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Review

Thiol-based Oxidative Posttranslational Modifications (OxiPTMs) of Plant Proteins

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The thiol group of cysteine (Cys) residues, often present in the active center of the protein, is of particular importance to protein function, which is significantly determined by the redox state of a protein's environment. Our knowledge of different thiol-based oxidative posttranslational modifications (oxiPTMs), which compete for specific protein thiol groups, has increased over the last 10 years. The principal oxiPTMs include *S*-sulfenylation, *S*-glutathionylation, *S*-nitrosation, persulfidation, *S*-cyanylation and *S*-acylation. The role of each oxiPTM depends on the redox cellular state, which in turn depends on cellular homeostasis under either optimal or stressful conditions. Under such conditions, the metabolism of molecules such as glutathione, NADPH (reduced nicotinamide adenine dinucleotide phosphate), nitric oxide, hydrogen sulfide and hydrogen peroxide can be altered, exacerbated and, consequently, outside the cell's control. This review provides a broad overview of these oxiPTMs under physiological and unfavorable conditions, which can regulate the function of target proteins.

Keywords: Persulfidation • *S*-cyanylation and *S*-acylation • *S*-glutathionylation • *S*-nitrosation • *S*-sulfenylation

Introduction

Once synthesized in ribosomes, proteins can undergo numerous posttranslational modifications (PTMs) involving chemical changes in specific amino acid residues, which are mediated by enzymatic or nonenzymatic additions of certain chemical groups. These additional regulatory mechanisms, many of which have a very significant impact on cellular signaling, affect the chemical properties of target proteins and, consequently, their spatial conformation, stability, folding properties, subcellular location and biological function (Navrot et al. 2011, Friso and van Wijk 2015).

This highly diverse range of PTMs includes phosphorylation, ubiquitination, SUMOylation, γ-carboxylation, poly(ADP-ribosyl)ation, acetylation, redox modification, methylation, glycosylation, acylation, alkylation, hydroxylation, nitration and nucleotide addition, among others (Vu et al. 2018, Arefian et al. 2021, Gough and Sadanandom 2021, Péter et al. 2021, Wang et al. 2021c). The UniProtKB/Swiss-Prot databases have identified over 450 different PTMs (Conibear 2020, Zhang and Zeng 2020, Wang et al. 2021b), which demonstrates the complexity of cellular proteomes. The identification of a single PTM in a specific protein requires specific experimental techniques, which, in many cases, involve complex technical protocols, including chemoselective reactions, in order to label specific amino acid residues, combined with tandem mass spectrometry analyses (Chuh and Pratt 2015, Shortreed et al. 2015, Aslebagh et al. 2019). Additionally, although developed to identify protein PTMs in different databases (Li and Tang 2016, Audagnotto and Dal Peraro 2017, Xie et al. 2018), bioinformatic tools need to be corroborated by experimental techniques.

This review provides a comprehensive and updated overview of the major oxidative posttranslational modifications (oxiPTMs), which affect thiol groups of protein cysteine residues and their functioning in plant cells.

Overview of the thiol-based oxiPTMs: a mechanism of protein regulation

Thiol (-SH) groups of cysteine (Cys) residues, which are involved in the protein's active center and folding, are essential for the functioning and regulation of many proteins (Poole 2015, Ulrich and Jakob 2019). These thiol groups can be deprotonated to a negatively charged thiolate (Cys-S^-), resulting in enhanced reactivity. Furthermore, the oxidation of the thiol group can involve either one- or two-electron oxidation events, leading to the formation of thiyl radicals (Cys-$S^\bullet$) and sulfenic acids (Cys-SOH), respectively (Trujillo et al. 2016, Turell et al. 2020). Under cellular oxidant conditions, sulfenic acid (-SOH) is oxidized to sulfinic acid (-SO_2H) and then to sulfonic acid (-SO_3H), the latter being an irreversible process, which usually triggers the inactivation of the target protein (**Fig. 1**).

On the other hand, it should be noted that the acid dissociation constant (pKa) of the thiol group is typically close to the physiological pH (7.0–7.4). The thermodynamics and kinetics of

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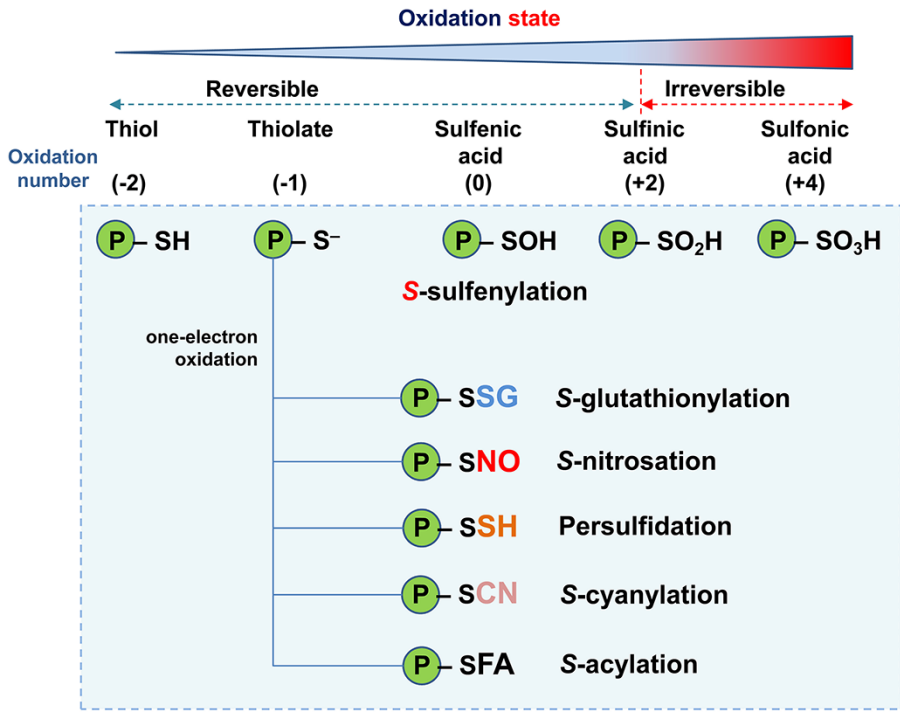

