^{2+} influx in a hydroxyl radical- (Laohavisit *et al.*, 2012) or peroxide-dependent manner (Richards *et al.*, 2014). *AtANN1*-deficient plants are impaired in the ROS-mediated increase in the cytoplasmic Ca^{2+} concentration (Laohavisit *et al.*, 2012). This process is of particular importance for root growth, since it largely relies on cell expansion, which is dependent on the activation of Ca^{2+} channels downstream of the NADPH oxidase RbohC (Foreman *et al.*, 2003). Besides impaired root development, *ann1* mutants are hypersensitive to drought (Konopka-Postupolska *et al.*, 2009). ANNEXIN1 is *S*-glutathionylated on both Cys residues (Cys111 and Cys239), resulting in a 50% decrease in Ca^{2+} affinity (Konopka-Postupolska *et al.*, 2009). This mechanism probably serves to restrict the AtANN1 membrane association and inhibit ROS-mediated Ca^{2+} fluxes in a negative feedback loop.

Salicylic acid (SA) signalling

SA regulates both local and systemic acquired resistance to limit the progress of pathogen infections. SA governs a transcriptional response that is largely mediated by the coordinated action of the NONEXPRESSER OF PR GENES 1 (NPR1) transcriptional co-activator and interacting TGA transcription factors (Fig. 3). Both NPR1 and TGAs are regulated by changes in the cellular redox status that occur upon pathogen infection (Mou *et al.*, 2003; Tada *et al.*, 2008). Normally, NPR1 localizes to the cytoplasm in the form of oligomers formed through intramolecular R-S-S-R bonds involving the residues Cys82 and Cys216 (Mou *et al.*, 2003). SA triggers perturbation of the cellular redox status, which leads to a Trx-h3/h5-dependent reduction of disulphides and subsequent monomerization of NPR1 and nuclear import. This activation process directly competes with oligomerization promoted by *S*-nitrosylation of Cys156. The interplay between these two modifications provides a tight control for NPR1-dependent signalling (Tada *et al.*, 2008). Recent discovery of NPR1 denitrosylation by a Trx-h5/NTRA system revealed a double role for Trx-h5 in the control of NPR1 cytoplasmic retention (Kneeshaw *et al.*, 2014). Upon import into the nucleus, NPR1 interacts with TGA basic leucine-zipper transcription factors to promote transcription of pathogenesis-related (*PR*) genes. Clade 2 TGA transcription factors (TGA2/5/6) act as constitutive repressors of *PR* transcription. Binding of NPR1 to TGA2 results in the co-activation of transcription and depends on NPR1 Cys521 and Cys529, which are required for activation of the NPR1 *trans*-activation domain (Rochon *et al.*, 2006). It has been demonstrated that NPR1 is an SA receptor and that these two Cys residues are involved in the stabilization of copper atoms that are necessary for SA binding (Wu *et al.*, 2012). Furthermore, TGA2/6 interact with glutaredoxin GRX480 (ROXY 19), suggesting that GRX480 might control their redox status. This complex might also involve NPR1, which implies the role of GRX480 in the regulation of the interaction between TGA and NPR1 (Ndamukong *et al.*, 2007); however, the precise function of GRX480 remains to be elucidated.

The interaction of NPR1 with clade 1 TGA transcription factors (TGA1/4) relies on their redox status. When oxidized, TGA1 and/or TGA4 form an intramolecular disulphide bridge (Cys260–Cys266) that hinders interaction with NPR1. Reduction of this disulphide stimulates the formation of a complex with NPR1, and subsequent binding to the *as-1* element for activation of *PR* genes (Després *et al.*, 2003). Recent data indicate that the two remaining TGA1 Cys residues, Cys172 and Cys287, are also involved and act as a regulatory disulphide (Lindermayr *et al.*, 2010). Furthermore, all four residues were found to undergo *S*-nitrosylation/*S*-glutathionylation upon treatment with *S*-nitrosoglutathione. These modifications serve to protect the Cys residues from oxidation and

