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., 2013a, 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; Gsr, 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; Pxx, 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-SO-H, sulfenic acid; R-SO_{2}H, sulfenic acid; R-SO_{3}H, sulfonic acid; RNS, reactive nitrogen species; ROS, reactive oxygen species; R-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 Rio, 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’; Glathionylation, R-S-SG; sulfenylation, R-SO2H; and sulfonylation, R-SO2H).

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 (Glarign 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 (H2O2)-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 Rio, 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 pKa (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 pKa, i.e. the ability to form the anionic form of the sulfur, called thiolate (R-S¯), which is much more reactive than the thiol. If the pKa of the sulfur atom is higher than the pH of the solution, the protonated thiol will be the dominant species. However, if the pKa is lower than the pH, the majority of the thiols will be present as a thiolate (Cys prone to oxidation). The pKa 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 pKa of reactive Cys residues. In general, the more hydrogen bonds a Cys-sulfur receives, the lower the pKa 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...
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-\(\text{SO}_2\)H), and subsequently to irreversible sulfonic acid (R-\(\text{SO}_3\)H; Roos and Messens, 2011). The reversion of the R-\(\text{SO}_2\)H modification is catalysed by an ATP-dependent sulfiredoxin enzyme (Srx) that is capable of reducing R-\(\text{SO}_2\)H to R-SOH in Arabidopsis (Rey et al., 2007). However, thus far, R-\(\text{SO}_2\)H 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 PrxIIIF (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
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/REGULATOR Y COMPO slows 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 Ca2+ 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 Ca2+ 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 H2O2-dependent inhibition (Fig. 2). Oxidizing conditions led to the
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 H2O2 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 H2O2-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 H2O2-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 Ca2+ fluxes in Arabidopsis roots (Laohavisit et al., 2012). ANNEXIN1 binds to lipid membranes in a Ca2+-dependent manner and stimulates Ca2+ 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 Ca2+ 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 Ca2+ channels downstream of the NADPH oxidase RbohC (Foreman et al., 2003). Besides impaired root development, ann1 mutants are hypersensitive to drought (Konopka-Postupska et al., 2009). ANNEXIN1 is S-glutathionylated on both Cys residues (Cys111 and Cys239), resulting in a 50% decrease in Ca2+ affinity (Konopka-Postupska et al., 2009). This mechanism probably serves to restrict the AtANN1 membrane association and inhibit ROS-mediated Ca2+ 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 (Ndumukong 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-I 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...
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 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...
subjected to direct regulation by CPKs (Xie et al., 2014b) 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$_2$O$_2$ (Jammes et al., 2009), although the exact mechanism of this regulation needs further investigation. Similarly, H$_2$O$_2$ 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-INDUCIBLE (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., 2014a).

In alfalfa (Medicago sp.), the H$_2$O$_2$-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$_2$O$_2$ 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$_2$O$_2$-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$_2$O$_2$-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 phosphorytrosine 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$_2$O$_2$ treatment both in vitro and in vivo. Furthermore, this inactivation is positively correlated with MAPK6 activation by H$_2$O$_2$, 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$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ 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
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 disulfide 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$_2$O$_2$ 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$_2$O$_2$ 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) (Lorkovic, 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 (PLSPI), which functions in the removal of the thylakoid-transferred 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 PTPs 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 recombantly 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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