Fig. 1 Outline of the main thiol-based oxiPTMs. The upper side of the panel indicates the oxidation states of sulfur (S) in proteins, which can be from thiol (−2) to sulfonic acid (+4). Under cellular oxidant conditions, the oxidation from sulfinic acid to sulfonic acid could take place, the latter being an irreversible process. The principal reversible oxiPTMs result from the interaction between thiolate with either hydrogen peroxide (H_2O_2; *S*-sulfenylation); glutathione (GSH; *S*-glutathionylation), nitric oxide (NO; *S*-nitrosation), hydrogen sulfide (H_2S; persulfidation), cyanide (HCN; *S*-cyanylation) or fatty acid (FA; *S*-acylation) are also displayed in the lower side of the panel.

protein thiol oxidation *in vivo* are affected by thiol acidity, which varies according to the protein microenvironment in a specific subcellular location and protein folding (Roos et al. 2013, Zhang et al. 2021). This is particularly important when the cellular redox state is susceptible to modification under stressful conditions during which the generation of oxidant molecules is uncontrollably boosted and initiates a cascade of cellular signals (Chung et al. 2013, Castro et al. 2021). In *Arabidopsis*, under oxidative stress conditions, the peroxidatic Cys of the chloroplast 2-Cys peroxiredoxin (2-Cys Prx) can be oxidized to sulfinic acid causing its inactivation; however, the activity is restored when this Cys is reduced back to sulfenic acid by a sulfiredoxin enzyme (Iglesias-Baena et al. 2010), a process in which NADPH thioredoxin reductase C appears to be involved (Puerto-Galán et al. 2015).

The change in the redox state of a specific thiol group can either increase or decrease the biological function of the target protein (**Table 1**). Proteins in any cellular context are prone to be exposed to different potential agents, which can oxidize or reduce the thiol group. The final impact will therefore depend on the accessibility of these potential thiol targets to different potential oxidants or reducing molecules such as certain free radical species, including hydroxyl ($^{\bullet}OH$), peroxyl ($ROO^{\bullet}$), superoxide ($O_2^{\bullet-}$), nitric oxide ($^{\bullet}NO$) and nitrogen dioxide ($^{\bullet}NO_2$), as well as nonradical molecules such as hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$) and hydrogen sulfide (H_2S). Furthermore, certain pair molecules like reduced/oxidized glutathione [GSH/glutathione disulfide (GSSG)] and $NADPH/NADP^+$ (reduced and oxidized nicotinamide adenine dinucleotide phosphate, respectively), which are involved in numerous metabolic pathways either as substrates or cofactors and whose reduced/oxidized ratio can be significantly affected under nitro-oxidative conditions, also play an important role in cellular redox homeostasis (Møller et al. 2020, Vogelsang and Dietz 2020, Corpas et al. 2021b). Additionally, the involvement of other thiol-based redox compounds such as *S*-nitrosothiols (SNO), polysulfide (H_2S_n), thioredoxins (Trxs), glutaredoxins (Grxs) and peroxiredoxins (Prxs) also needs to be taken into account (Rouhier et al. 2015, Knuesting and Scheibe 2018, Benchoam et al. 2020, Kimura 2021, Sánchez-Guerrero et al. 2021, Takata et al. 2021). This complex redox regulation under physiological and stress conditions has mostly been described in chloroplasts (Yoshida et al. 2018, Liebthal et al. 2020, Yokochi et al. 2021) and mitochondria (da Fonseca-Pereira et al. 2019, Martí et al. 2020). Thus, chloroplastic Prx II E is involved in peroxynitrite reductase activity, which is inhibited by *S*-nitrosation (Romero-Puertas et al. 2007), while mitochondrial PrxII F through *S*-nitrosation prevents the thermal aggregation of citrate synthase (Camejo et al. 2015).