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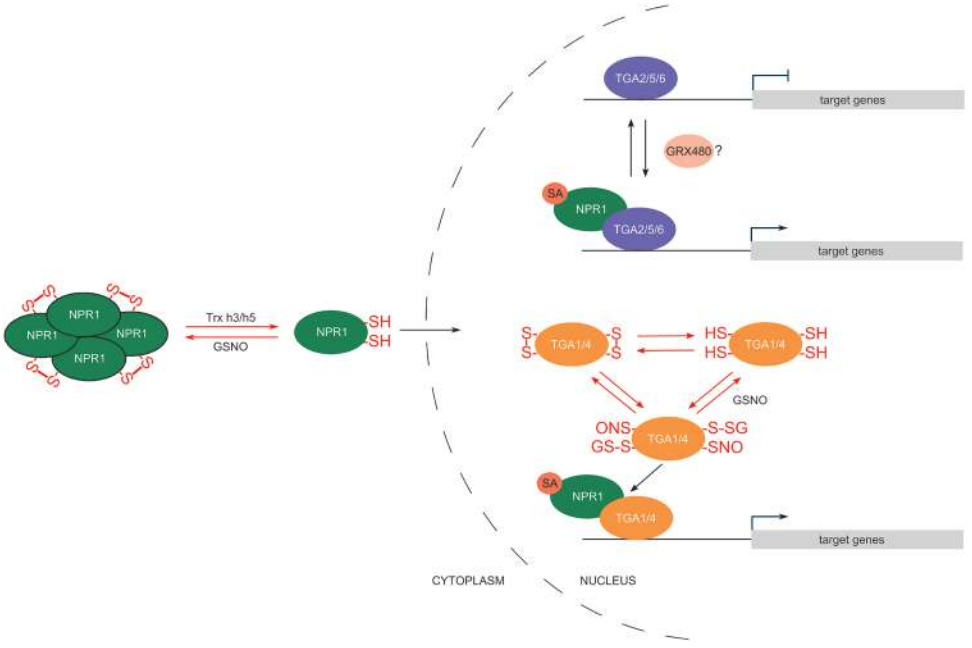

Fig. 3. Schematic representation of NPR1-based plant immune signalling. Upon pathogen infection and initial oxidative burst, the cytoplasm gets reduced by cell antioxidant systems. This leads to the monomerization of transcriptional co-activator NPR1 and its subsequent translocation to the nucleus. Upon import, NPR1 interacts with transcriptional repressors TGA2/5/6 and derepresses the expression of pathogenesis-related (*PR*) genes. Binding with TGA1/4 is promoted by *S*-glutathionylation/*S*-nitrosylation of their Cys residues and results in the activation of *PR* gene expression. Red arrows indicate reactions involving Ox-PTMs of Cys residues.

consequently lead to an increase in the DNA-binding activity of TGA1 (Lindermayr *et al.*, 2010).

Jasmonate (JA) signalling

The biologically active form of JA, the JA–Ile conjugate, triggers the 26S proteasome-mediated proteolysis of the JASMONATE ZIM-domain (JAZ) transcriptional repressors by mediating their interaction with the F-box protein CORONATINE INSENSITIVE1 (COI1), which is part of the Skp1/Cullin/F-box^{COI1} ubiquitin E3 ligase complex. Proteolysis of JAZ proteins derepresses multiple transcription factors (i.e. MYC2) and leads to panoramic changes in gene expression (Pauwels and Goossens, 2011). Besides JA derivatives, JA precursors, such as 12-oxo-phytodienoic acid (OPDA), are also able to exert transcriptional responses (Taki *et al.*, 2005). A recent study has extended our knowledge about OPDA signalling by the identification of its chloroplastic receptor CYCLOPHYLIN 20–3 (CYP20-3; Park *et al.*, 2013). Cyclophilins are characterized by a highly conserved peptidyl-prolyl isomerase domain that, if functional, assists proper folding of their target proteins (Trivedi *et al.*, 2012). CYP20-3 is reduced by *m*-type Trx (Motohashi *et al.*, 2001) and was demonstrated to undergo oxidative inhibition mediated by the formation of two intramolecular R-S-S-R bonds (Cys53–Cys170 and Cys128–Cys175; Motohashi *et al.*, 2003). Thus far, in *Arabidopsis*, the only identified target of CYP20-3 is the chloroplast SERINE ACETYL-TRANSFERASE1 (SAT1), which catalyses the rate-limiting step in Cys synthesis. The physical interaction of CYP20-3 with SAT1 is crucial for optimal synthesis of Cys. Consequently, *cyp20-3* mutant plants exhibit low thiol content and are impaired in light-dependent stress responses (Dominguez-Solis *et al.*, 2008). Direct binding of OPDA to CYP20-3 was shown to stimulate this interaction and ultimately promote the production of cellular antioxidants (Park *et al.*, 2013). Therefore, CYP20-3 is a redox-sensitive cross-talk point linking OPDA signalling with maintenance of the cellular redox balance.