Other molecules with antioxidant capacity, such as ascorbate and melatonin, are also involved in this pool of regulatory molecules (Tan et al. 2015, Zechmann 2018, Aghdam et al. 2021). All these elements broaden the network of interactions, which are difficult to decipher if a holistic analysis is not used to examine all the complex interactions. The oxiPTM mechanism for the regulation of signaling protein functions requires that: (i) the oxiPTM-promoted change in protein function is caused by cellular stimuli; (ii) the modification be rapid and be

Table 1 Number of identified plant proteins susceptible to undergo oxiPTMs according to proteomic analyses in different plant species and organs.

oxiPTM	No of identified proteins	Plant species/organ	Reference
S-sulfenylation	1,394[a]	*Arabidopsis* cell culture treated with H_2O_2	Huang et al. 2019
	132 [b]	*Arabidopsis* plastids	De Smet et al. 2019
S-glutathionylation	79[c]	*Arabidopsis* cell culture	Dixon et al. 2005
	25[d]	Wheat seedlings	Gietler et al. 2016
S-nitrosation	63[e]	*Arabidopsis* cell exposed to the NO donor *S*-nitrosoglutathione	Lindermayr et al. 2005
	52[e]	*Arabidopsis* leaves of plants exposed to NO gas	
	46[f]	Endogenous *S*-nitrosated proteins in *Arabidopsis* cells	Fares et al. 2011
	44[f]	Endogenous *S*-nitrosated proteins in *Arabidopsis* plantlets	Puyaubert et al. 2014
	32[e]	*Populus* × *canescens*	Vanzo et al. 2014
	926[g]	*Arabidopsis*	Hu et al. 2015
	402	Peanut root tips	Pan et al. 2021
	35[h]	*Arabidopsis* guard cells treated with flg22	Lawrence et al. 2020
Persulfidation	106[i]	*Arabidopsis* leaves	Aroca et al. 2015, Aroca et al. 2017
	2015		
S-cyanylation	163	*Arabidopsis* leaves	García et al. 2019
S-acylation	600	*Arabidopsis*	Hemsley et al. 2013; Kumar et al. 2020
	2643[j]		
	450 [k]	Poplar cell culture	Srivastava et al. 2016

[a]BTD-based probe.
[b]YAP1C probe.
[c]GS-biotin-labeling studies, 2D-PAGE followed by MALDI-MS.
[d]Immublot probe with anti-GSH antibodies and identified by MALDI-TOF and LC–MS/MS.
[e]BSM and LC–MS/MS.
[f]BSM and labeling with isotope-coded affinity tags.
[g]Site-specific nitrosoproteomic approach.
[h]Iodo tandem mass tag™ labeling.
[i]Modified BSM.
[j]Acyl resin-assisted capture assay.
[k]Acyl-Biotin Exchange method.

mediated by an enzyme and (iii) the modification be specific and reversible (Shelton et al. 2005). In addition, the effectiveness of these PTMs can vary depending on such factors as the accessibility of the potential target Cys' thiol group to each molecule incorporated. For example, NO and H_2S, being smaller than GSH (reduced glutathione), are expected to access a larger number of Cys residues. Nevertheless, the proteins corresponding to each oxiPTM, whose number and specificity are expected to increase with the development of new techniques, have been identified using proteomic analysis (Alcock et al. 2018, Shi and Carroll 2020, Wang et al. 2021b). **Fig. 1** shows the principal oxiPTMs examined in this review.

S-sulfenylation

S-sulfenylation involves the reaction of H_2O_2 with redox-sensitive Cys in proteins to form cysteine sulfenic acid (Cys-SOH), as well as the reversible covalent addition of one oxygen atom to the thiol group. If the thiol oxidation state persists over time, two or three oxygen atoms can be added to generate an irreversible covalent addition until sulfonic acid is formed (**Fig. 1**). This generally results in protein degradation or inactivation and is usually associated with nonfunctional proteins and stressful conditions (Filipovic et al. 2018).

With the aid of a dimedone-based DYn-2 probe, initial *in vitro* studies of protein *S*-sulfenylation, which used the human colon carcinoma (RKO) cell line treated with 500 μM H_2O_2, identified over 778 *S*-sulfenylated proteins following detection with liquid chromatography–tandem mass spectrometry (LC–MS/MS) (Yang et al. 2014). These *S*-sulfenylated proteins, approximately 92% of which contained only one or two *S*-sulfenylated residues, include protein kinases, phosphatases, acetyltransferases, deacetylases and deubiquitinases. This suggests that *S*-sulfenylation regulates these proteins and may mediate other additional PTMs (Yang et al. 2014).

In *Arabidopsis thaliana* cell cultures treated with 100 μM H_2O_2, an initial analysis of sulfenome identified roughly 100 potential *S*-sulfenylated proteins (Waszczak et al. 2014). Later, 1394 proteins susceptible to *S*-sulfenylation were identified using a more reactive 1-(pent-4-yn-1-yl)-1 H-**b**enzo[c][1,2]**t**hiazin-4(3H)-one 2,2-**d**ioxide (BTD) based probe (Fu et al. 2019) than the DYn-2 probe to detect *S*-sulfenylated residues (Huang et al. 2019), which had previously identified 200 *S*-sulfenylated proteins (Akter et al. 2015) (**Table 2**). These proteins included mitogen-activated protein kinase 4 (MAPK4), which was *S*-sulfenylated at Cys181, leading to a decrease in kinase activity (Huang et al. 2019). A more in-depth analysis of *Arabidopsis* cells treated with 1 mM H_2O_2, using sulfenic acid yeast ACTIVATOR PROTEIN 1 containing single redox-active Cys598 (YAP1C) trapping technology tagged to the plastids, identified 132 *S*-sulfenylated proteins (De Smet et al. 2019). More recently, a new noninvasive strategy using a

Table 2 Representative examples of plant proteins that undergo several oxiPTMs.

NADP-DH/redox PTMs	Effects	Reference
Ferredoxin-NADP reductase		
S-nitrosation	Unknown	Begara-Morales et al. 2013; Holzmeister et al. 2011[b]; Niu et al. 2019[b]
Persufidation	Unknown	Aroca et al. 2017[b];
Glyceraldehyde-3-phosphate dehydrogenase (NADP-GAPDH)		
S-nitrosation	Inhibition	Lindermayr et al. 2005[b]; Abat et al., 2008; Romero-Puertas et al. 2008[b]; Wang et al. 2017; Vanzo et al. 2014[b]; Niu et al. 2019[b]; Tanou et al. 2009[b] ; Hu et al. 2015[b]; Gong and Shi 2019
S-cyanylation	Unknown	García et al. 2019
Persulfidation	Activation	Aroca et al. 2015[b,c]
Glutathionylation	Inhibition	Zaffagnini et al. 2007[c]
Catalase		
S-nitrosation	Inhibition	Begara-Morales et al. 2013; Aroca et al. 2015; Rodríguez-Ruiz et al. 2019b[a,c]; Palma et al. 2020
Persulfidation	Inhibition	Aroca et al. 2015[b]; Corpas et al. 2019[c]
S-acylation	Unknown	Kumar et al. 2020[b]
APX		
S-nitrosation	Activation	Begara-Morales et al. 2014; Yang et al. 2015
Persulfidation	Activation	Aroca et al. 2015
S-cyanylation	Unknown	García et al. 2019
S-acylation	Unknown	Kumar et al. 2020[b]
NADP-malic enzyme		
S-nitrosation	Inhibition	Hu et al. 2015[b]; Muñoz-Vargas et al. 2020[c]
Persulfidation	Inhibition	Aroca et al. 2017[b]; Muñoz-Vargas et al. 2020[b,c]
S-cyanylation (NADP-dependent malic enzyme NADP-ME2)	Unknown	García et al. 2019[b]
S-acylation	Unknown	Kumar et al. 2020[b]
NADP-isocitrate dehydrogenase		
S-nitrosation	Inhibition	Fares et al. 2011[b]; Leterrier et al. 2012[c]; Puyaubert et al. 2014[b]; Hu et al. 2015[b]; Muñoz-Vargas et al. 2018[c]; Niazi et al. 2019[c]
Persulfidation	Inhibition	Aroca et al. 2017[b]; Muñoz-Vargas et al. 2018[a,c]
Glutathionylation	Inhibition	Leterrier et al. 2012[c]; Niazi et al. 2019[c]
S-cyanylation	Unknown	García et al. 2019
Sulfenylation	Unknown	De Smet et al. 2019[b]
S-acylation	Unknown	Kumar et al. 2020[b]
NADPH oxidase		
S-nitrosation	Inhibition	Yun et al. 2011
Persulfidation	Activation	Shen et al. 2020

[a]Computational prediction.
[b]Proteomic identification.
[c]Activity in vitro assay.

disulfide-linked peptide reporter has been implemented to identify *S*-sulfenylated proteins in *Arabidopsis* cells (Wei et al. 2020).

These studies using cell cultures treated with high concentrations of H_2O_2 have provided a large number of candidate proteins for *S*-sulfenylation. However, more in-depth analyses need to be carried out on other plant species under both physiological and stressful conditions in order to evaluate the role of *S*-sulfenylation.

S-glutathionylation

Glutathione (GSH; γ-L-glutamyl-L-cysteinyl-glycine) is a non-protein thiol compound, which, along with ascorbate, is the most abundant soluble antioxidant present in plant cells (Foyer and Noctor 2011). With its pKa of roughly 8–9 and at physiological pH, the -SH group of GSH is highly protonated. Under optimal physiological conditions, although mainly found in a reduced form of GSH, in oxidative stress environments, free glutathione is oxidized to glutathione disulfide (GSSG) (Airaki et al. 2011; Diaz-Vivancos et al. 2015). GSH is required in a diverse range of detoxification pathways including the ascorbate-glutathione cycle for H_2O_2 regulation, the glyoxalase pathway for methylglyoxal (MG) detoxification (Kharbech et al. 2020, Dorion et al. 2021), and also for the biosynthesis of phytochelatins, which are a group of low molecular weight polypeptides involved in heavy metal and metalloid detoxification (Gupta et al. 2013, Rodríguez-Ruiz et al. 2019a, Bhat et al. 2021). Some evidence indicates that stress-induced reductions in the GSH/GSSG ratio in different cellular systems promote the formation of mixed disulfide bridges between