Cys Ox-PTMs: control switches in plant signal transduction

Protein tyrosine phosphatases (PTPs) and mitogen-activated protein kinases (MAPKs)

MAPKs are involved in the regulation of almost every aspect of plant growth, development and stress responses (Takahashi *et al.*, 2007; Beckers *et al.*, 2009; Pitzschke *et al.*, 2009; Kosetsu *et al.*, 2010; Betsuyaku *et al.*, 2011). The three-component MAPK signalling cascades are initiated by stimulus-triggered activation of MAPK kinase kinases (MAPKKKs) that in turn phosphorylate MAPK kinases (MAPKKs), which phosphorylate specific MAPKs. The *Arabidopsis* genome encodes more than 60 MAPKKKs, 10 MAPKKs, and 20 MAPKs (Ichimura *et al.*, 2002), which, depending on the environmental and developmental stimuli, regulate respective cellular processes. The potential complexity of signalling combinations that could arise from numerous members of MAPKKK/MAPKK/MAPK families suggests that these proteins might function as convergence points linking multiple rather than single hormonal pathways. Moreover, recent data indicate that, apart from the classical MAPK signalling cascades, MAPKs might be

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subjected to direct regulation by CPKs (Xie *et al.*, 2014*b*) or ROS, providing a multi-level control of their activity.

In yeast and mammalian cells, several MAPKs undergo Ox-PTMs (Cross and Templeton, 2004; Day and Veal, 2010; Templeton *et al.*, 2010). In plants, the evidence for direct perception of the ROS signal by MAPKs is starting to emerge. There are several indications that plant MAPKs are activated in response to oxidative stress. The ABA-dependent stomatal closure is positively regulated by MAPK9 and MAPK12, which both function downstream of ROS in the signalling cascade (Fig. 2). The kinase activity of MPK12 is stimulated by both ABA and H_2O_2 (Jammes *et al.*, 2009), although the exact mechanism of this regulation needs further investigation. Similarly, H_2O_2 was demonstrated to induce MAPKKK1 (ANP-1), which in turn activates MAPK3 and MAPK6 (Kovtun *et al.*, 2000). Importantly, the activation of both MAPKs requires a functional OXIDATIVE SIGNAL-INDUCIBLE1 (OXI1) kinase that itself undergoes ROS-dependent activation (Rentel *et al.*, 2004). However, the redox sensing capabilities of MAPKKK1, OXI1, or their interacting partners are yet to be demonstrated. The redox regulation of MAPK signalling cascades is likely to be evolutionary conserved, since tomato (*Solanum lycopersicum*) MPK1/2, which are orthologous to *Arabidopsis* MAPK6, also undergo oxidative activation (Zhou *et al.*, 2014). Recently, redox control was demonstrated for rice (*Oryza sativa*) OsMPK3 and OsMPK6. *In vitro*, the activity of both proteins is negatively regulated by rice thioredoxin h (OsTrx23) and depends on the redox status of Cys179 and Cys210, respectively (Xie *et al.*, 2009). According to the proposed model, under oxidative stress both MAPKs are activated via sulfenylation of their redox-sensitive residues, and subsequent reduction, probably involving an *S*-glutathionylated intermediate, renders them inactive (Xie *et al.*, 2014*a*).

In alfalfa (*Medicago* sp.), the H_2O_2-dependent cell death is controlled by the oxidative stress-activated MAP triple-kinase 1 (OMTK1)–MMK3 pathway (Nakagami *et al.*, 2004). Strikingly, the authors demonstrated that H_2O_2 treatment is crucial for the activation of the OMTK1–MMK3 signalling, even if both proteins are abundantly expressed, and therefore regulation of OMTK1 at the post-translational level was proposed (Nakagami *et al.*, 2004).

Recently, three members of the *Arabidopsis* MAPK family, MAPK2, -4, and -7, have been found to undergo H_2O_2-dependent sulfenylation (Waszczak *et al.*, 2014). The discovery of MAPK4 sulfenylation is of particular importance, since the MEKK1–MKK1/MKK2–MPK4 cascade has been implicated in the control of ROS homeostasis (Pitzschke *et al.*, 2009), plant cell death, and immunity (Kong *et al.*, 2012). Recently, the *Brassica napus* orthologue of AtMAPK4 (BnMPK4) has been demonstrated to undergo H_2O_2-dependent aggregation, which was abolished by mutation of Cys232. Interestingly, the aggregation did not affect the protein kinase activity (Zhang *et al.*, 2015). Further research is needed to elucidate the biological significance of these modifications *in vivo*.