glutathione and protein thiols. *S*-glutathionylation, a reversible PTM (**Fig. 1**), occurring spontaneously between glutathione and a protein thiolate, regulates protein function in different subcellular compartments (Shelton et al. 2005, Mailloux and Treberg 2016, Zhang et al. 2018, Kalinina and Novichkova 2021). This nonenzymatic interaction can be through several mechanisms: (i) protein thiol (P-SH) is oxidized by reactive oxygen species (ROS) to a sulfenic acid (P-SOH) which then rapidly reacts with GSH to form P-SSG; or (ii) exchange of thiol-disulfide between P-SH with glutathione disulfide (GSSG) (Zhang et al. 2018). There is also another proposed mechanism mediated enzymatically by a glutaredoxin (GRX) where P-SH is oxidized to a thiyl radical ($PS^{\bullet}$), which then quickly reacts with GSH to form a thiyl radical glutathionyl intermediate ($PSSG^{\bullet-}$), which then reacts with O_2 to form PSSG (Beer et al. 2004). Another example in which *S*-glutathionylation is mediated by a GRX has been described in the *Arabidopsis* brassinosteroid insensitive 1-associated receptor-like kinase 1 (BAK1), in which this PTM causes the inhibition of its kinase activity (Bender et al. 2015). Under certain adverse environmental conditions, *S*-glutathionylation can prevent protein Cys overoxidation, which causes its inactivation through the formation of sulfonic residues (Zaffagnini et al. 2007, 2012, Gurrieri et al. 2019). For example, 2-Cys Prx undergoes deglutathiolation in the presence of sulfiredoxin in pea chloroplasts, which does not occur in the mitochondrial Prx IIF. Thus, it has been suggested that glutathionylation/deglutathionylation is associated with changes in redox state during plant development or in response to stress conditions (Calderón et al. 2017).

S-glutathionylated proteins have been detected using techniques such as the use of GSH antibodies and labeling with the aid of ^{35}S radiolabeling and biotinylation (Ito et al. 2003, Gao et al. 2009). Thus, in *Arabidopsis* cell cultures, approximately 79 *S*-glutathionylated proteins have been identified using a combination of GSH biotin labeling, 2D-PAGE and matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) (Dixon et al. 2005). In wheat seedlings, 25 *S*-glutathionylated proteins were identified using 2-D electrophoresis, immunoblot tests, anti-GSH antibodies, MALDI-TOF (time-of-flight) and LC–MS/MS (Gietler et al. 2016) (**Table 2**).

As mentioned above, given its antioxidant properties, interest in the involvement of GSH in *S*-glutathionylation processes has been growing in plant research. For example, GSH regulates ethylene biosynthesis in *Arabidopsis* by modulating 1-aminocyclopropane-1-carboxylate oxidase (ACO) activity through *S*-glutathionylation. With the aid of *in silico* docking analysis, Cys63 has been identified as the best candidate for *S*-glutathionylation of ACO (Datta et al. 2015). In *Arabidopsis*, *S*-glutathionylation can trigger the inhibition of chloroplastic NADP-glyceraldehyde-3-phosphate dehydrogenase (AtGAPA1) at Cys149 (Zaffagnini et al. 2007). *S*-glutathionylation has also been found to facilitate the insoluble aggregation of *S*-glutathionylated AtGAPA1, which appears to be irreversible. On the other hand, the aggregation process has been reported to be halted by Trx h1 (Zaffagnini et al. 2019). Another target protein for *S*-glutathionylation, α-amylase 3 (AtAMY3), which catalyzes the cleaving of α-1,4-glucosidic bonds in starch, is involved in the response to osmotic stress and stomatal opening and is also regulated by Trxs (Gurrieri et al. 2019). A pKa analysis of catalytic cysteine residues Cys499 and Cys587 suggests that one of these residues, which are susceptible to *S*-glutathionylation, can be deprotonated. This mechanism prevents overoxidation under stress conditions when high H_2O_2 content is present (Gurrieri et al. 2019). Cytosolic NADPH-generating isocitrate dehydrogenase (NADP-ICDH) in *Arabidopsis* has also been shown to be *S*-glutathionylated at Cys363, which is located outside the active center. Although it does not directly affect NADP-ICDH activity, *S*-glutathionylation appears to mediate inhibition caused by *S*-nitrosation in the presence of *S*-nitrosoglutathione (GSNO) under in vitro conditions (Niazi et al. 2019). *S*-glutathionylation appears to play a major role in certain organelles. Accordingly, in an analysis of nine photosynthetic species from streptophyte algae to angiosperms, Müller-Schüssele et al. (2021) have identified 364 proteins susceptible to undergo *S*-glutathionylation, of which 151 have a plastid location.

S-nitrosation

Nitric oxide ($^{\bullet}NO$) is a free radical with signaling functions found in higher plants. It mediates a wide variety of plant processes ranging from seed germination to fruit ripening and is involved in mechanisms of response to biotic and abiotic stresses, either directly or through its interaction with other growth regulators (González-Gordo et al. 2019, 2020a, Kolbert et al. 2019, Mishra et al. 2021, Corpas et al. 2022a, 2022b). Much of this regulation is carried out through NO-derived PTMs such as tyrosine nitration (Corpas et al. 2021a), metal nitrosylation and *S*-nitrosation (Corpas et al. 2020, Gupta et al. 2020). *S*-nitrosation, previously referred to as *S*-nitrosylation, is a covalent reaction involving one-electron oxidation of thiol groups. Thus, the presence of the thiol group enables GSH to react with NO to generate *S*-nitrosoglutathione (GSNO), regarded as a low molecular *S*-nitrosothiol, which can mediate transnitrosation reactions (Corpas et al. 2013). GSNO content is controlled by GSNO reductase (GSNOR), an enzyme that is inhibited by *S*-nitrosation (Sakamoto et al. 2002, Leterrier et al. 2011, Guerra et al. 2016, Tichá et al. 2017).