Apart from being regulated by ROS, MAPKs also control regulatory events upstream of ROS accumulation. During the wounding response, MAPK8 serves as a regulatory hub converging both the canonical MAPKK/MAPK-dependent as well as the Ca^{2+}-dependent activation mode to regulate ROS homeostasis via negative regulation of the *RbohD* transcript level (Takahashi *et al.*, 2011).

The well-established link between the cellular redox status and activity of MAPKs is the oxidative stress-related negative regulation of MAPK repressors. The activity of MAPKs depends positively on the phosphorylation status of threonine and tyrosine residues within the conserved TXY motif in the activation loop (Zhang *et al.*, 1994; Canagarajah *et al.*, 1997). As such, the PTPs have been implemented in the control of various processes that involve MAPK signalling components such as guard-cell signalling (MacRobbie, 2002), oxidative stress tolerance (Lee and Ellis, 2007), SA homeostasis (Bartels *et al.*, 2009), disease responses (Lumbreras *et al.*, 2010), and development (Strader *et al.*, 2008; Walia *et al.*, 2009). ROS mediate the inactivation of PTPs and double-specificity PTPs (DsPTPs) that act on both phosphotyrosine and phosphothreonine residues and repress MAPKs (Gupta *et al.*, 1998; Xu *et al.*, 1998). The activity of PTPs and DsPTPs, which, apart from their active-site motif, do not share any sequence similarity, requires a highly conserved Cys residue: Cys265 in AtPTP1 (Xu *et al.*, 1998) and Cys135 in AtDsPTP1 (Gupta *et al.*, 1998). The activity of AtPTP1 is negatively influenced by H_2O_2 treatment both *in vitro* and *in vivo*. Furthermore, this inactivation is positively correlated with MAPK6 activation by H_2O_2, indicating that AtPTP1 acts as a redox sensor linking oxidative stress with MAPK activity (Gupta and Luan, 2003).

The soybean (*Glycine max*) GmPTP has a low sensitivity to inhibition with H_2O_2 and displays hypersensitivity towards GSSG-induced *S*-glutathionylation. GmPTP activity is governed by two redox-active Cys residues (Cys78 and Cys176) that control the catalytic Cys266, which itself is not a primary target for oxidation. *S*-Glutathionylation of Cys176 leads to rapid inactivation of the enzyme. This is followed by *S*-glutathionylation of Cys78, and further leads to the formation of Cys78–Cys266 intra/intermolecular disulphide(s), probably protecting the Cys266 from oxidation (Dixon *et al.*, 2005).

The general paradigm of oxidative inactivation of PTPs has recently been challenged by the discovery of a reductant-inhibited PTP from maize (*Zea mays*; ZmRIP1). ZmRIP1 phosphatase activity is insensitive to H_2O_2 and decreases irreversibly upon reduction of Cys181 with dithiothreitol (Li *et al.*, 2012). It has been suggested that Cys181 might be involved in the formation of intramolecular disulphide; however, the exact mechanism of this redox regulation needs to be elucidated. Interestingly, upon H_2O_2 treatment, ZmRIP1 undergoes chloroplast-to-nucleus translocation, which is indicative of a role in signal transduction; however, the target proteins of ZmRIP1 are currently not known.

Cys Ox-PTMs control protein translation and stability

Next to the regulation of transcription, the control of mRNA translation by *trans*-acting factors is crucial for fine-tuning

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protein expression. Thus far, the best-studied case of redox-regulated control of mRNA translation occurs during expression of *Chlamydomonas reinhardtii* photosystem II reaction centre protein D1, which is encoded by the chloroplast *psbA* gene (Kim and Mayfield, 1997; Yohn *et al.*, 1998; Alergand *et al.*, 2006). This two-component system involves the polyadenylation-binding protein RB47 and the protein disulphide isomerase RB60. Binding of RB47 to the 5′-untranslated region of the *psbA* mRNA is required for translation and depends on RB60, which regulates this process in a light-dependent manner. During the photosynthetic light reactions, a reducing environment is generated by photosystem I. The reducing equivalents are relayed through the Fdx/FTR/Trx system to the chloroplast RB60. RB60 contains two Trx-like CGHC-sites that serve to interact with Cys143 or Cys259 of RB47. This interaction results in the reduction of the RB47 regulatory disulphides (Cys259–Cys143 or Cys259–Cys55) and activates the binding of RB47 to the *psbA* mRNA (Alergand *et al.*, 2006). Upon translation, the D1 protein is incorporated into photosystem II complexes. Importantly, under oxidizing conditions, RB60 facilitates the conversion of reduced RB47 to its inactive oxidized form (Kim and Mayfield, 1997). An analogous system could control the *psbA* mRNA translation in *Arabidopsis*, since two currently uncharacterized RNA-binding proteins have been found to interact with the *psbA* mRNA in a redox-dependent manner (Shen *et al.*, 2001).