Most research on *S*-nitrosation, which is one of the most studied oxiPTMs, has been carried out in *A. thaliana*. Thus, using the biotin switch method (BSM), 63 candidates for *S*-nitrosation have been identified in *Arabidopsis* cells treated with the NO donor GSNO (Lindermayr et al. 2005). In the same study, using *Arabidopsis* plants exposed to NO gas, 52 *S*-nitrosated proteins were detected in leaves. Using an alternative technique based on a combination of BSM and labeling with isotope-coded affinity tags, a total of 46 endogenous proteins, which appeared to be *S*-nitrosated, were identified in *Arabidopsis* cells (Fares et al. 2011). With the aid of a similar approach, 44 endogenous *S*-nitrosated proteins, 11 of which were overnitrosated

under cold stress conditions, were also identified in *Arabidopsis* plantlets (Puyaubert et al. 2014). The number of *Arabidopsis* *S*-nitrosated proteins identified was subsequently extended to 926 with the aid of a site-specific nitrosoproteomic approach (Hu et al. 2015). More recently, the *S*-nitrosoproteome in *Arabidopsis* guard cells has been studied in response to the bacterial peptide flagellin using an iodo tandem mass tag labeling technique, which enabled 35 *S*-nitrosated proteins to be identified (Lawrence et al. 2020).

A study of poplar leaves has identified 172 *S*-nitrosated proteins, under ozone stress conditions; 32 *S*-nitrosated proteins were differentially affected, 9 of which showed a higher degree of *S*-nitrosation, with lower S-nitrosation observed in 23 of these proteins; this suggests the presence of a de-nitrosation mechanism to regulate these proteins (Vanzo et al. 2014). These authors, who specifically studied phenylalanine ammonia-lyase 2 involved in lignin biosynthesis, observed that, under ozone stress, enzyme activity increased due to de-nitrosation. On the other hand, an *S*-nitrosoproteome analysis of the root tips of peanut plants under aluminum stress, causing programmed cell death, has identified 402 *S*-nitrosated proteins, which closely correlated with an increase in GSNO content as a consequence of the inhibition of GSNOR activity by *S*-nitrosation (Pan et al. 2021). A site-specific nitrosoproteomic study of tomato plants under sodium alkaline stress has identified 334 *S*-nitrosated proteins in 425 different *S*-nitrosated loci. These proteins were involved in a wide range of metabolic processes, such as NO homeostasis and ROS metabolisms, as well as Ca^{2+}, ethylene and mitogen-activated protein kinase (MAPK) signaling. In this study, potential key target proteins, including ACO, ascorbate peroxidase (APX) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were selected (Gong and Shi 2019). *In silico* analyses have been used to complement the study of these phenomena, while different bioinformatic platforms have also been developed for these purposes, although it will eventually be necessary to experimentally confirm all the theoretical data reported (Kolbert and Lindermayr 2021).

Persulfidation

H_2S is a gasotransmitter that is endogenously generated as part of the sulfur and Cys metabolism (González-Gordo et al. 2020b). This molecule in plant cells has been shown to be involved in a myriad of physiological and stressful processes through multiple interactions with phytohormones and other signaling molecules, including H_2O_2, NO and melatonin (Corpas 2019, Corpas and Palma 2020). At the protein level, H_2S mediates a PTM called persulfidation, previously known as *S*-sulfhydration, which involves H_2S interactions with a thiol group of susceptible Cys (Aroca et al. 2015, Aroca et al. 2018, 2021a, 2021b, Wang et al. 2021a).

H_2S is a gas, but in an aqueous solution, it is dissociated to hydrosulfide (HS^-) and sulfide (S^{2-}) anions according to the following reaction: $H_2S_{(aqueous\ solution)} \leftrightarrow HS^- + H^+ \leftrightarrow S^{2-} + 2\,H^+$. As H_2S has an estimated pKa value of 6.8 at 37°C, the hydrosulfide anion (HS^-) predominates at the physiological pH of 7.4. Using a modified BSM combined with LC–MS/MS analysis, a total of 106 putative persulfidated proteins were initially identified in *Arabidopsis* leaves (Aroca et al. 2015). Later, using the tag-switch method, the number of persulfidated proteins identified increased to 2015 in the same plant species (Aroca et al. 2017) (**Table 2**). Like *S*-glutathionylation, persulfidation has been considered a protection mechanism that generates resistance to irreversible protein oxidation (Pantaleno et al. 2021).

Analysis of the oxiPTM persulfidation, whose importance in plant physiology has been demonstrated with the aid of new methodological approaches, has highlighted the role of H_2S under physiological and stressful conditions (Kouroussis et al. 2019, Fu et al. 2020, Zhao et al. 2020). A comparative analysis of protein persufidation, *S*-glutathionylation and *S*-nitrosation has shown that persulfidation plays a more prominent role than the other two oxiPTMs in *Arabidopsis* (Aroca et al. 2018). The physiological importance of persulfidation has been demonstrated in processes such as ROS metabolism regulation in which certain antioxidant enzymes such as APX and catalase have been reported to be persulfidated (**Table 1**) (Aroca et al. 2015, Corpas et al. 2019). In tomato (*Solanum lycopersicum*) plants, the H_2S-producing enzyme L-cysteine desulfhydrase (SlLCD1), a nuclearly encoded isozyme, is involved in the regulation of fruit ripening (Hu et al. 2020). Persulfidation is also involved in *Arabidopsis* stomatal closure through the persulfidation of different proteins in the cascade of signals, including SnRK2.6 (Chen et al. 2020), ABAI4 (Zhou et al. 2021), RBOHD and the L-cysteine desulfhydrase (Shen et al. 2020), with the latter two proteins playing a role in the generation of $O_2^{\bullet-}$ and H_2S, respectively. Furthermore, autophagy is regulated through the persulfidation of ATG4 (Laureano-Marín et al. 2020) and ATG18a (Aroca et al. 2021b).

S-cyanylation

Cyanide (HCN), which inhibits mitochondrial cytochrome oxidase and, consequently, the respiratory pathway, is known to have a negative impact on cellular metabolism and was used as a poison during the First and Second World Wars, as well as a further chemical terrorism weapon. HCN can also inhibit other key metalloproteins such as the antioxidant enzymes copper-zinc superoxide dismutase and catalase (Corpas et al. 1998).

Endogenous HCN is part of the cell metabolism as a result of cyanogenic glycosides hydrolysis by β-glycosidases and α-hydroxynitrile lyase (Arenas-Alfonseca et al. 2018, Cressey and Reeve 2019, Gotor et al. 2019) and is also released as a co-product of ethylene biosynthesis (Ansari et al. 2019). Cyanide is mainly detoxified by mitochondrial β-cyanoalanine synthase (CAS-C1) in the following reaction: L-cysteine + HCN → β-cyano-L-alanine + H_2S (Machingura et al. 2016). HCN is associated with several physiological regulatory functions such as seed germination, nitrate assimilation and root growth, as well as a co-product of ethylene biosynthesis; HCN is also accumulated

as a defense mechanism against plant pathogens and herbivores (Miller and Conn 1980, Siegień and Bogatek 2006, Zidenga et al. 2017). More recently, HCN has gained more prominence as a mediator of the PTM *S*-cyanylation due to its interaction with thiol groups (**Fig. 1**). In human blood and plasma, HCN has been observed to interact with albumin disulfide, as well as with heavy and light IgG chains (Fasco et al. 2007, 2011). Given that HCN does not react with free sulfhydryl groups, it has been suggested that *S*-cyanylation occurs in free Cys residues from GSH or other small molecules such as mixed disulfides.

In higher plants, our limited knowledge of *S*-cyanylation mainly comes from a pioneer study of *Arabidopsis* wild-type plants and a CAS-C1 knockout mutant. This proteomic analysis of *Arabidopsis* roots and leaves identified 163 proteins susceptible to *S*-cyanylation (García et al. 2019). As HCN detoxification by CAS-C1 also generates H_2S, the correlation between both these molecules, which mediate S-cyanylation and persulfidation, raises new questions about their mutual interactions as a mechanism of metabolic regulation (García et al. 2019). Enzymes that are targeted by *S*-cyanylation include APX and NADP-ICDH (**Table 1**) that participate also in the regulation of the levels of H_2O_2 and NADPH, respectively.

S-acylation

This reversible PTM, frequently known as *S*-palmitoylation, enables palmitate (C16 fatty acid) to be added to the thiol group of a specific Cys from soluble or peripheral membrane proteins through a thioester bond (Hurst and Hemsley 2015, Li and Qi 2017). This reaction is catalyzed by a family of protein *S*-acyl transferases containing a Asp-His-His-Cys motif in a Cys-rich domain (Batistič 2012, Yuan et al. 2013, Hemsley 2013, 2020). *S*-acylation increases the hydrophobicity of target proteins, which facilities their attachment to the membrane and, consequently, trafficking regulation (Hemsley 2020) and can be reversed by thioesterases (Zheng et al. 2019).

Proteomic analyses of *Arabidopsis* using a biotin switch isobaric tagging for relative and absolute quantification-based approach have identified 600 putative *S*-acylated proteins (Hemsley et al. 2013) and, in poplar cells, several hundred potential *S*-acylated proteins (Srivastava et al. 2016) (**Table 1**). Representative proteins that illustrate the importance of *S*-acylation include Ca^{2+}-dependent protein kinases, calcineurin-B-like proteins, MAPKs, receptor-like kinases, integral membrane transporters, ATPases and soluble *N*-ethylmaleimide-sensitive factor-activating protein receptors (Zheng et al. 2019). A more recent proteomic analysis of *Arabidopsis* using an acyl resin-assisted capture assay has expanded the list of potential *S*-acylated proteins to 2643 (Kumar et al. 2020). The importance of protein *S*-acylation has been highlighted in meiotic *Arabidopsis*, which is involved in the development of male and female sporophyte reproductive structures and associated gametophytes (Li et al. 2019).

Table 1 shows the number of identified proteins susceptible to all the oxiPTMs in higher plants described above.