In addition, mRNA stability might be subjected to redox control. *SALT OVERLY SENSITIVE 1* (*SOS1*) transcripts are stabilized after H_2O_2 treatment. *SOS1* codes for a plasma membrane Na^+/H^+ antiporter crucial for the maintenance of ion homeostasis in saline stress conditions. Under normal conditions, *SOS1* mRNA is highly unstable, but stress-induced production of H_2O_2 positively influences its stability and promotes salt stress tolerance (Chung *et al.*, 2008). Although the molecular mechanisms behind this process are unknown, a plausible scenario could involve redox-regulated RNA-binding protein(s) (Lorković, 2009).

A recent study demonstrated that the proteolytic control of protein maturity can also be subjected to redox regulation. The activity of the PLASTIDIC TYPE I SIGNAL PEPTIDASE 1 (PLSP1), which functions in the removal of the thylakoid-transfer signal upon import of proteins from the chloroplast stroma into the thylakoid lumen, depends on the regulatory disulphide bond between Cys166 and Cys286. Formation of the regulatory disulphide leads to the activation of proteolytic activity *in vitro*, probably via facilitating the entry of substrate proteins into the protease-binding pocket (Midorikawa *et al.*, 2014).

Proteolytic regulation of protein stability is gaining recognition in the field of plant cell death research (Coll *et al.*, 2010; Wrzaczek *et al.*, 2015). However, it is not yet clear whether any of the signalling components involved in these processes are directly targeted by ROS.

Perspectives

The portfolio of proteins that potentially undergo Ox-PTMs is continuously growing. Many studies have been performed to understand reversible Cys Ox-PTMs, and the repertoire of techniques that enable the identification of these PTMs is continuously expanding (Montrichard *et al.*, 2009). We have recently identified a set of sulfenylated proteins, which are potential ROS sensors, in *Arabidopsis thaliana* (Waszczak *et al.*, 2014; S. Akter *et al.*, unpublished data). Besides the Cys Ox-PTMs, formation of methionine sulfoxide and tyrosine nitration are emerging redox PTMs affecting protein structure and function (Jacques *et al.*, 2013). In this relatively new field, we expect that, in the near future, many more proteins undergoing Ox-PTMs at Cys, Met, and tyrosine residues will be identified.

Now we face the challenge of validating and functionally characterizing these proteins in terms of their biochemical, functional, and structural aspects in order to get extra insights into the ROS signal transduction mechanism at the molecular level. The validation of proteomic results requires a dedicated effort that in most cases focuses on a single protein at a time. A closer look at the results of current investigations reveals an apparent huge gap between the number of identified proteins and the number of proteins for which the occurrence of Ox-PTMs has been validated. This is caused by the difficulty in recombinantly expressing plant proteins, the lack of suitable activity assays, the absence of mutant phenotypes hampering the complementation studies, and, finally, the scarce information about the role and function of the identified proteins. We anticipate that future efforts in the field of plant redox biology will explore the sets of already identified but not yet validated proteomic findings. Furthermore, it is important to realize that proteins identified by redox proteomics approaches might be secondary, rather than primary targets for Ox-PTMs due to their relatively low reactivity with ROS when compared with, for example, thiol peroxidases. As discussed earlier, the thiol–disulphide relays initiated by thiol peroxidases have been demonstrated for multiple ROS sensory modules (Delaunay *et al.*, 2002; Miao *et al.*, 2006), which, complemented with more recent discoveries (Sobotta *et al.*, 2015), argues for an universal nature of such systems. Finally, in view of the future potential of redox sensory systems for manipulation of plant stress tolerance, we believe that, whenever possible, the relevance of all results obtained from *in vitro* experiments should be assessed *in vivo*.

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