How oxiPTMs are integrated in the metabolism of plant cells?

As previously described, nucleophilic thiol groups of protein Cys residues facilitate redox PTMs, which modify the function of the affected proteins. Research has mainly focused on specific oxiPTMs through the identification of potential targets with the aid of proteomic and LC–MS analyses adapted to each PTM. Each modification is evaluated in relation to its specific functional effect (an increase, decrease, or no effect on purified proteins) usually under *in vitro* conditions. This key step in the analysis needs to evaluate the effect of each oxiPTM under all cellular conditions, as specific thiol groups can be targeted by several competing PTM-promoting molecules according to their specificity, concentration, microenvironmental conditions and subcellular location, which will finally determine their role in the process.

In higher plants, protein *S*-nitrosation is the most studied oxiPTM, with available information mainly obtained from *Arabidopsis* plants. However, other oxiPTMs are attracting increasing attention given the interactions among them and the signaling properties of H_2O_2, NO and H_2S involved in regulating the final function of target proteins. **Table 2** shows different proteins regulated by several oxiPTMs. Examples of multiply regulated proteins include NADP-GAPDH, NADP-ICDH and NADP-malic enzyme, which are involved in generating NADPH (Hildebrandt et al. 2015, Niazi et al. 2019, Corpas et al. 2021b), a key molecule for the maintenance of redox homeostasis. On the other hand, biomolecules such as GSH, Prxs and Trxs, which, in turn, buffer cellular redox status, are also involved in regulating oxiPTMs. All these elements provide a detailed picture of complex redox equilibria, which finally determine the effect on the target protein.

Recently, there has been increasing interest in the potential physiological role of the *S*-nitrosothiol, thionitrous acid (HSNO), in cellular redox regulation, as a consequence of the interplay between NO and H_2S (Cortese-Krott et al. 2015, Nava et al. 2016, Chen et al. 2019, Marcolongo et al. 2019, Marozkina and Gaston 2020). The detection of this compound in biological systems whose chemistry is more complex than expected has been a major challenge. In plants, very little is known about HSNO, which, to our knowledge, has only been studied when applied exogenously to alfalfa plants under drought conditions using NOSH-aspirin, which simultaneously releases NO and H_2S to generate HSNO. Despite the beneficial effect of this *S*-nitrosothiol, little information is available concerning any potential oxiPTMs (Antoniou et al. 2020).

Summary and future perspectives

Interest in the different oxiPTMs has been growing of late due to their major physiological role in a wide range of higher plant processes. Over the last 10 years, significant advances have been made in this field with the development of appropriate technical approaches to specifically identify each redox modification. The methodology most commonly used involves proteomic

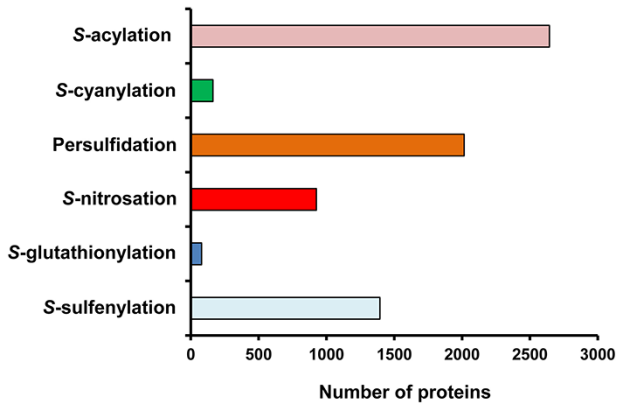

Fig. 2 Comparative analysis of the number of proteins identified that could be potentially targeted by one of the main thiol-based oxiPTMs in the model plant *Arabidopsis thaliana*. These PTMs include 2643 proteins for *S*-acylation (Kumar et al. 2020), 163 for *S*-cyanylation (García et al. 2019), 2015 for persulfidation (Aroca et al. 2017), 926 for *S*-nitrosation (Hu et al. 2015), 79 for *S*-glutathionylation (Dixon et al. 2005) and 1394 for *S*-sulfenylation (Huang et al. 2019).

analysis combined with chemoselective probes and mass spectrometry techniques (Yang et al. 2016, Shi and Carroll 2020, Zhang et al. 2021). **Fig. 2** shows a comparative analysis of proteins identified, which could be targeted by the principal oxiPTMs of cysteine thiols in the model plant *A. thaliana*, with a combination of three PTMs, persulfidation, *S*-sulfenylation and *S*-nitrosation, affecting over 690 proteins (Aroca et al. 2018).

Although some oxiPTMs have been studied in the algal model *Chlamydomonas* (Berger et al. 2016, De Mia et al. 2019), most studies have been carried out on *Arabidopsis* plants. Our knowledge of these oxiPTMs, therefore, needs to be extended to other plant species, especially those of agronomic interest under adverse environmental conditions, for crop improvement and biotechnological purposes. This is crucial given that signaling molecules, such as H_2O_2, NO and H_2S, and molecules with antioxidant capacity, such as GSH, are directly involved in these mechanisms. Although cross talk between the different oxiPTMs clearly exists, our knowledge concerning this phenomenon remains limited, and more research will need to be carried out in order to boost the potential of these molecular events.

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The authors have no conflicts of interest to declare.

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